Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3740–3744, April 1993
Neurobiology

Activated estrogen receptor mediates growth arrest and differentiation of a neuroblastoma cell line

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Communicated by Bert W. O'Malley, December 31, 1992

ABSTRACT **Several reports demonstrate estrogen receptor involvement in specific brain functions. In addition, estrogen receptors are expressed at early stages of brain development, suggesting that estrogens or related molecules may play an instructive role in the differentiation of specific brain areas. The lack of model systems in which these phenomena could be studied prompted us to develop a neuroblastoma cell line expressing the estrogen receptor. The cell line expresses the hormone receptor at levels compatible with a physiological activity. The activated estrogen receptor is capable of blocking proliferation of the cells without exerting toxic effects. Following growth arrest, the cells display a neuron-like morphology and express τ and synaptophysin, two proteins synthesized in differentiating neurons. The cell line generated will provide a valuable model system for molecular and biochemical studies of the activity of estrogens in neural-derived cells.**

Estrogens are important for regulation of cell growth and maturation of several tissues (e.g., uterus, liver, and brain). With regard to nerve cells, a series of *in vivo* and *in vitro* studies have demonstrated that 17β-estradiol promotes neuronal survival (1–3), neurite extension and arborization (4–6), and synapse formation (7, 8). In these systems, however, the limited number of cells expressing the estrogen receptor (ER) hampers a detailed analysis of the biological and biochemical mechanisms involved in estrogen activity. Such studies would be facilitated considerably by access to neuronal cells that uniformly express the ER and show biological responses to the cognate hormone.

Several studies have demonstrated that when cells are stably transfected with genes coding for steroid receptors they acquire responsiveness to specific hormones, indicating that a cell can be reprogramed to respond to external stimuli (9). All of these investigations, however, were carried out with nonneuronal cells.

In the present study, an expression plasmid containing the human ER was stably introduced into a neuroblastoma cell line. The results presented demonstrate that this system can be exploited to investigate biological functions mediated by the ER. In addition, the results described suggest a direct involvement of the activated ER in a series of morphological and biochemical changes that lead the transfected cells toward a differentiated state.

MATERIALS AND METHODS

Gene Transfer. The human neuroblastoma cell line SK-N-BE (10) was kindly provided by G. Melino (University of Rome). Cells were routinely cultured in 25-cm^2 plastic flasks in RPMI-1640 medium without phenol red (Sigma) supplemented with 10% charcoal-stripped fetal calf serum (DDC serum) (11). The expression vectors containing the human ER cDNA or the human progesterone receptor (PR) cDNA, kindly provided by G. Greene (12), were transfected into SK-N-BE cells by electroporation (450 V, 250 μF) using the Gene Pulser apparatus provided with a capacitance extender (Bio-Rad). The selection of successfully transfected cells was done in the presence of 300–400 μg of geneticin (G-418) per ml (GIBCO) for a minimum of 4 weeks. At their appearance, isolated colonies of geneticin-resistant cells were taken and cultured separately.

Enzymatic immunoassay (EIA) and Western Blotting. To identify ER-positive pools of cells, immunohistochemical analysis was performed using the rat monoclonal antibody H_{222} against the human ER (ER ICA, Abbott). ER expression was quantitated by an EIA (ER EIA, Abbott). For this purpose, the cytosolic fraction was prepared as described (11). Protein content was determined by Bradford's method (13).

Western analysis was done as described (14). Briefly, about 30×10^6 cells were trypsinized, washed, and resuspended in 200 μl of homogenization buffer (10 mM Tris·HCl/1 mM EDTA/1 mM dithiothreitol/5 mM $NaMoO_4$). The ER was immunoprecipitated by incubation with 12 μg of H_{222} purified antibody per ml for 3 h at 4°C and subsequent addition of protein A-Sepharose (Pharmacia) for 1 h at 4°C. The resin was washed with the homogenization buffer three times and the receptor was eluted with 2 M sodium thiocyanate. After precipitation with 8% (wt/vol) trichloroacetic acid and 0.1% (wt/vol) sodium deoxycholate, samples were resuspended in Laemmli sample buffer, boiled, and loaded on a discontinuous polyacrylamide gel (3% stacking and 10% separating gel). After blotting, the band corresponding to the ER was detected by incubating the nitrocellulose filter with the H_{222} specific antibody (2 μg/ml) followed by the peroxidase-conjugated anti-IgG secondary antibody (Vector Laboratories). The peroxidase-dependent colorimetric reaction was performed using the Vectastain peroxidase kit (Vector Laboratories).

Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. One day before transfection, 2–3 $\times 10^6$ cells were seeded in 10-cm^2 dishes in RPMI medium without phenol red supplemented with 10% DCC serum. The medium was replaced by Dulbecco modified Eagle medium (DMEM; GIBCO) 3 h prior to addition of the calcium phosphate/DNA coprecipitate. The reporter plasmid utilized contains the sequence −331/−87 of the *Xenopus* vitellogenin A_2 gene, upstream of the herpes simplex virus thymidine kinase (*tk*) promoter, fused to the CAT gene (15). The transfection was performed using 5 μg of the reporter plasmid. Sixteen hours after addition of the precipitate, the cells were rinsed and shocked for 2 min with 15% glycerol in phosphate-buffered saline (PBS). After a few washes with RPMI medium, cells were cultured in 10 ml of RPMI medium containing 10% DCC serum in the presence or absence of 17β-estradiol (0.01 μM). After 48 h, cells were trypsinized and suspended in 1 ml of TEN buffer (60 mM NaCl/40 mM Tris·HCl/1 mM EDTA, pH

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Abbreviations: ER, estrogen receptor; CAT, chloramphenicol acetyltransferase; EIA, enzymatic immunoassay; PR, progesterone receptor.

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7.5, at 4°C). Lysates from the cells were obtained by three cycles of freezing and thawing followed by centrifugation (12,000 × g, 5 min at 4°C) in an Eppendorf centrifuge. The supernatants were analyzed for CAT activity. Three hundred micrograms of protein extract was generally utilized to obtain <50% conversion of the substrate and to remain in the linear range of the CAT assay (16).

CAT activity was determined by incubating the enzyme extract in the presence of 2 μl of D-threo[*dichloroacetyl*-1,2-^{14}C]chloramphenicol per ml (25 μCi/ml; 1 Ci = 37 GBq; Amersham), 10 mM acetyl coenzyme A, and 40 mM Tris·HCl at pH 7.5 for 2 h at 37°C. The incubation was terminated by addition of ethyl acetate. The acetylated products, extracted by the organic solvent, were separated by thin-layer chromatography and then examined by autoradiography. The spots corresponding to the acetylated compounds were scraped, dissolved in Atomlight (New England Nuclear/DuPont), and assayed for radioactivity using a Packard Tricarb 1600 scintillation-β counter. The activity of the enzyme was determined by calculating the percentage of substrate converted into the acetylated form.

Cell Count and [^{3}H]Thymidine Incorporation. Cells (1.5 × 10^4) were plated in triplicate in 25-cm^2 flasks. Cell number and viability in the presence or absence of 17β-estradiol (1 nM) were evaluated using 0.4% trypan blue stain (Sigma) according to the technical instructions.

[^{3}H]Thymidine incorporation was estimated by plating 10^4 cells in a six-well dish. The cells were grown for 12 days in the presence of the indicated hormones. [^{3}H]Thymidine (Amersham; 1.0 μCi/ml of medium) was added 3 h before harvesting the cells. Cells were lysed and assayed for radioactivity by addition of scintillation fluid.

Immunocytochemistry and Morphometric Analysis. SK-ER3 cells were grown on coverslips pretreated with 5% gelatin in the presence or absence of 17β-estradiol (1 nM). Cells were then fixed at room temperature with 4% paraformaldehyde in 200 mM borate buffer for 10 min. After a few rinses the cells were incubated for 5 min in the presence of 2% horse serum and 0.1% Triton X-100 in PBS. Anti-τ (ICN) and anti-synaptophysin (Boehringer Mannheim) at 1:100 and 1:5 dilutions, respectively, were applied and incubated overnight at 4°C in a humidified chamber. After several rinses in PBS, cells were incubated with a 1:100 dilution of a fluorescein-conjugated horse anti-mouse secondary antibody (Vector Laboratories) for 2 h at room temperature. After several washes, coverslips were mounted onto slides using Perma-Fluor (Helena Laboratories).

Mean neuritic length of individual neurons was quantified using an automatic image analyzer equipped with IMAGE software developed and kindly provided by Wayne Rosband (National Institutes of Health, Bethesda, MD). A total of 60 cells was analyzed for each experimental group. Statistical analysis was performed using ANOVA. $P \leq 0.05$ was taken as the level of statistical significance.

RESULTS

Gene Transfer and ER Expression. The human ER-containing plasmid pHERT-3 (12) and pSV2-neo (17) were transfected into SK-N-BE cells and colonies were selected for resistance to the neomycin analogue G-418. To avoid the presence of estrogens, all steps were carried out in medium containing charcoal-stripped serum and free of phenol red (because of its well-described estrogen-like activity) (18). The pools of neomycin-resistant colonies were tested for the expression of the receptor by EIA to select the transfectants expressing the ER at levels compatible with a physiological activity. It was determined that in the pool SK-ER3 the level of expression of the transgene was about 400 fmol/μg of cytosolic protein. This concentration is comparable to the levels found in target organs (19) and in other cell lines of tumor origin expressing this steroid receptor (20).

Western analysis showed that the expressed receptor had the expected size (67 kDa) (Fig. 1 *Left*) and there was no evidence of proteolytic fragments even after a prolonged colorimetric reaction (data not shown).

Biological Activity of the Human ER in SK-ER3 Cells. To confirm that the transfected human ER displayed the biological activity of the wild-type ER, its ability to activate an estrogen-responsive target gene in a hormone-dependent manner was analyzed. A reporter gene containing the estrogen response element (see *Materials and Methods*) was introduced into the SK-ER3 cells or into the parental cell line. A marked increase in CAT activity was observed in the transfected cells only when 17β-estradiol was added to the culture medium. As shown in Fig. 1 *Right*, 0.01 μM 17β-estradiol was capable of generating a 5-fold induction of CAT activity in the transfected SK-ER3 cells but not in the parental SK-N-BE line.

Since a similar level of induction is observed in other cell lines expressing ER (21), we concluded that, as expected, the transfected receptor was biologically active.

Estrogen-Mediated Blockade of Cell Proliferation. As is well known, estrogens control the proliferation rate of several cell types (22, 23). Therefore, we analyzed the effects of 17β-estradiol on cell number and thymidine incorporation in SK-ER3. Prior to performing the experiments, we attempted to synchronize the cells by culturing them in a medium containing low DCC serum (0.4%) for 48 h. Complete medium (10% DCC serum) was then added. Twenty-four hours later, the cells were incubated in the presence of 17β-estradiol (1 nM) or vehicle. A significant growth arrest was noticeable after 5 days of continuous presence of the hormone (Fig. 2). Over the following days, the cell number of this experimental group remained constant. At the 12th day in culture, 15 times fewer cells were found in the presence of estrogens (0.9×10^6 versus 13.9×10^6 cells). This arrest did not appear to be due to a toxic effect of the hormone because no obvious sign of cell death could be detected in the culture.

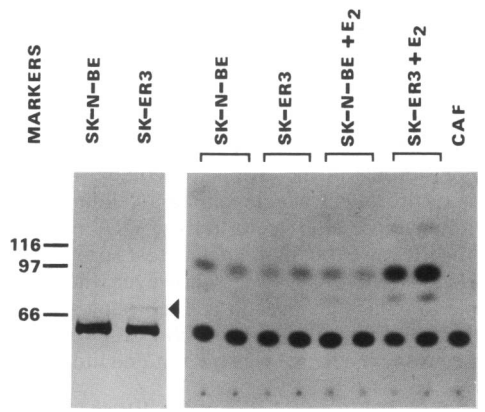

FIG. 1. ER analysis in SK-ER3 cells. (*Left*) Western analysis with anti-ER monoclonal antibody (H_{222}). Equal amounts of proteins from parental (SK-N-BE) and transfected (SK-ER3) cells were separated by SDS/PAGE, blotted, and immunostained. The band at 67 kDa visible in SK-ER3 extracts (arrowhead) corresponds to ER. The band at about 50 kDa is due to the recognition, by the secondary antibody, of the immunoglobulins used to precipitate the ER. The molecular mass standards are β-galactosidase (116 kDa), phosphorylase B (97 kDa), and bovine serum albumin (66 kDa). (*Right*) Autoradiograms of standard thin-layer chromatographic assay of CAT activity. Each CAT assay was performed with 300 μg of proteins from SK-N-BE and SK-ER3 cells treated with vehicle or with the hormone. The lower spot corresponds to nonacetylated substrate. The induction factors of cells treated with 17β-estradiol (+ E_2) were 1 and 5 ± 0.3 for SK-N-BE and SK-ER3, respectively. CAF, chloramphenicol incubated in the absence of enzyme extracts.

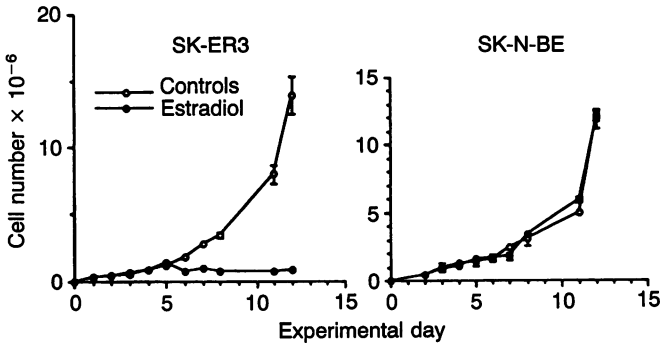

FIG. 2. Effect of 17β-estradiol on SK-N-BE and SK-ER3 growth rate. Cells were plated as described in the text and treated with 17β-estradiol (1 nM, final concentration). At the indicated times, cells from three different flasks were counted by trypan blue exclusion (0.4%) in a Burker chamber. Data represent the mean ± SD of three separate determinations.

Further analysis by trypan blue exclusion and [^{3}H]thymidine incorporation led us to conclude that the effect of 17β-estradiol was to inhibit cell proliferation. Indeed, as shown in Table 1, [^{3}H]thymidine incorporation was drastically reduced after 12 days in the continuous presence of estrogens (195 cpm/μg of protein versus 2209 cpm/μg of protein). The estrogen-induced growth arrest and decrease in [^{3}H]thymidine incorporation could be prevented when the specific ER antagonist LY 117018 (0.1 μM) was added to the culture medium at the same time as 17β-estradiol (2600 cpm/μg of protein). The estrogen antagonist by itself did not have any measurable effect (2219 cpm/μg of protein).

Analysis of SK-ER3 Morphology. The most striking effect of the addition of the hormone, however, was on cell morphology. Untreated SK-ER3 cells had a flat perikaryon with very short processes (Fig. 3, SK-N-BE). Once treated with the hormone for a prolonged period of time, these cells assumed a morphology reminiscent of neurons. The cell body decreased in size and became more refractive, and long neurite-like processes were formed. Such an effect was linked to the presence of the transfected ER, as no change in morphology was noticeable after hormonal treatment in the parental cell line (Fig. 3, SK-N-BE + E_2). As shown in Fig. 3 (SK-ER3), the mere presence of ER was sufficient to cause some morphological modification. This may have been due to the presence of trace amounts of steroids in the stripped medium. To confirm the specificity of this effect, we generated a cell line stably transfected with an expression vector for the PR. In these cells the levels of the PR were 280 fmol/μg of cytosol protein. As shown in Fig. 3 (SK-N-BE PR + Prog.), however, the activated PR was unable to induce changes in cell morphology.

The effect of different doses of 17β-estradiol on the length of the processes of SK-ER3 was quantitated utilizing an image analyzer. After 12 days in culture, the mean length (±SEM) of all the processes per cell was 8.9 ± 0.14 μm in SK-ER3, whereas in cells treated with 1 nM and 0.01 nM 17β-estradiol it was 17.6 ± 0.3 μm and 11.3 ± 0.3 μm, respectively. In cells exposed to 17β-estradiol (1 nM) plus the estrogen antagonist LY 117018 (0.1 μM), this value was considerably reduced, 6.7 ± 0.12 μm. The antagonistic activity of LY 117018 is also apparent in Fig. 3 (SK-ER3 + E_2 + Ly).

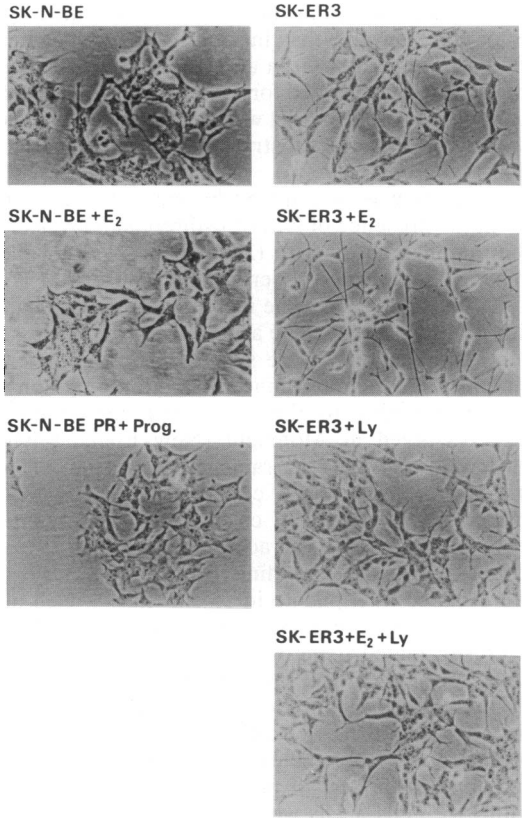

FIG. 3. Estrogen and estrogen antagonist effect on the morphology of parental SK-N-BE and ER- (SK-ER3) expressing cells. Cells were plated as described in the text. Before performing the morphological analysis, cells were incubated for 12 days in the presence of vehicle (SK-N-BE, SK-ER3), 1 nM 17β-estradiol (SK-N-BE + ER_2, SK-ER3 + E_2), 0.1 μM estrogen antagonist (SK-ER3 + Ly), 0.1 μM estrogen antagonist plus 17β-estradiol (SK-ER3 + E_2 + Ly), or 1 nM progesterone (SK-N-BE PR + Prog.). (×130; Nikon microscope.)

On the basis of the results presented above, we concluded that the changes in SK-ER3 morphology were specifically due to the presence of activated ER.

Antigenic Properties of SK-ER3 Cells. Several experimental manipulations of the cell culture medium (e.g., exposure to

Table 1. Effect of 17β-estradiol on SK-N-BE and SK-ER3 growth rate and estrogen antagonist on cell number and thymidine incorporation

Treatment	SK-ER3		SK-N-BE	
	Cell number × 10^{-6}	[^{3}H]Thymidine incorporation, cpm/μg of protein	Cell number × 10^{-6}	[^{3}H]Thymidine incorporation, cpm/μg of protein
Vehicle	4.7 ± 0.14	2209 ± 120	4.9 ± 0.01	—
17β-Estradiol	0.03 ± 0.006*	195 ± 86*	4.7 ± 0.14	2209 ± 120
LY 117018	5.3 ± 0.11	2219 ± 235	4.8 ± 0.01	—
17β-Estradiol + LY 117018	3.1 ± 0.02	2600 ± 831	4.9 ± 0.02	—

For analysis of the growth rate, cells were plated as described in the text. Synchronized cells were treated with 17β-estradiol (1 nM, final concentration). The experiment was performed twice. For analysis of estrogen and estrogen antagonist effects on SK-ER3 growth, 17β-estradiol (1 nM) and LY 117018 (0.1 μM) were added to the cells. The [^{3}H]thymidine incorporation assay was performed in triplicate on cells grown for 12 days in the presence or absence of the indicated hormone or anti-hormone. The experiment was performed twice. Data represent the mean ± SD of six separate determinations.

*$P \leq 0.001$ versus vehicle-treated SK-ER3.

low serum, phorbol esters, dibutyryl cAMP, etc.) have been shown to induce morphological differentiation of neuroblastoma cells (24). However, these changes are not always associated with the acquisition of neuronal-specific antigenic properties (24). A series of immunocytochemical studies were carried out to determine whether the 17β-estradiol-dependent morphological modifications were correlated with the expression of key proteins in the differentiation of neuronal cells. In particular, we studied the expression of two proteins, τ and synaptophysin, known to be present in outgrowing axons and synaptic vesicles, respectively (25, 26). After 12 days in culture in the presence of the hormone, τ and synaptophysin were expressed (Fig. 4). As expected, synaptophysin had a punctate appearance, whereas τ was mainly associated with the cytoskeletal components of the cells.

Estrogen as a Primer in SK-ER3 Differentiation. ER effects appear to be mediated by a series of proteins whose mRNAs can be detected as early as 30 min after the administration of the hormone (27–30). Some reports suggest that once these mRNAs are induced the ER is no longer required. We therefore tested whether the morphological changes in SK-ER3 cells required continuous exposure to 17β-estradiol. This was tested with a pulse experiment. The SK-ER3 cells were incubated in the presence of 1 nM 17β-estradiol for different lengths of time (Fig. 5) and then grown until day 12 in the absence of the hormone. We found that a 16-h exposure time was sufficient to lead to the same morphological differentiation as that observed in the continuous presence of the hormone. A slight change in morphology could also be detected with an exposure time as short as 30 min (Fig. 5).

This effect was blocked by α-amanitin at a concentration known to inhibit RNA polymerase II activity (2 μg/ml) (data not shown).

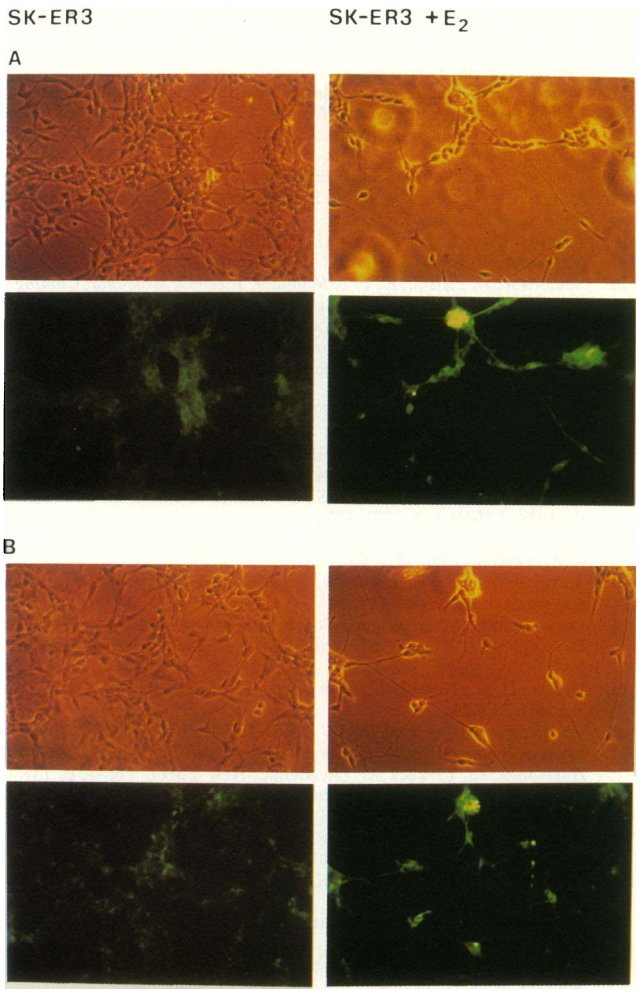

FIG. 4. τ and synaptophysin immunoreactivity in SK-ER3 cells treated with 17β-estradiol. (*A Upper*) Phase-contrast pictures of cells grown for 12 days in the presence of vehicle or 1 nM 17β-estradiol. (*A Lower*) Immunofluorescence pictures showing τ expression in the neuritic processes of the 17β-estradiol-treated SK-ER3 cells. (*B Upper*) Phase-contrast. (*B Lower*) Fluorescence pictures showing synaptophysin immunoreactivity. A dotted labeling scattered in the cytoplasm and in the neurites is clearly visible in the 17β-estradiol-treated cells. (×200.)

DISCUSSION

The goal of the present study was to generate an *in vitro* system that allows the biological functions of estrogens on the nervous system to be studied. Previous reports have indicated that the acquisition of a specific steroid receptor is a condition that is both necessary and sufficient for cell responsiveness to a hormone (9). Therefore, a neuroblastoma cell line expressing the human ER was produced. Care was taken to generate a cell line in which expression of the receptor was at levels considered optimal for its physiological activity, since the possibility of toxic effects of 17β-estradiol in cells expressing supraphysiological levels of the cognate receptor has been reported (16). On the other hand, some authors have ascribed a lack of hormone-responsiveness to low levels of expression of the transfected receptor gene (31, 32). In the SK-ER3 neuroblastoma cells, the levels of receptor were comparable to those measured in other tumor-derived estrogen-responsive cell lines (20).

The results presented here indicate that activation of the transfected receptor causes a growth arrest of the neuroblastoma cells. This finding might be considered surprising as estrogens are generally thought to induce cell proliferation. This has been shown *in vivo* in target organs (e.g., uterus) as well as *in vitro* in cells of tumoral origin derived from tissues expressing the hormone receptor (e.g., mammary gland and pituitary) (22, 23). Our results, however, are in agreement with the data reported by Rasmussen *et al.* (33), which demonstrated that estrogens act as an antimitotic agent in simian virus 40-transformed hypothalamic cell lines. Our study further extends this observation, describing the involvement of 17β-estradiol in the morphological differentiation of the transfected cells.

A question could be raised as to whether or not growth arrest and morphological differentiation are independent events. To try to answer this question, SK-ER3 cells were incubated for 24 h with the mitotic inhibitor 1-β-D-arabinofuranosylcytosine (50 μM). The surviving cells (16%) exhibited growth arrest without showing changes in cell morphology, indicating that the blockade of cell proliferation is not in itself sufficient to elicit differentiation of the cells (data not shown).

During maturation of the nervous system, changes in the antigenic properties of neuroblasts have been described and the appearance of specific proteins has been correlated with a particular stage of nerve cell differentiation. The expression of τ and synaptophysin in the estrogen-treated SK-ER3 cells indicates that these cells had entered a developmental program leading toward neuronal differentiation. The effect of 17β-estradiol on τ expression in SK-ER3 cells reproduces that described by Ferreira and Caceres (5) in dissociated cell cultures from fetal rat hypothalamus.

Consideration of all of the effects observed suggests that the system generated may be a valuable tool in investigating the involvement of the ER in the proliferation and differentiation of nervous cells. How the described effects are elicited by the hormone remains to be established. Several reports

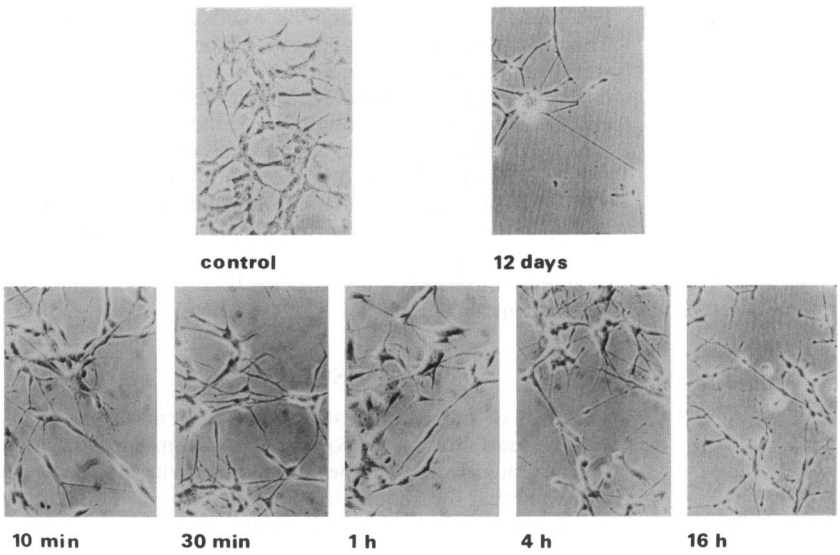

FIG. 5. Effect of pulse exposure to 17β-estradiol on SK-ER3 morphology. Following synchronization in RPMI medium containing 0.4% DCC-stripped serum, the cells were switched to RPMI medium containing 10% DCC-stripped serum plus 1 nM 17β-estradiol or vehicle. At the indicated times, the 17β-estradiol-containing medium was replaced with RPMI medium supplemented with 10% DCC-stripped serum. After 12 days the cells were photographed and counted by trypan blue exclusion. The cell numbers ($\times 10^6$) were as follows: controls, 4.7 ± 0.14; 17β-estradiol for 12 days, 0.026 ± 0.006; for 10 min, 2.2 ± 0.26; for 30 min, 1.9 ± 0.22; for 1 h, 1.6 ± 0.05; for 4 h, 1.9 ± 0.11; and for 16 h, 0.025 ± 0.005. (×130 Nikon microscope.)

have indicated that cooperation among hormones and growth factors plays an important role in neuronal differentiation (34, 35). In our study, the results of transient treatment with 17β-estradiol would argue in favor of 17β-estradiol acting as the initial step of a series of events leading to differentiation. Subsequent steps could be the result of responses to other molecules present in the culture medium or possibly the result of an ER-activated genetic cascade. Candidates for these cooperating activities are growth factors (e.g., insulin growth factors). Synthesis of these molecules as well as expression of their receptors have been described in neuroblastoma cell lines (36). In addition, other studies in organotypic cultures derived from fetal rat hypothalamus have demonstrated that interaction between estrogens and growth factors induces outgrowth of neurites (37). Here we demonstrate that estrogens may influence not only the morphological characteristics of neuronally derived cells but also their antigenic properties and growth pattern.

Finally, the growth arrest and differentiation elicited by estrogens reported herein might suggest new therapeutic approaches for the treatment of specific cancers. Therapies based on the utilization of differentiating agents for cancer treatment are currently exploiting the effects of retinoids on the hemopoietic cells [e.g., for the treatment of promyelocytic leukemia (38)]. ER-positive neuroblastomas (39) may therefore represent a potential target for estrogen therapy.

We are indebted to G. Greene for his generous gift of the ER and PR expression vectors and to E. Boncinelli, S. Denis-Donini, N. Weigle, and T. Hayes for critically discussing the results here presented. The invaluable help of David McEnzie in restyling the manuscript is greatly appreciated. This work was supported by the European Economic Community (BRIDGE Programme T-Project Animal Cell Biology BIOT-CT92-0308), by the Italian National Council for Research (P. F. Ingegneria genetica e basi molecolari delle malattie ereditarie, P. F. Biotecnologie e bioinstrumentazione to A.M.), and by the Hereditary Disease Foundation, the European Science Foundation (Twinning Grant 9114), and a North Atlantic Treaty Organization Collaborative Research Grant (to E.C.).

1. Faivrebauman, A., Rosenbau, E., Pujmirat, J., Grousell, D. & Tixiervidal, A. (1981) *Dev. Neurosci.* **4**, 118–119.
2. Gahr, M. & Konishi, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7380–7383.
3. Rasmussen, J. E., Torres-Aleman, I., MacLusky, N. J., Naftolin, F. & Robbins, R. J. (1990) *Endocrinology* **126**, 235–240.
4. Toran-Allerand, D. (1976) *Brain Res.* **106**, 407–412.
5. Ferreira, A. & Caceres, A. (1991) *J. Neurosci.* **11**, 392–400.
6. Uchibori, M. & Kawashimi, A. S. (1985) *Int. J. Dev. Neurosci.* **3**, 381–388.
7. Frankfurt, M., Gould, E., Woolley, C. S. & McEwen, B. S. (1990) *Neuroendocrinology* **51**, 530–535.
8. Arai, Y. & Matsumoto, A. (1978) *Psychoneuroendocrinology* **3**, 31–45.
9. Strahle, U., Boshart, M., Klock, G., Stewart, F. & Schutz, G. (1989) *Nature (London)* **339**, 629–632.
10. Biedler, L., Roffler-Tarlov, S., Schachner, M. & Freedman, L. S. (1978) *Cancer Res.* **38**, 3751–3757.
11. Vegeto, E., Cocciolo, M. G., Raspagliesi, F., Piffanelli, A., Fontanelli, R. & Maggi, A. (1990) *Cancer Res.* **50**, 5291–5295.
12. Kushner, P. J., Hort, E., Shine, J., Baxter, J. D. & Greene, G. L. (1990) *Mol. Endocrinol.* **4**, 1465–1473.
13. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–259.
14. Maggi, A., Susanna, L., Bettini, E., Mantero, G. & Zucchi, I. (1989) *Mol. Endocrinol.* **3**, 2505–2510.
15. Klein-Hitpass, L., Ryffel, G. U., Heitlinger, E. & Cato, C. B. (1988) *Nucleic Acids Res.* **16**, 647–662.
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY), 2nd Ed.
17. Wermette-Hammond, M. E., Laurer, S. J., Corsini, A., Walker, D. & Taylor, J. M. (1989) *J. Biol. Chem.* **264**, 9094–9101.
18. Berthois, Y. & Katzenellenbogen, B. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2496–2500.
19. Clark, J. H. & Peck, E. J. (1979) *Female Sex Steroids: Receptors and Function* (Springer, New York).
20. Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M. & McGuire, W. L. (1978) *Cancer Res.* **38**, 2434–2437.
21. Tora, L., Gaub, M. P., Mader, S., Dierich, A., Bellard, M. & Chambon, P. (1988) *EMBO J.* **7**, 3771–3778.
22. Kiss, R., de Launoit, Y., Danguy, A., Paridaens, R. & Pasteels, J.-L. (1989) *Oncology* **46**, 391–399.
23. Sorrentino, J. M., Kirkland, W. L. & Sirbasku, D. A. (1976) *J. Natl. Cancer Inst.* **56**, 1149–1153.
24. Denis-Donini, S. & Augusti-Tocco, G. (1980) in *Current Topics in Developmental Biology*, ed. Moscona, A. & Hunt, R. (Academic, New York), Vol. 16, pp. 323–348.
25. Drubin, D. G., Kobayashi, S. & Kirschner, M. (1986) *Ann. N.Y. Acad. Sci.* **466**, 257–268.
26. Wiedenmann, B. & Franke, W. (1985) *Cell* **41**, 1017–1028.
27. Katzenellenbogen, B. S. & Gorski, J. (1975) in *Biochemical Action of Hormones*, ed. Litwack, G. (Academic, New York), Vol. 3, p. 187.
28. Loose-Mitchell, D. S., Chiappetta, C. & Stancel, G. (1988) *Mol. Endocrinol.* **2**, 946–951.
29. Weisz, A. & Bresciani, F. (1988) *Mol. Endocrinol.* **2**, 816–824.
30. Cattaneo, E. & Maggi, A. (1990) *Eur. J. Pharmacol.* **188**, 153–159.
31. Watts, C. K., Parker, M. G. & King, R. J. B. (1989) *J. Steroid Biochem.* **34**, 483–490.
32. Touitou, I., Mathieu, M. & Rocheford, H. (1990) *Biochem. Biophys. Res. Commun.* **169**, 109–115.
33. Rasmussen, J. E., Torres-Aleman, I., MacLusky, N. J., Naftolin, F. & Robbins, R. J. (1990) *Endocrinology* **126**, 235–240.
34. Cattaneo, E. & McKay, R. D. G. (1990) *Nature (London)* **347**, 762–765.
35. Birren, S. J. & Anderson, D. J. (1990) *Neuron* **4**, 189–201.
36. Martin, D. M., Yee, D., Carlson, R. O. & Feldman, E. L. (1992) *Mol. Brain Res.* **15**, 241–246.
37. Toran-Allerand, C. D., Ellis, L. & Pfenninger, K. H. (1988) *Dev. Brain Res.* **41**, 87–100.
38. Cheson, B. D. (1992) *N. Engl. J. Med.* **327**, 422–424.
39. Fujimoto, M., Yoshino, E., Hirakawa, K., Fujimoto, J. & Tamaja, T. (1984) *Clin. Neuropharmacol.* **7**, 357–362.