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Short- and Long-term Estrogen Deprivation of T47D Human Breast Cancer Cells in Culture

CATHERINE S. MURPHY,* LORRAINE F. MEISNER,† SHI QI WU† and V. CRAIG JORDAN*

**Department of Human Oncology, University of Wisconsin Clinical Cancer Center, University of Wisconsin, Madison, WI 53792, U.S.A. and †State Laboratory of Hygiene, University of Wisconsin, Madison, WI 53792, U.S.A.*

Abstract—*The effects of short- and long-term estrogen deprivation on T47D human breast cancer cells was studied. Cells were routinely grown in an estrogenized environment in media containing fetal bovine serum with phenol red indicator. Cells were estrogen deprived (grown in media containing dextran-coated charcoal-stripped fetal bovine serum without phenol red) for either 10 days or at least 8 months, and effects on genotype, receptor content, and cell growth responsiveness were studied. Cells grown in an estrogenized environment are hypertetraploid, whereas long-term estrogen-deprived cells have become hyperdiploid. Short-term estrogen-deprived cells exhibit a decreased growth rate and progesterone receptor (PgR) content, while estrogen receptor (ER) content is not significantly altered. ER mRNA levels are significantly decreased in these cells. Incubation of these cells with estradiol (10^{-10} M) for 6 days causes a 5-fold stimulation in cell growth and this stimulation can be inhibited by the antiestrogens 4-hydroxytamoxifen (4-OHT), ICI 164,384, and RU 39411.*

Cells cultured under long-term estrogen deprivation exhibited an increased growth rate and were refractory to the effects of estradiol and of 4-OHT on cell growth. These cells were ER negative with low levels of PgR; however, one clone of this line was found to be ER and PgR negative. No mRNA for the ER was detected in this line or its clone. With these cell lines it is possible to study the biological characteristics necessary for the outgrowth of a receptor negative, hormone nonresponsive cell population from a receptor positive, hormone-responsive population grown in a estrogen-free environment.

INTRODUCTION

VARIOUS estrogen-responsive and nonresponsive human breast cancer cell lines have been established to study the effects of steroid hormones and growth factors on cell growth *in vitro*. Estrogen-responsive cell lines, such as MCF-7 [1] and T47D [2], have been studied extensively for their growth responsiveness in the presence of estrogens and antiestrogens [3–5]. However, it has been necessary to re-evaluate the biological properties of these cell systems in light of the finding that a contaminant of phenol red (the standard pH indicator present in cell culture media) is estrogenic and stimulates the growth of receptor-positive breast cancer cells [6–8]. Removal of phenol red from cell culture media has permitted the quantitation of cell growth responses to estrogens and to various growth factors [9]. However, the long-term culture of MCF-7 cells in phenol red-free media has resulted in significant changes in morphology, growth rate, receptor content, and response to hormones and antihormones [10, 11]. In the present study, we have investigated the short- and long-term effects of estrogen deprivation on T47D cells in culture.

There are many conflicting reports in the literature that describe the T47D breast cancer cell line. The cells were first described by Keydar *et al.* as estrogen receptor (ER)-positive and progesterone receptor (PgR)-positive [2], but it has subsequently been reported that T47D lines contain high constitutive levels of PgR, low-to-negative levels of ER, and are unresponsive to estrogens for growth and PgR production [12–15]. However, other laboratories have described ER-positive, PgR-positive (uncloned or clonal) lines that are responsive to estrogen and antiestrogen treatment [5, 16–20]. It appears that many variant lines can develop

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Send correspondence and reprint requests to: Dr V. Craig Jordan, Department of Human Oncology, University of Wisconsin Clinical Cancer Center, 600 Highland Avenue, Madison, WI 53792, U.S.A.
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depending on the conditions under which these cells are kept [21].

We used an uncloned line of T47D cells which is ER- and PgR-positive and highly responsive to estrogenic stimulation for growth and PgR production. This line has been routinely carried in a fully estrogenized state [i.e. in media containing unstripped fetal bovine serum (FBS) and phenol red]. We describe the short- and long-term effects of estrogen deprivation on the genotype, receptor and ER messenger RNA levels, and growth responsiveness of these cells to estrogen and antiestrogens. We report, for the first time, the development of a hormone-nonresponsive/receptor-negative cell population from a hormone-responsive/receptor positive line in response to long-term estrogen deprivation *in vitro*.

MATERIALS AND METHODS

Tissue culture

T47D cell line A (T47D:A) was originally obtained at passage 81 from the American Type Culture Collection (Rockville, MD). All cell stocks were kept in RPMI 1640 media supplemented with 10% (v/v) FBS (heat inactivated), 6 ng/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), and Penicillin (100 units/ml)/Streptomycin (100 μg/ml). All tissue culture reagents were obtained from Gibco Laboratories (Grand Island, NY) unless otherwise stated. T47D cell line B (T47D:B), obtained from T47D:A, was routinely cultured as above but with media containing 10% (v/v) dextran-coated charcoal-stripped FBS (DCC-FBS). T47D cell line C (T47D:C), also obtained from T47D:A, was cultured as above, but in phenol red-free RPMI 1640 media with 10% (v/v) DCC-FBS. Cell stocks were kept in T150 flasks (Corning, Park Ridge, IL) in a humidified atmosphere of 95% air:5% CO_2 at 37°C. All cells were removed from flasks with 0.25% trypsin/EDTA solution when passaged. All experiments were conducted on cells between passage numbers 133 and 191 for T47D:A and T47D:B and between numbers 123 and 173 for T47D:C. All lines were routinely tested and found to be free of mycoplasma.

Cell lines were cloned by dilution method into 96 well culture dishes (0.5 cell/well final dilution) and only wells containing single cell colonies were used.

Hormones

17β-Estradiol was purchased from Sigma Chemical Co. 4-Hydroxytamoxifen (4-OHT) and ICI 164,384 were obtained from ICI Pharmaceuticals (Macclesfield, U.K.). RU 39411 was obtained from Roussel-UCLAF (Romainville, France). [^{3}H]Promegesterone (17α-methyl [^{3}H]R5020) (86.2 Ci/mmole) and [^{3}H]estradiol (2,4,6,7-[^{3}H]estradiol-17β) (95.4 Ci/mmole) were purchased from NEN Research Products (Boston, MA). All compounds were prepared in a concentrated form in 100% ethanol and diluted in cell culture media. Final ethanol concentrations in the media never exceeded 0.2%.

Karyotyping analysis

For analysis of the cell karyotypes, cells were treated with 0.1 μg/ml colcemid in fresh media and incubated for 4 h at 37°C. Cells in mitosis were shaken from the T150 flasks and collected. Cells were incubated for 20 minutes at 37°C with 0.075 M KCl solution, washed, and fixed in a mixture of 75% MeOH/25% glacial acetic acid. G-banding was performed using Trypsin and Giemsa stain.

Cell flow cytometry

Cells were trypsinized and washed 2 × with phosphate buffered saline (PBS). Cells were kept at 5×10^6 in 0.5 ml of a 0.1% triton X/0.5 ml PBS mixture to which 2 ml ethanol was added, and samples were kept at 4°C until analyzed. Just prior to cell flow cytometry analysis, cells were treated with RNAase (180 U/ml in PBS) (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C and incubated with propidium iodide dye (50 μg/ml in PBS) (Sigma Chemical Co.) for 1 h. Samples were read and analyzed on a FACStar-Plus machine (Becton-Dickinson, Mountain View, CA). Histograms are representative of at least three samplings of each test condition studied.

Estrogen and progesterone receptor analysis

To determine the receptor contents of the cell lines tested, Scatchard analysis [22] was performed on cells that had been plated in test media for 48 h. For total binding determinations, cells were washed once with warm Hank's balanced salt solution (HBSS) and then incubated at 37°C for 1 h with radiolabelled ligand. For PR determination, 1 ml/well of [^{3}H]R5020 (at seven concentrations) in HBSS-complete containing 25 nM dexamethasone was used. [^{3}H]Estradiol in HBSS-complete was used for ER determinations. For determination of nonspecific binding, complementary wells were incubated with the above hot solutions plus 100-fold excess cold ligand. Cells were rinsed 3 × with HBSS-complete containing 1 mg/ml bovine serum albumin (BSA) and once with HBSS-complete without BSA. Cells were treated with 1 ml/well 0.1× calcium/magnesium-free-HBSS (CMF-HBSS) and sonicated for 12 s/well with a Kontes Ultrasonic Cell Disrupter. Samples (500 μl) were taken in triplicate from both total and nonspecific wells, and samples were counted in a Tracor Analytic Mark III scintillation counter. Specific binding was determined from total and nonspecific counts. Samples

(50–100 μl) were taken from the same wells for protein determinations as described below.

For analysis of PR synthesis in cells pretreated with various compounds, a single point assay was used. After 6 days of treatment with compounds, cells were rinsed once with warm HBSS-complete and treated as above with 2 nM [^{3}H]R5020 ± 100-fold excess cold ligand. Each point represents triplicate determinations.

Estrogen receptor/progesterone receptor enzyme immunoassay (EIA)

Cells were harvested from T150 flasks by trypsinization and homogenized in buffer containing 0.4 M KCl to extract nuclear receptor. Homogenization was performed on ice using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) with 5 s bursts. Previous studies have shown that high salt buffer does not interfere with the enzyme immunoassay [23, 24]. ER-EIA and PR-EIA analysis was performed using an Abbott ER-EIA or PR-EIA monoclonal antibody kit (Abbott Laboratories, North Chicago, IL). Samples were read on a Quantum II Analyzer (Abbott Laboratories) using Module 8, Mode 1.9. Triplicate determinations were made.

ER–messenger RNA analysis

RNA purification and Northern blotting were performed as described previously [25]. For Northern blot analysis, RNA was transferred from a formaldehyde gel to Hybond N membrane (Amersham, Arlington Heights, IL) as per manufacturer's instructions. Lambda cut with Hind III was used as a molecular weight marker. Blots were analyzed using a ^{32}P-labelled nick translated EcoRI insert from the plasmid HEO which was graciously supplied by Professor Pierre Chambon, Strasbourg, France [26]. HEO contains the 1.8 kbp open reading frame of the human estrogen receptor cDNA. After autoradiography, blots were stained with methylene blue stain (0.02%) to determine accuracy of sample loading [27].

Growth response studies

For growth response studies, cells were plated into T150 flasks containing the appropriate test media for 10–12 days prior to plating into 24-well dishes. Media was changed every 3 days. On day 0 of the experiment, cells were plated into 24-well dishes at 2×10^4 to 2×10^5 cells per well. Media containing compounds was added on day 1 and cells were allowed to grow for 6 days, with fresh media containing compounds added on day 4. On day 7, cells were washed with warm HBSS-complete and harvested for DNA and protein assays, or radiolabelled ligand receptor analysis was performed as described above.

DNA and protein determinations

Cells were treated with 1 ml/well 0.1 × CMF-HBSS and sonicated as described above. Samples (50–100 μl) were taken for DNA and protein determinations. DNA was detected by incubation of the samples with Hoechst Dye 33258 (Calbiochem-Behring Corp., La Jolla, CA) based on a method by LaBarca and Paigen [28]. Samples were read on a SLM-Aminco Fluoro-Colorimeter III.

Protein was determined using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) based on a method by Bradford [29]. Samples were read on a Bausch & Lomb spectronic 601 spectrophotometer. All points for DNA and protein measurements represent a mean of 3–4 sampled wells with error bars indicating standard error of the mean (S.E.M.).

RESULTS

Karyotype analysis

Cells from T47D:A, T47D:B and T47D:C were karyotyped at passage numbers 138, 137 and 126 respectively (Table 1). At the time of karyotype analysis I, T47D:A had been carried in phenol red containing media with unstripped FBS for one year. T47D:B had been carried in media containing phenol red and DCC-FBS for 9 months, and T47D:C had been kept in phenol red-free media containing DCC-FBS for 4 months. Cytogenetic analysis revealed that T47D:A was 100% 'hypertetraploid', whereas T47D:B was approximately 96% 'hyperdiploid' and T47D:C was approximately 1/3 'hypertetraploid' and 2/3 'hyperdiploid'. Cells were rekaryotyped at a later passage number (152, 152, and 138 respectively for T47D:A, T47D:B and T47D:C) (analysis II). T47D:A was 100% 'hypertetraploid' and T47D:B and T47D:C were both approximately 96% 'hyperdiploid'. All cells contained an abnormal chromosome number 1 identical to that seen in the T47D line first described by Keydar *et al.* [2]. In addition to the shared T47D markers, unique marker chromosomes were found for each line, indicating that lines T47D:B and T47D:C were genotypically distinct even though they displayed a similar chromosome number (data not shown). Similar genotypic divergence has been reported in other cells of the T47D lineage; however, those cells were kept under estrogenized conditions [21]. Lines T47D:A, T47D:B, and T47D:C have been analyzed several times at later passage numbers, and their karyotypes appear to be consistent with the data presented in analysis II.

Cell flow cytometry analysis

In order to confirm the results from karyotype analysis, cell flow cytometry analysis was performed on the three cell lines (Fig. 1). Cells from T47D:A

Table 1. Karyotype analyses of cell lines used in this study. Cells were karyotyped at two different intervals as indicated by analysis I and II and as described in the Results section. T47D:A = cultured in media containing phenol red and unstripped FBS; T47D:B = cultured in media containing phenol red and DCC-FBS; T47D:C = cultured in media without phenol red, containing DCC-FBS

Cell line	Passage No. at time of karyotyping	Number of chromosomes*	Percentage of modal cell	Number of normal chromosomes	Number of marker chromosomes
Analysis I					
T47D:A†	138	113	100	82	31
T47D:B‡	137	56	96	40	16
T47D:C	126	a§ 114	33	83	31
		b 59	67	42	17
Analysis II					
T47D:A	152	115	100	83	32
T47D:B	152	60	96	42	18
T47D:C	138	55	96	40	15

*Due to instability and random loss or gain of either normal chromosomes or markers, these numbers are the most representative of the modal karyotypes.
†Line T47D:A is almost an exact duplicate of the original ATCC line which has become tetraploid, with duplication of all the chromosomes. Two markers were present in only a single copy.
‡Line T47D:B retains almost all of the markers seen in the parent line, except they are generally present as a single copy. However, approximately three markers per cell are present in duplicate.
§a and b represent two populations found within line T47D:C at the time of first analysis.

displayed a G_0/G_1 fluorescence peak consistently at approximately channel number 243. T47D:B and T47D:C displayed peaks at approximately channel numbers 139 and 133 respectively, indicating a chromosome number which was approximately half that of T47D:A. These results therefore support the karyotype analysis.

Analysis of receptor content

A tritium-labelled ligand binding assay was performed in order to determine the effects of short-term estrogen deprivation of the ER and PgR content of these cells. Cells from T47D:A, which were kept under either fully estrogenized conditions or which were cultured in estrogen-deprived conditions for 10 days, were tested. Also, T47D:C and one clone (clone 4) from T47D:C were assayed for their ER and PgR levels (Table 2). The ER content of the fully estrogenized line did not change significantly after short-term estrogen deprivation; however, PgR levels dropped dramatically, exhibiting at least an 80% decrease in receptor content. Unlike other T47D cell lines described by Horwitz *et al.* [12–15], the PgR production in these cells *could* be re-stimulated by exposure to estradiol at 10^{-10} M for 6 days (Fig. 2). This 3–4-fold stimulation by estradiol could be inhibited to control levels by increasing concentrations of the antiestrogen 4-OHT. The antiestrogen alone did not significantly inhibit or stimulate PgR production in these cells.

Interestingly, T47D:C under long-term estrogen deprivation appeared to be ER-negative and PgR-positive. No estrogen receptor could be detected using the ligand binding assay; however, approximately 61 fmole/mg protein of PgR was detected. Incubation of these cells with estradiol (10^{-10} M) for 6 days did not significantly increase PgR levels (data not shown). Clone 4 of this cell line (T47D:C4) was found to be *both* ER- and PgR-negative by Scatchard analysis. Enzyme immunoassays were performed to determine if the receptor is truly absent in these lines, or if the receptor is present but not binding to the labelled ligands.

Abbott ER and PR enzyme immunoassays were utilized to confirm the findings of the ligand binding assay. T47D:A kept in the presence and short-term

Table 2. Estrogen and progesterone receptor analysis of cell lines using radiolabelled ligand binding assay. T47D:A was studied both when under fully estrogenized conditions and under short-term estrogen deprivation conditions (10 days)

Cell line	ER (fmol receptor/mg protein)	PgR (fmol receptor/mg protein)
T47D:A	40 K_d = 0.123 nM	2059 K_d = 0.362 nM
T47D:A after short-term estrogen deprivation	43 K_d = 0.772 nM	309 K_d = 0.204 nM
T47D:C	0	61 K_d = 0.22 nM
Clone 4 of T47D:C	0	0

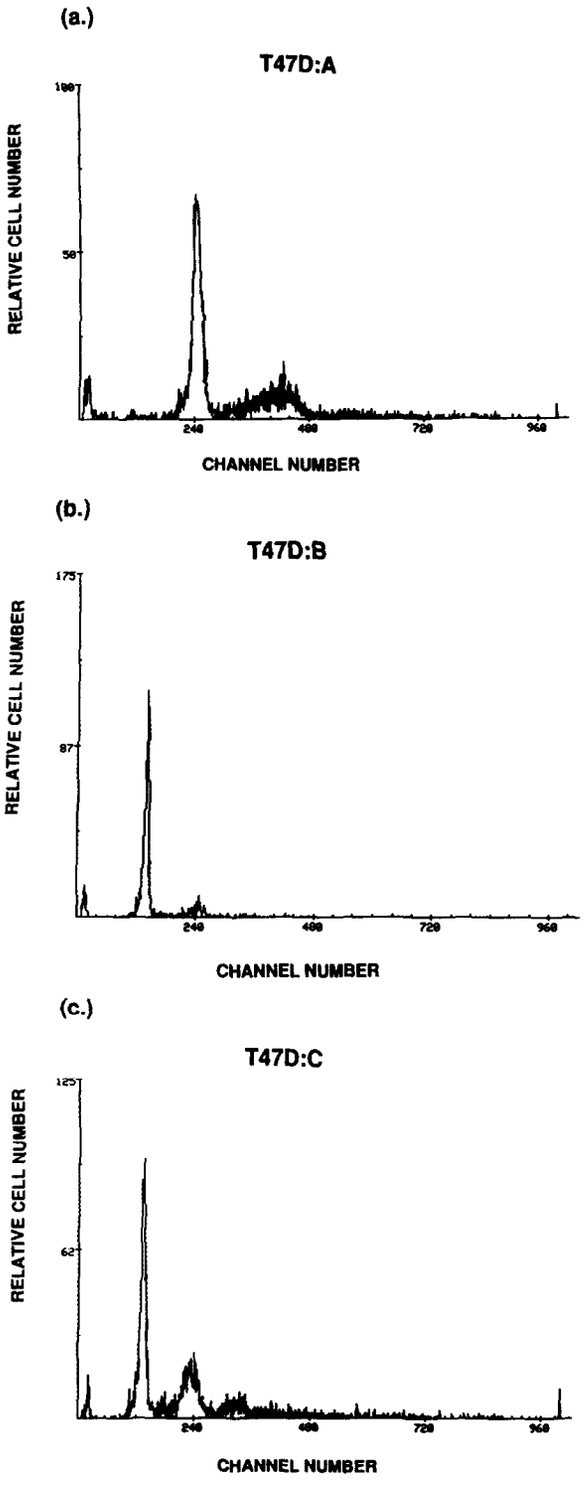

Fig. 1. Cell flow cytometric histograms of cell lines T47D:A, T47D:B and T47D:C. Mean channel number for the G_0/G_1 peak for T47D:A is 243, whereas the mean channel number for T47D:B and T47D:C are 139 and 133 respectively. (a) T47D:A; (b) T47D:B; (c) T47D:C.

absence of estrogen (10 days) displayed positive ER levels (>10 fmole receptor/mg protein) and highly positive PgR levels, whereas T47D:C was again found to be ER negative (<10 fmole receptor/mg protein) and PgR positive. Clone T47D:C4 was negative for ER and PgR by EIA analysis, thus confirming the results of the ligand binding assay.

ER mRNA analysis

Cells were analyzed for the presence of the messenger RNA for the ER. RNA Northern blot analysis was performed using the ^{32}P-labelled nick translated EcoRI insert from HEO plasmid as a probe. The results are presented in Fig. 3. Analysis shows that the ER-cDNA probe is binding to a specific band at the 6.2 kb position in T47D lines T47D:A and T47D:B and in the positive control preparation of MCF-7 cells. This band corresponds to the mRNA for the ER [26].

Interestingly, T47D:A which had been estrogen-deprived for 10 days displayed very low levels of ER-mRNA in comparison to when it is fully estrogenized. The ER-negative T47D:C and ER-negative control line MDA-MB-231 did not contain any measurable ER-mRNA. Also, clone T47D:C4 did not contain any ER message (data not shown). Methylene blue staining of the blot demonstrated that these differences cannot be accounted for by differences in gel loading (Fig. 3).

Cell growth studies

Cell growth was also studied in these cells, and the impact of phenol red on the growth of T47D:A was determined. Cells were incubated for 10 days in media containing 10% DCC-FBS in the presence or absence of phenol red (5 mg/l) and subsequently exposed to hormones for 6 days. As shown in Fig. 4(a), cells incubated with phenol red showed only a slight increase in cell growth with exposure to estradiol. At the highest concentrations used, 4-OHT inhibited cell growth to at least 50% below control levels both in the presence or absence of estradiol (10^{-10} M). The control growth level of cells that were kept under short-term phenol red-free conditions [Fig. 4(b)] was significantly lower

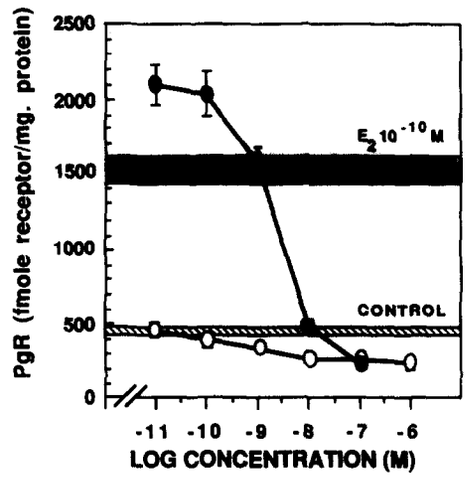

Fig. 2. Progesterone receptor synthesis response of line T47D:A incubated with compounds for 6 days. Cells were kept in estrogen-free media for 10 days before incubation with compounds. Cells were then plated at 2 × 10^5 cells/well and incubated with compounds for 6 days. Hatched bar, control; stippled bar, estradiol 10^{-10} M; (○) 4-OHT; (●) 4-OHT + estradiol 10^{-10} M.

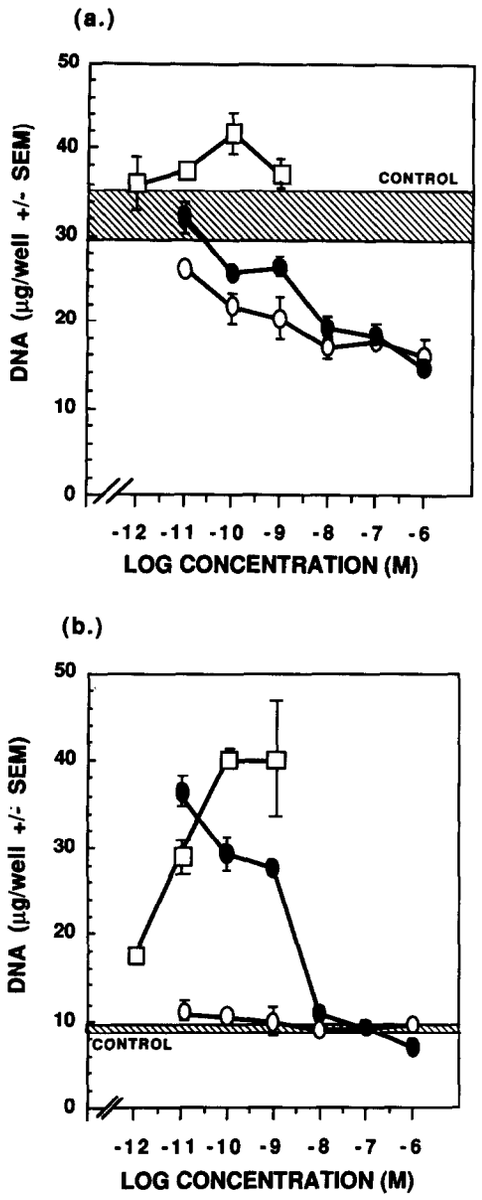

Fig. 4. Growth response to T47D:A to compounds in media with and without phenol red. Cells were cultured for 10 *days in media containing estrogen-free serum with or without phenol red. Cells were then plated at* 1 × 10^5 *cells/well and incubated with compounds for* 6 *days. (a) Cell growth response in media containing phenol red; (b) cell growth response in media without phenol red. Hatched bar, control;* (□) *estradiol;* (○) 4-*OHT;* (●) 4-*OHT* + *estradiol* 10^{-10} M.

than that of control cells in phenol red-containing media, thereby allowing for the detection of a growth stimulation by incubation with increasing concentrations of estradiol. The cells displayed a 4-fold increase in growth when estradiol (10^{-10} M) was used. Again, 4-OHT effectively inhibited estradiol-stimulated growth, but did not inhibit growth to below the levels of the control group. Studies utilizing cell number as an end point corresponded well with the DNA studies reported; therefore, the initial inhibition and subsequent growth stimulation by estradiol was not a result of a short-term decrease and subsequent increase in DNA content per cell. This was also supported by cell flow cytometric analysis (data not shown).

Comparison of growth response curves for T47D:A kept in short-term estrogen-free conditions to T47D:C (kept under long-term estrogen deprivation) is shown in Fig. 5. Again, estradiol could stimulate the growth of T47D:A 4–6-fold, and this could be effectively inhibited by incubation with increasing concentrations of 4-OHT. However, T47D:C was unresponsive to estradiol stimulation, and 4-OHT also had no effect on the growth of

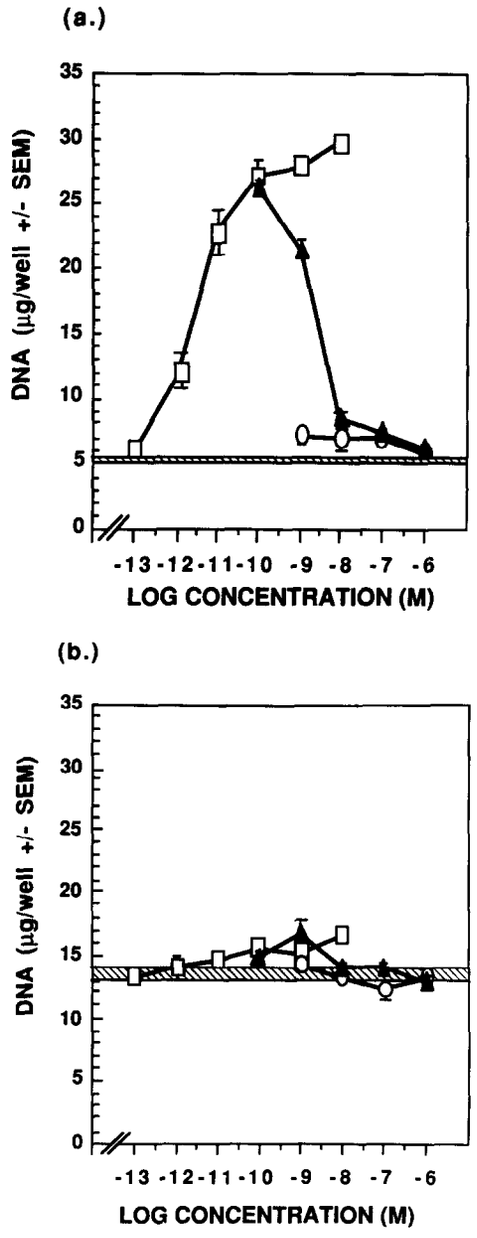

Fig. 5. Comparison of the growth response of T47D:A to the growth response of T47D:C after incubation with estradiol and 4-OHT. Cells from T47D:A were kept under short-term estrogen deprivation (10 *days). Both cell types were plated at* 7.5 × 10^4 *cells/well and incubated for* 6 *days with compounds. (a) T47D:A; (b) T47D:C; hatched bar, control;* (□) *estradiol;* (○) 4-*OHT;* (▲) 4-*OHT* + *estradiol* 10^{-10} M.

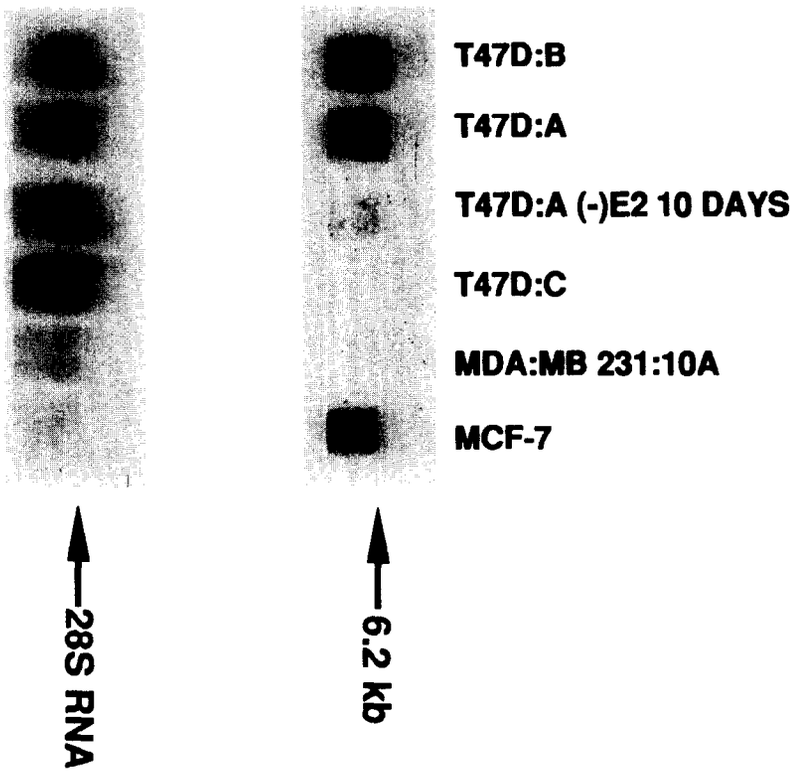

Fig. 3. Northern blot analysis of mRNA for the estrogen receptor. The blot was hybridized to ^{32}P*-labelled HEO probe (as described in Materials and Methods section). RNA samples harvested from cell lines are depicted in lanes 1–6 (left to right) as follows: lane 1, T47D:B; lane 2, T47D:A; lane 3, T47D:A after estrogen-deprivation for 10 days; lane 4, T47D:C; lane 5, MDA-MB-231 cell line clone 10A; lane 6, MCF-7 cell line.*