Journal of Neurochemistry
Raven Press, Ltd., New York

Estrogen Receptor Is Expressed in Different Types of Glial Cells in Culture

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Abstract: Estrogens derived from the aromatization of androgens are believed to be responsible for the induction of the sexual differentiation of the CNS interacting with specific estrogen receptors (ER) present in developing neurons. However, the brain cellular distribution of ER is not so well documented. The aim of this study was to investigate the qualitative and quantitative expression of ER mRNA in well characterized cultures of rat type 1 and type 2 astrocytes and of oligodendrocytes by polymerase chain reaction. A series of amplifications with a set of primers spanning along the entire ER mRNA was utilized in the different types of glial cells, in a positive control (uterus), and in a negative control (SK-N-BE cell line) previously shown to be devoid of ER. The data obtained show that ER mRNA is expressed in all three types of glial cell analyzed in almost equal amounts, which are 25–50 times lower than those in the uterus. The mRNA expressed in the glia is homologous with that expressed in the uterine tissue. **Key Words:** Estrogen receptor—Glial cell—Astrocytes—Oligodendrocytes.
J. Neurochem. **63,** 2058–2064 (1994).

The CNS is a well known target for the activity of sex steroids. A series of studies, in fact, has demonstrated that both estrogens and androgens participate in the induction of the sexual differentiation of the brain towards a masculine pattern. Estrogens are believed to be particularly responsible for this effect, because they are synthesized locally by a specific aromatase, which converts into estrogens the androgens secreted by the testis in the fetus and in the neonate. In addition, a series of studies carried out in rodents have demonstrated that, at an early time during the development, estrogen receptors (ER) are expressed in numerous brain areas. Interestingly, in selected brain regions (e.g., hippocampus and cortex) the number of ER increases during the maturation of the embryo, reaches the highest concentration at birth, and then rapidly starts to decrease to attain the low levels typical of adulthood. In other brain nuclei, the high levels of ER reached during brain maturation are maintained for the entire life span of the animal. These observations, together with a series of more recent studies on the effect of estrogens on nervous cells in culture, suggest a direct effect of estrogens on the differentiation of brain cells (Ma et al., 1993).

To understand better the role exerted by estrogens in the development of the brain, it is important to define which cells of the CNS express its cognate receptor. It is well known that throughout brain development neurons express ER, as well as the enzyme aromatase, which is responsible for the synthesis of estrogens from testosterone. In contrast, glial cells appear to be devoid of aromatase activity (Negri Cesi et al., 1992) in spite of the fact that in vivo (Luquin et al., 1993) and in vitro (Garcia-Segura et al., 1989; Jung-Testas et al., 1991, 1992*a*) studies have demonstrated that these cells respond to estrogens. For instance, it has been reported that estrogens added to glial cultures increase cell proliferation (Jung-Testas et al., 1992*a*), produce alterations of astrocyte and oligodendrocyte morphology (Garcia-Segura et al., 1989; Jung-Testas et al., 1992*a*), affect the expression of specific cellular proteins, such as the myelin basic protein (MBP) in oligodendrocytes and the glial fibrillary acidic protein (GFAP) in astrocytes (Jung-Testas et al., 1992*a*), and induce progesterone receptors in mixed glial cell cultures (Jung-Testas et al., 1991). Moreover, preliminary studies performed by [^{3}H]tamoxifen binding in rat mixed glial cells in culture (Jung-Testas et al., 1992*a*) and by immunocytochemistry (Langub and Watson, 1992) on sections of the brain of adult guinea pig seem to suggest that ER can be synthesized in glial cells.

Received February 15, 1994; revised manuscript received April 29, 1994; accepted May 2, 1994.

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Abbreviations used: DIV, days in vitro; DMEM, Dulbecco's modified Eagle medium; ER, estrogen receptor(s); GFAP, glial fibrillary acidic protein; MBP, myelin basic protein.

The aim of this study was to investigate the expression of ER mRNA in well characterized cultures of rat type 1 and type 2 astrocytes and oligodendrocytes. The qualitative and quantitative determinations of ER mRNA were performed utilizing competitive PCR, a method previously demonstrated to be very sensitive and highly reliable for assessing the expression of ER mRNA in the hippocampus and the hypothalamus dissected from the female rat brain (Bettini et al., 1992; Santagati et al., 1993).

MATERIALS AND METHODS

Animals

The animals (Sprague–Dawley rats; Charles River, Calco, Italy) were housed in an environmentally controlled room (lights on from 6:00 a.m. to 8:00 p.m., temperature 20°C).

Cell cultures

Cell cultures of oligodendrocytes and type 1 and 2 astrocytes were obtained from the brains of neonatal rats as previously described.

Type 1 astrocytes. Primary cultures of mixed glia were obtained from the cerebral cortex of 1–2-day-old rats, utilizing the method of McCarthy and de Vellis (1980), with slight modifications (Melcangi et al., 1993). The cells were plated at high density (20×10^6 cells) in 75-cm^2 dishes and grown for 2 weeks. The bedlayer, which consists of type 1 astrocytes, was separated by shaking. The separated type 1 astrocytes were resuspended with trypsin and replated in Dulbecco's modified Eagle medium (DMEM)/10% fetal calf serum (FCS) in 6-cm petri dishes at a density of about 1.5×10^6 cells/dish.

Type 2 astrocytes. Primary cultures of mixed glia were grown for 2 weeks, and then the cells of the top layer were collected by gentle washing (Aloisi et al., 1988). For further purification, these cells were filtered through a 25-μm nylon mesh and further purified by incubation in a flask placed in a CO_2 incubator (37°C, 95% air/5% CO_2) for half an hour. The cells that detached following gentle shaking were seeded at low density (2×10^4 cells/cm^2) into petri dishes precoated with poly-L-lysine (10 μg/ml). The subcultures were maintained under the same conditions as the primary cultures (DMEM supplemented with 10% heat-inactivated FCS, with 50 U/ml penicillin and 50 μg/ml streptomycin). The cultures were assayed for the presence of ER mRNA 5 days later.

Oligodendrocytes. Primary cultures of mixed glia were obtained as before from whole brain, but calf serum (10%) was used instead of FCS (Besnard et al., 1989). The cellular suspension was diluted with the culture medium up to a ratio of 30 ml/brain; 10 ml of these fractions was dispensed in 10-cm petri dishes. After about 2 weeks, the cells were dislodged, filtered, and concentrated as previously described for type 2 astrocytes. After 1 h of incubation in a flask placed in a CO_2 incubator (37°C, 95% air/5% CO_2), the cells were collected as previously described and seeded at a high density (5×10^4 cells/cm^2) into petri dishes precoated with poly-L-lysine (10 μg/ml). After 24 h, the medium containing serum was removed, and a chemically defined medium was used (5 μg/ml insulin and 10 μg/ml transferrin in DMEM with 50 units/ml penicillin and 50 μg/ml streptomycin at pH 7.2). This medium was changed every 2 days; cells were grown for 5 days and then collected for mRNA preparation.

Validation of cell cultures by immunocytochemistry

Type 1 and type 2 astrocytes were labeled using a monoclonal antibody raised against GFAP (anti-GFAP), diluted 1:400 (BioMakor, Rehovot, Israel), utilizing the following procedure: the cells were washed three times with DMEM/20m*M* HEPES at pH 7.2–7.4 supplemented with 15% horse serum (Biochrom K. G., Berlin, Germany), and fixed with 5% acetic acid in 95% ethanol for 12 min at −20°C. The cells were washed three times and incubated for 90 min at room temperature in DMEM/HEPES with anti-GFAP. The cells were then washed with DMEM/HEPES and incubated for 60 min with anti-mouse fluorescein isothiocyanate-labeled IgG (Boehringer Mannheim, Milan, Italy) diluted 1:20. After two washings with DMEM/HEPES and two with phosphate-buffered saline, the cells were mounted using 87% glycerol in phosphate-buffered saline. Pictures were taken using a photo microscope Zeiss III with epicondenser (light to xenon, excitation filter BP 485/20, barrier filter BP 520–560, suppressing red filter BG 38, heat filter KG 1).

Type 2 astrocytes were labeled using the A2B5 monoclonal antibody (1:2); the same procedure described above was adopted with the difference that the cultures were fixed with 4% paraformaldehyde for 10 min at room temperature before the addition of the antibody. The A2B5 monoclonal antibody was a gift from Dr. G. Levi (Istituto Superiore di Sanità, Rome, Italy).

Oligodendrocytes were labeled using monoclonal antibody raised against MBP (anti-MBP; Boehringer Mannheim, Milan, Italy) diluted 1:20, following the procedure previously described for astrocytes.

RNA extraction

Total RNA was extracted from tissues (rat uterus) or cells by the 4 *M* guanidine thiocyanate method previously described (Santagati et al., 1993). Qualitative and quantitative analyses of the extracted RNA were assessed by measuring the absorbance at 260, 280, and 320 nm. All the preparations had an A_{260}/A_{280} ratio of 1.8 or higher.

Preparation of the internal standard

The pGEM3Z plasmid (Promega, Madison, WI, U.S.A.) containing the *Eco*RI fragment of the ER cDNA (Koike et al., 1987), corresponding to the 1,800 bp of the coding region, 210 nucleotides of the 5′-untranslated region, and 74 nucleotides of the 3′-untranslated region, was digested with *Not*I (Boehringer Mannheim, Mannheim, Germany) in order to delete 66 bp corresponding to nucleotides 212–278 of the receptor coding region. Following purification via agarose gel electrophoresis, the digested plasmid (pGEM3Z-ERmut) was ligated on itself. The pGEM3Z-ERmut was used as a template for transcription by the T7 polymerase according to the Promega transcription protocol. Synthesis of the RNA (standard RNA) was performed in the presence of 30 μCi of [^{3}H]UTP. The efficiency of the reaction was calculated on aliquots by scintillation counting on trichloroacetic acid-precipitable material. The template was then digested with RNase-free DNase. After phenol/chloroform extraction, the newly synthesized ^{3}H-labeled standard RNA was ethanol-precipitated, resuspended in water, and stored in aliquots at −70°C.

cDNA synthesis

Single-stranded cDNA was synthesized from total RNA isolated from rat uterus, oligodendrocytes, and type 1 and

TABLE 1. *Oligonucleotide sequences of PCR primers*

Primer	Sequence	Position	Code present in Fig. 5
1/1a	5′-TGCTCGAATGATGCAGTGGCG-3′	179	A
1b	5′-CAGAGCCTTCTCCATGGGCAT-3′	117	
3a	5′-GTTGTGCTGGGGGTTTGCCCC-3′	300	B
3b	5′-GCTGGAGAGTCTCTCTCGGCC-3′	516	
5a	5′-GCAACTCTTCCTCCGGTTCTT-3′	705	C
5b	5′-CTTAGTGTGCTTGATCACAAG-3′	921	
7a	5′-CTCCAGAAGGTGAACTTGATC-3′	1,134	D
7b	5′-CTCTTCTCCCTGCAGGTTCAT-3′	1,347	
9a	5′-GCCTTTGTTACTCATGTGCCG-3′	1,557	E
9b	5′-GTTGGGGAAGCCCTCTGCTTC-3′	1,793	

Oligonucleotides a are sense primers. Oligonucleotides b are antisense primers. Position refers to 5′ end of the oligonucleotide relative to the coding sequence of ER mRNA.

type 2 astrocytes. As a negative control, RNA was also extracted from the human neuroblastoma cell line SK-N-BE, which was shown to be devoid of ER in previous studies (Ma et al., 1993). For the cDNA synthesis, 30 μg of total RNA was digested with 10 units of RNase-free DNase (Boehringer Mannheim, Milan, Italy) for 40 min at 25°C, then extracted with phenol chloroform, and precipitated in 70% ethanol at −70°C for 1 h. The RNA was resuspended in water. For the annealing of the RNA to the oligo(dT) primer, 10 μl of the RNA solution was incubated with 2.5 μg of oligo(dT)$_{12-18}$ (Pharmacia, Milan, Italy) for 5 min at 65°C. The annealing was then carried out at room temperature for 30 min. The synthesis of the cDNA was done in a final volume of 25 μl in the presence of 40 m*M* KCl, 6 m*M* $MgCl_2$, 20 m*M* dithiothreitol, 1.2 m*M* dA,T,GTP, 50 μCi of [^{3}H]dCTP, 35 units of AMV reverse transcriptase (P. H. Stehelin, Basel, Switzerland), and 50 m*M* Tris-HCl, pH 8.2. The reaction was carried out for 60 min at 42°C. The yield of the reaction was calculated by trichloroacetic acid precipitation and scintillation counting of aliquots of the reaction mixture before and after incubation.

For the quantitative analysis, the reverse transcription of the cell mRNA and standard RNA was performed using as primers a mixture of four ER-specific oligonucleotides (Santagati et al., 1993). The primer mixture was added to 1 μg of cell mRNA and reverse-transcribed with a series of dilutions of standard RNA. Aliquots containing $\frac{1}{10}$ of the cDNA mixtures were amplified for a fixed number of cycles (25). The exact concentration of cell ER mRNA could be determined by direct comparison of cell and standard amplified cDNAs (Santagati et al., 1993).

Amplification of ER by PCR

Generally, $\frac{1}{10}$ of the cDNA reaction mixture was combined with 10 μl of 10 × PCR buffer (Promega), 200 μ*M* deoxynucleotides (Boehringer Mannheim, Mannheim, Germany), 2.5 units of *Taq* polymerase (Promega), and 50 m*M* oligonucleotide synthetic primers to a final volume of 100 μl. The sequence of the primers utilized in the various amplifications is reported in Table 1. The amplification was carried out for 30 cycles in the Hybaid DNA thermal cycler (Teddington, U.K.). The thermal profile of the DNA amplification was as follows: *denaturation:* 1 min 15 s at 94°C; *primer annealing:* 1 min 15 s at 42°C; *extension:* 2 min 30 s at 72°C. At the 30th cycle, the reaction was terminated by addition of 50 μl of DNA dye [50% (vol/vol) glycerol, 0.25% (wt/vol) bromphenol blue, and 0.25% (wt/vol) xylene cyanol]. Fifteen microliters of the incubation mixture were then loaded onto a 3% agarose gel, together with the appropriate molecular weight markers. The bands corresponding to the amplified DNA were then visualized by ethidium bromide staining. In parallel, we amplified sample where the cDNA had been omitted. No amplification products were observed in these control samples.

Quantitative analysis

For the quantitative analysis, an aliquot of the cDNA reaction mixture was amplified with primers that could recognize both standard and cell cDNA (2a: 5′-GCTCTGGGCGAGGTGTACGTG-3′; 2b: 5′-AGCCCCCAGACTATTGGACC-3′). Each PCR reaction mixture (20 μl) was subjected to electrophoresis in 3% (wt/vol) agarose gel in TBE buffer (90 m*M* Tris, 90 m*M* borate, 2 m*M* EDTA) to separate the two DNA species amplified (standard DNA: 126 bp; cell ER DNA: 192 bp). Gels were stained with ethidium bromide and photographed with Polaroid type 667 film. The photographic negatives were scanned by the AppleScanner laser densitometer (Apple Computer, Cupertino, CA, U.S.A.) and the data analyzed by AppleScan, ImageFolder, and CricketGraph programs on a Macintosh computer (Apple Computer). The linearity range of the curve linking the absorbance reading to the concentration of DNA stained with the ethidium bromide stain was determined experimentally.

Amplified DNA sequence analysis

The amplified DNA sequences were cloned into pGEM3Z plasmids following addition of appropriate linkers. The double-stranded DNA sequencing was performed by the Sanger dideoxy method (Sanger et al., 1977) using Sequenase T7 DNA polymerase (Promega) following the instructions provided by the supplier.

ER mRNA quantitative analysis

The quantitative analysis of ER mRNA was done by the competitive PCR method recently perfected in our laboratory (Santagati et al., 1993).

RESULTS

The purity of type 1 astrocytes has been evaluated at 5 days in vitro (DIV) with an anti-GFAP monoclonal antibody; as shown in Fig. 1, after shaking of the cul-

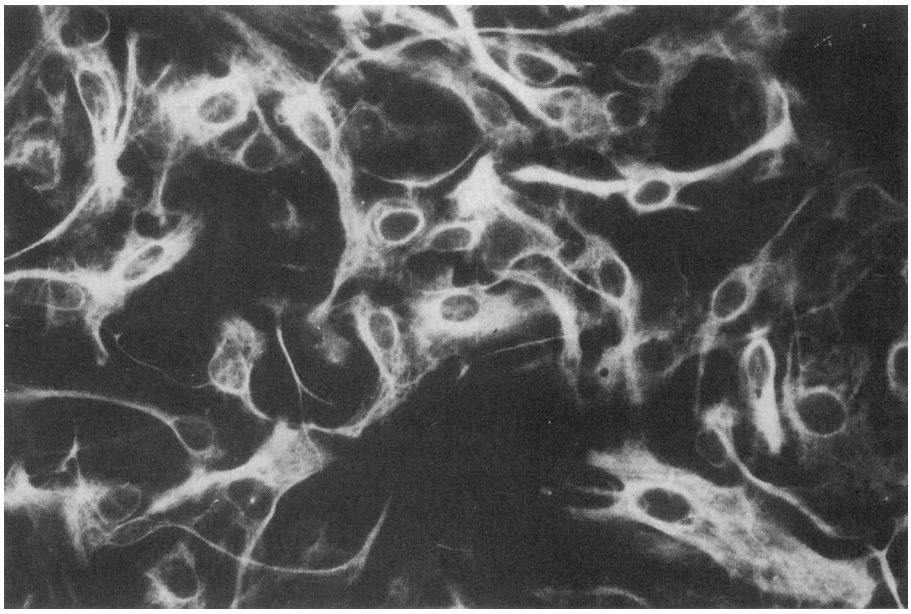

FIG. 1. Immunofluorescence photomicrograph of type 1 astrocytes at 5 DIV labeled with anti-GFAP monoclonal antibody. Magnification ×305.

tures, almost all the cells are GFAP-positive and no dark cells (usually type 2 astrocytes or oligodendrocytes) are present on the top of the cultures. When the immunostaining of cultures of type 1 astrocytes was performed with the A2B5 antibody, no cell appeared to be positive. Enriched cultures of type 2 astrocytes were tested at 5 DIV against A2B5 and GFAP antibodies; as shown in Figs. 2 and 3, almost all the cells were A2B5- and GFAP-positive. Enriched cultures of oligodendrocytes at 5 DIV were positive to an anti-MBP antibody (Fig. 4).

A series of amplifications was devised in order to demonstrate the presence of ER-like transcripts in glial cells grown in culture, as specified in Materials and Methods. To prove the specificity of the amplification procedure, a positive and a negative control were amplified in parallel with the RNA extracted from the glial cells. Tissue dissected from the rat uterus, a well known target for estrogen activity, was used as the positive control; the negative control was represented by a cell line, SK-N-BE, that previous experiments had shown to be devoid of ER protein (Ma et al., 1993). Once the presence in glial cells of ER-like mRNA sequences had been proved with a set of primers aimed at amplifying the ER cDNA coding for the hormone binding site (7a–9b), a second round of am-

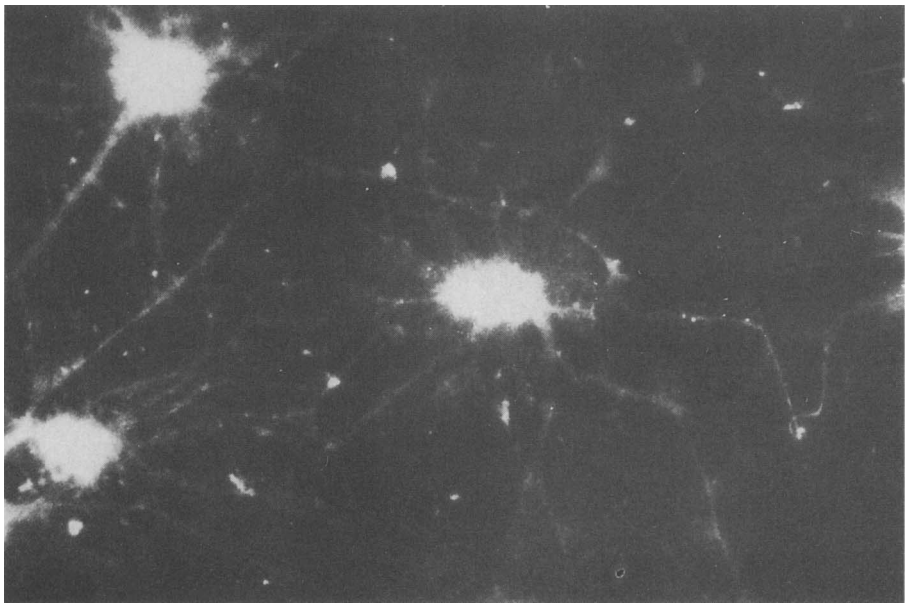

FIG. 2. Immunofluorescence photomicrograph of type 2 astrocytes at 5 DIV labeled with anti-A2B5 monoclonal antibody. Magnification ×580.

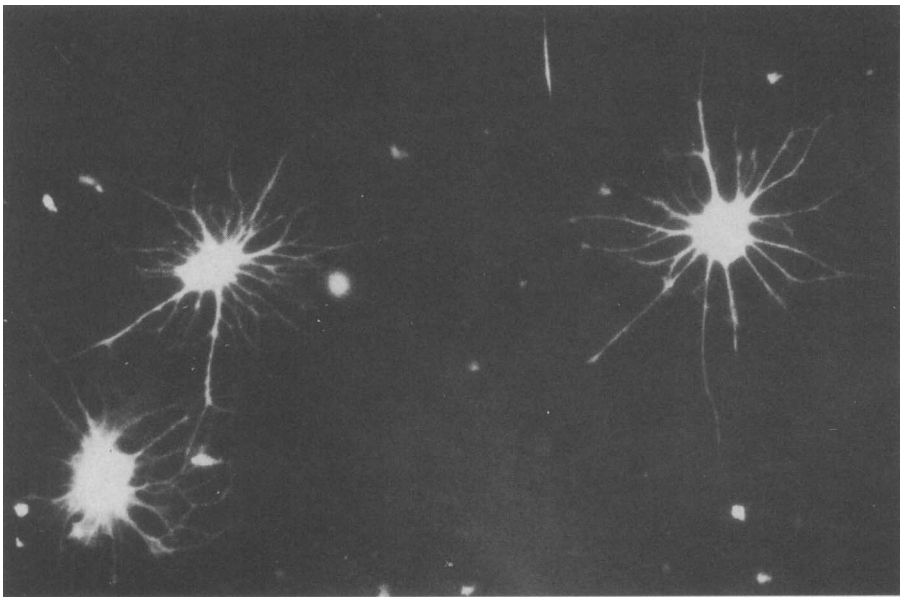

FIG. 3. Immunofluorescence photomicrograph of type 2 astrocytes at 5 DIV labeled with anti-GFAP monoclonal antibody. Magnification ×340.

plifications was performed using primers spanning the entire ER mRNA (Table 1), to demonstrate that the entire ER mRNA was expressed in the various cell preparations. In Fig. 5 are shown the products of this series of amplifications done with the following primers: A: 1a–1b, expected size 296 nucleotides; B: 3a–3b, expected size 218 nucleotides; C: 5a–5b, expected size 210 nucleotides; D: 7a–7b, expected size 213 nucleotides; and E: 9a–9b, expected size 236 nucleotides.

These experiments demonstrated the presence of ER-like cDNA in all the primary cultures examined. Furthermore, these studies did not show any sign of gross structural modification of the receptor mRNA in the preparations examined. This observation was confirmed by subsequent experiments in which much larger fragments of the ER cDNA were amplified (data not shown). With the SK-N-BE cells, a very limited amount of amplified DNA of the expected molecular weight could be observed in some of the experiments performed. The lack of reproducibility of this observation had to be attributed to the very low abundance of the ER mRNA expressed by these cells in the undifferentiated state.

To prove that the amplified products indeed represented the ER cDNA, several of the amplified cDNA fragments were cloned into pGEM3Z and sequenced.

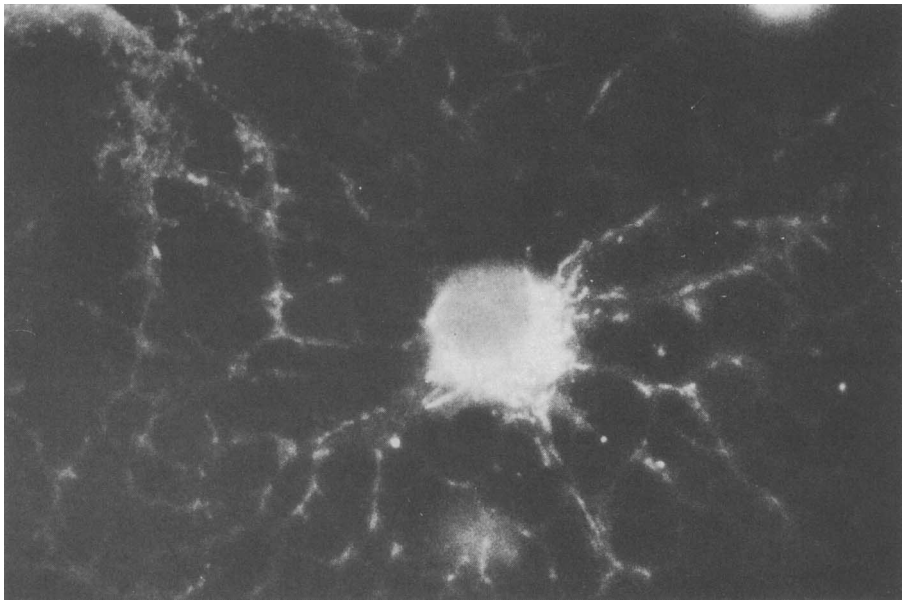

FIG. 4. Immunofluorescence photomicrograph of oligodendrocytes at 5 DIV labeled with anti-MBP monoclonal antibody. Magnification ×1,445.

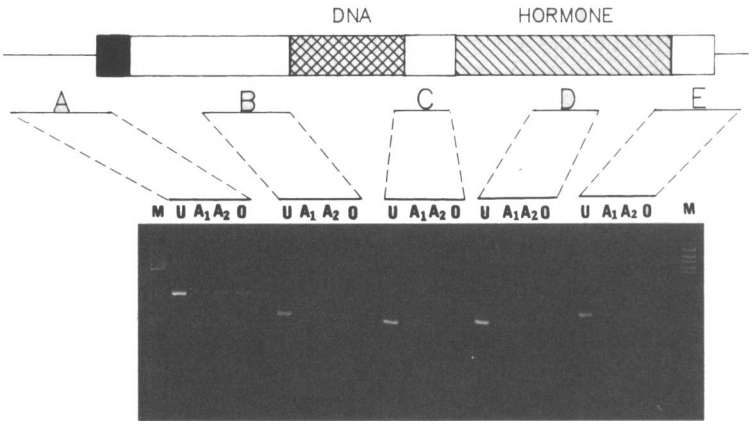

FIG. 5. Electrophoretic analysis of ER cDNA amplification products: 3% agarose gel electrophoresis of the products of PCR amplification of the cDNAs synthesized on a template of total RNA extracted from rat uterus (U), oligodendrocytes (O), and astrocytes type 1 (A_1) and type 2 (A_2). The sequences of the primers utilized for the amplification of the various segments of the ER cDNA are shown in Table 1. The first and last lanes are MW markers: pBr322 DNA *Hae*III cut. The amplification was carried out as described in Materials and Methods.

The analysis of these sequences demonstrated 100% homology between ER cDNA of the different glial preparations and that of the uterus.

Finally, the concentrations of ER mRNA in the glial cell culture and in the uterine tissue were measured using the competitive PCR assay, a methodology recently adapted to ER mRNA quantitation by our laboratory (Santagati et al., 1993). As shown in Table 2, the level of ER mRNA detected in the glial cells was ~0.04 pg/μg of total RNA; this level was much lower than the value found for the uterus (1.5 pg/μg of total RNA).

DISCUSSION

The data presented here indicate that the ER mRNA is expressed in all three types of glial cells analyzed: type 1 and type 2 astrocytes and oligodendrocytes. The experiments performed prove that the ER mRNA expressed in the glial cells is structurally indistinguishable from the message expressed in the uterine tissue. It is concluded, therefore, that the glial cells grown in culture may synthesize the ER.

To the authors' knowledge, no previous evidence for the presence of the ER or its mRNA in primary cultures of different types of glial cells has been reported. However, a study by Jung-Testas et al. (1992*a*) indicated the existence of [^{3}H]tamoxifen binding in primary cultures of mixed glial cells.

The levels of the ER mRNA detected appear to be very similar in the three types of primary cultures and are much lower than those found in the positive control, the rat uterine tissue. The low abundance of these messages raises the question of the function really played by estrogens in glial cells. It has to be emphasized that, most likely, not all the cells (see below) in culture express ER mRNA and, under our assay conditions, it is obviously impossible to determine the number of cells actually expressing the ER mRNA. The first report on the distribution of [^{3}H]estrogen in the brain demonstrated that only rare astrocytes are able to concentrate estrogens (Pfaff and Keiner, 1973); more recent studies performed on the guinea pig brain by immunostaining have shown that a variable number (10–46%, depending on the brain area analyzed) of GFAP-immunoreactive cells (type 1 astrocytes) are ER-positive (Langub and Watson, 1992). On the contrary, the great majority of the oligodendrocytes and microglial cells show no evidence of labeling (Langub and Watson, 1992). This observation, which represents an apparent discrepancy with the homogeneous distribution of the ER message in the different types of glial cells observed in the present study, might be explained by the different methodological approach (immunohistochemistry in tissues versus reverse-transcription PCR in cells in culture), or by the different species utilized (guinea pig versus rat).

TABLE 2. *Quantitation of ER mRNA in glial cells*

cDNA preparation	ER mRNA (pg/μg of total RNA)
Uterus	1.5
Astrocytes, type 1	0.03–0.06
Astrocytes, type 2	0.03–0.06
Oligodendrocytes	0.03–0.06

The finding of ER mRNA expression in glial cells is in line with the observation of their responsiveness to estrogens, as indicated in the introductory section. In conclusion, our results indicate that the glial cells, grown in well characterized cultures, express definite amounts of ER. The observation is in substantial agreement with previous results obtained with different receptor visualizing methods and with the functional response of glial cells to estrogens.

The estrogen sensitivity of glial cells might be an important issue in brain development, because it is known that astrocytes establish highly dynamic reciprocal interactions with neurons in several processes, such as migration, maturation, development of the appropriate neuronal circuits, and alteration of synaptic relationships (LoPachin and Aschner, 1993), which are key steps in the sexual differentiation of the brain.

As it is now well established that estrogens are the most important triggers of the sexual differentiation of the brain, an effect usually attributed to their known direct effect on neurons, it might be speculated also that the direct actions of estrogens on glial cells might contribute to the modulation of the action of these steroids on the brain. Furthermore, the presence of ER in oligodendrocytes, the myelinating cells of the CNS, is worthy of attention, because brain myelination is affected by estradiol (Curray and Heim, 1966) and estradiol stimulates proliferation of rat sciatic nerve Schwann cells, the cell type involved in the myelination of peripheral nerves (Jung-Testas et al., 1992*b*).

Further studies are obviously needed to substantiate the effects of estrogens on glial cells and, in particular, on the glial–neuronal interactions; however, the existence of ER in all the types of glial cells so far studied, as shown in this article, is a promising indication.

Acknowledgment: This work was supported by the EEC (BRIDGE Programme, T-project Animal Cell Biology BIOT-CT92-0308, to A. M.), by the Italian National Council of Research (P.F: Ingegneria genetica e basi molecolari delle malattie ereditarie, to A.M., and P.F: Biotecnologie e biostrumentazione, to L.M. and A.M.), and by AIRC (to A.M.).

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