Effect of Growth on the Estrogen Receptor Levels in MCF-7 Cells


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ABSTRACT

MCF-7 cells have been shown to contain estrogen receptor in several cell fractions following homogenization: nuclei, microsomes, and cytosol. The amount of 17β-estradiol-binding capacity found in each cellular compartment depended on the inclusion of detergent in homogenization buffers and on the use of 0.25 M sucrose in the nuclear washes. 17β-Estradiol receptor (E2R) associated with nuclei (whole nuclei exchange assay, 0.6 M KCl soluble, and that found on membranes sheared from crude nuclear pellets by centrifugation in 0.25 M sucrose buffer) displayed a dissociation constant (Kd) of 0.77 ± 0.01 (S.D.) nM (n = 7). Kd's of the cytoplasmic (microsomes and soluble) receptors were determined to be 0.33 ± 0.10 nM (n = 9). Exchangeable ligand on partially purified nuclei assumed its highest level in MCF-7 cells during logarithmic growth in serum-containing media (0.8 pmol/µg DNA) but declined after the culture reached confluency (0.2 pmol/µg DNA). Seventy-five % of the nuclear E2R (0.8 pmol/µg DNA) but declined after the culture reached confluency (0.2 pmol/µg DNA). Seventy-five % of the nuclear E2R declined linearly after feeding MCF-7 cells in logarithmic growth phase an estrogen- and serum-free medium (t½ = 3.5 days). Another class of salt-extractable nuclear receptor (0.2 pmol/µg DNA) persisted in postconfluent cultures whether fed estrogen (serum-containing media) or not (serum-free media). This residual binding capacity remained in nuclei of MCF-7 cells for an extended period of time. MCF-7 cells demonstrated functionality of E2R throughout their growth phases as evidenced by the replenishment of cytosolic E2R and the induction of progesterone receptor when given 17β-estradiol.

INTRODUCTION

The level of E2R in breast tumors has been shown to vary widely (18). For the most part, hormone-independent breast neoplasms are either devoid of or contain negligible E2R levels, while other tumors may display significant concentrations of estrogen-binding protein (two-thirds of such cancers may be hormone dependent). Numerous investigations have demonstrated that the concentration of receptor in E2R-positive breast cancers may depend on the age of the patient [i.e., pre- or postmenopausal (17, 20, 24)], the cellularity of the tumor (18), and the therapeutic history of the host (28).

The most actively utilized research model of human breast cancer, the MCF-7 cell culture, has been reported to contain a strikingly variable concentration of E2R. In these cells, the level of specific estrogen-binding capacity has been shown to depend on the hormonal exposure of the culture [prolactin (22), insulin (4), estrogen (14)] and on the laboratory source of the cells (15).

The quantitation of E2R in breast tumor cells depends greatly on the assay methodology and on the cell fractionation procedure. Confusion has resulted from reports of charged and uncharged forms of the nuclear receptor and from the character of the various cellular components isolated. A thorough investigation recently reported by Edwards et al. (8) showed that most of the E2R in MCF-7 cells can be found in the cytosol without bound ligand (uncharged) and in the purified nuclei with bound estrogen (charged), with very little nuclear E2R being uncharged (3%). While most of the nuclear binding capacity can be extracted with 0.6 M KCl, there remains a persistent level (0.06 pmol/µg DNA) of estrogen high-affinity binding which is exchangeable in the salt-extracted nuclear residue (11).

The effect of growth on the levels of E2R in the nuclear and extranuclear compartments of MCF-7 cells has not been reported. Variations in receptor content may be expected to result from growth or the cell cycle (5). The investigations reported herein were designed to examine the effects of the growth of MCF-7 cultures on their cellular E2R levels.

MATERIALS AND METHODS

Materials. 17β-[3H]Estradiol (91 to 113 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and purified on thin-layer chromatographic sheets (ITLC-SA; Gelman Instrument Co., Ann Arbor, MI) with CHCl3:CH3OH (97:3) as the developing solvent (11). Nonradioactive hormones were obtained from Research Plus Laboratories, Inc. (Denville, NJ).

Cell Culture and Harvesting. MCF-7 cells of human breast tumor origin were cultured in closed T25 flasks utilizing 20 ml Eagle’s minimal essential medium supplemented with Hanks’ balanced salts, nonessential amino acids, insulin, and antibiotics and made 10% with respect to calf serum (26). Cultures were refed every 2 to 3 days. Subculturing (after 2 weeks growth) involved trypsin digestion (26) to obtain monoclonal suspensions followed by the plating of 2 × 10⁶ cells/flask. Cultures were confluent after 1 week (approximately 20 × 10⁶ cells flask) and would grow for 1 to 2 more weeks (>50 × 10⁶ cells/flask).

In certain experiments, cells were grown for 3 days immediately after passage in 20 ml of Eagle’s minimal essential medium (supplemented as described above), after which the medium was then replaced with 20 ml of a serum-free CM prepared without insulin, thymidine, and cortisol (12). Bovine insulin (to a final concentration of 10 µg/ml; Sigma Chemical Co., St. Louis, MO) was added before use.

Preparation of Cell Fractions. All steps were carried out at 0°C. Following removal of medium, flasks were washed twice with 0.9% NaCl solution and once with Tris:EDTA buffer [10 mM Tris-HCl:1.5 mM EDTA (pH 7.4 at 0°C)]. The cells were then transferred from the flask using a rubber policeman with 2 ml of Tris:EDTA buffer made 8 mM with dithiothreitol and placed in a Dounce tube. Reducing agent was added to protect receptors in the event of cell rupture. The suspension of harvested cells from 2 flasks was homogenized in 4 ml of the buffer utilizing 15 strokes of a tightly fitting pestle. The homogenate was centrifuged at 800 × g for 10 min (4°C), and the supernatant was saved.
(crude cytosol). A more purified nuclear pellet was prepared in certain experiments by homogenizing cells in either or both of the following buffers: Tris:potassium:magnesium buffer with 0.1% saponin (10 mM Tris-HCl, pH 7.4, 0.5 mM KCl; 1 mM MgCl₂; and 8 mM dithiothreitol) or buffered sucrose solution (10 mM Tris-HCl, pH 7.6; 0.25 mM sucrose; 3 mM MgCl₂; and 8 mM dithiothreitol). When both buffers were used, the sucrose solution was used to wash the nuclei 3 times, and the supernatants obtained following the centrifugation were combined with the crude cytosol. The crude cytosol was centrifuged at 105,000 × g for 4 hr, yielding a supernatant (cytosol) and a high-speed pellet (particulate).

The partially purified nuclear and particulate pellets were extracted with Tris:EDTA:KCl buffer (10 mM Tris-HCl:1.5 mM EDTA:0.6 mM KCl; 1 mM thioglycerol;10% glycerol (pH 8.5, 0°) by triturating (with Dounce pestle) every 10 min for 1 hr. Solubilized proteins were separated from insoluble material by centrifugation at 105,000 × g for 30 min. The supernatant extracts were diluted with Tris:EDTA buffer to a concentration of less than 0.1 M KCl to enable precipitation of the protamine sulfate (11). DNA was determined from the residual nuclear or particulate pellet (105,000 × g pellet) by the diphenylamine method of Burton (3) using Sigma type I calf thymus DNA as a standard.

**Assay Procedures.** A modification of the method of Anderson et al. (1) was used for the exchange assay of estrogen binding in whole nuclei. Nuclei were isolated as described above, and a homogeneous suspension of nuclei was prepared in 15 ml of sucrose buffer. Twenty-four 0.5-ml aliquots were taken for the assay, and two 1-ml aliquots were taken for DNA determination. The 24 sample tubes were centrifuged at 800 × g for 10 min and decanted, and 0.3 ml Tris:EDTA buffer was added to each sample. Ten µl of the 6 different estrogen concentrations in ethanol (final concentration, 0.2 to 4.0 nM 17β-[3H]estradiol) were added in duplicate to 12 tubes. Nonspecific binding was determined by a parallel incubation of 12 similar tubes with 17β-[3H]estradiol plus 200-fold excess 17β-estradiol. The samples were incubated for 60 min at 23° with shaking (preliminary experiments had shown these incubation conditions to yield optimal receptor detection). Free estrogen was then washed out with 1.0 ml of Tris:EDTA buffer 3 times, centrifuging at 1000 × g for 10 min and decanting between washes. The pellet was extracted with 1.5 ml of ethanol overnight, and 1 ml of extract was counted in 10 ml of scintillation fluid on a scintillation spectrometer equipped with an absolute activity analyzer. The binding capacity and dissociation constants were determined from a Scatchard plot of the data (21).

The exchange assay on a suspension of whole cells was carried out by first detaching cells from T₂₅ flasks with trypsin, washing cells by centrifugation in medium containing 10% calf serum (for trypsin inhibition), and suspending them in COM. The receptor assay was carried out in a manner similar to that used for the exchange assay on whole nuclei described above.

The protamine sulfate method of Steggles and King (27) as modified by Zava et al. (29) was used to determine the 0.6 µ M KC1-solubilized nuclear receptor. Nuclei were isolated, and the 0.6 µ M KCl (2 ml) extract was obtained as described above. The supernatant was diluted to 13 ml with Tris:EDTA buffer, bringing the KCl concentration to below 0.1 M. Protamine sulfate solution (1 µg/ml, 0.3 ml) was added to each of 24 aliquots (0.5 ml) of the extract, and the mixture was allowed to stand for 10 min. Samples were centrifuged at 1000 × g for 10 min, and the supernatant was removed carefully by suction. To each tube were added 0.3 ml of Tris:EDTA buffer and 10 µl of different estrogen concentrations as described for exchange assay for whole nuclei. The samples were incubated (37° for 60 min or 0° for 15 hr) without shaking (11). Occupied nuclear receptor may be determined by equilibration at either of these temperatures (11). Tubes were then washed 3 times within 1 ml of Tris:EDTA which was removed by suction. The precipitate was extracted and counted, and the binding capacity and Kᵦ were determined as described for the exchange assay of whole nuclei.

The cytosolic ER was also determined by the above-described protamine sulfate procedure. Twenty-four 0.5-ml aliquots of the 105,000 × g supernatant were taken for the assay, 0.3 ml protamine sulfate solution (1 mg/ml) was added to each sample, and the assay was carried out as described for the 0.6 µ M KCl nuclear extract utilizing the 0° equilibration.

The PGR was determined by adaptations of a previously published method (2). The 105,000 × g supernatant was divided into 0.2-ml aliquots following the addition to a concentration of 3.3 µM cortisol. The aliquots were then added to 2 sets of 14 tubes. To the first set were added 3[H] progesterone standards in addition to 200-fold excess unlabeled progesterone. Following a 2-hr incubation, 0.2 ml Tris:EDTA buffer containing 60% glycerol and 1 mM dithiothreitol (pH 7.5) was added, and the tubes were incubated for 1 hr with frequent vortexing. Dextran-coated charcoal (1:10 in Tris:EDTA:dithiothreitol (0.4 ml) was then added; all tubes. This mixture was incubated for 30 min with vortexing at 10-min intervals. The tubes were centrifuged at 2000 × g for 10 min, and aliquots of 0.25 ml were taken for liquid scintillation counting in 4 ml ethanol plus 10 ml scintillation fluid. Binding capacities and Kᵦ's were calculated by Scatchard analysis.

The glucose-6-phosphatase assay to determine purity of nuclei was based on the incubation of cell fractions with the specific substrate, glucose 6-phosphate, and the determination of the liberated Pi (19). Preliminary studies on cell homogenates indicated the presence of this enzyme and showed the formation of phosphate to be linear for 4 hr at 37° with no degradation of the enzyme. To duplicate tubes (samples and control) containing 0.6 ml of a cell fraction were added 0.1 ml of 20 mM NaF, 0.1 ml of 40 mM EDTA, and 0.2 ml of 30 mM glucose 6-phosphate (all prepared in 0.1 M Tris-malate buffer, pH 6 at 0°). Tubes were incubated at 37° for 4 hr. One ml of 10% trichloroacetic acid was added to the sample tubes after incubation to stop the reaction. Tubes were centrifuged at 800 × g for 10 min, and phosphate was determined [using a modification of the method of Fiske and SubbaRow (9) on 1.5 ml of the supernatant diluted to 2 ml with distilled water. The amount of phosphate in the cell fraction was determined from the standard curve using the change of absorbance from the control to the test solution. The enzyme activity was reported in pmol phosphate formed/min/µl DNA.

**RESULTS**

**Distribution of ER at Confluence.** Utilizing the whole-cell assay technique, it was ascertained that at confluence of MCF-7 cells contained a total high-affinity saturable binding capacity of 1.66 pmol 17β-estradiol/mg DNA (Table 1). Following homogenization, the crude nuclei (1000 × g pellet) displayed a specific binding capacity of 0.87 pmol/mg DNA, the cytoplasmic particulate (105,000 × g pellet) yielded 0.14 pmol/mg DNA, and the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of ER at confluence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>1.00</td>
</tr>
<tr>
<td>Crude nuclei</td>
<td>0.77</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.48</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.42</td>
</tr>
</tbody>
</table>

* DNA value used is that found in whole cells from which the fraction was obtained.
cytosol (105,000 x g supernatant) yielded 0.57 pmol/mg DNA (Table 1). This process recovered 95% of the cellular binding capacity. The cytosolic and particulate fractions were relatively free of nuclear contamination since the amount of DNA assayed in these fractions was insignificant (less than 5% of total cellular DNA). The dissociation constants (Kd) of the receptors were of 2 separate magnitudes: 0.33 ± 0.10 (S.D.) nM for the cytoplasmic components; and 0.77 ± 0.01 nM for the nuclei. It is conceivable that, during the 1 hr at 23° incubation required for the whole-cell assay, the cytoplasmic E2R would be driven into the nucleus; therefore, the high Kd observed for this assay is understandably nearer that of E2NR (Table 1).

At the present time, it is not known whether the 17β-estradiol-binding activity displayed by the cytoplasmic particulate is that of cytoplasmic E2R artifactually trapped during the centrifugation of this fraction or if this receptor is specifically bound by the endoplasmic reticulum (microsomes) in situ. However, this E2R displayed a Kd similar to that of the cytosolic E2R and unlike that of the nuclear receptor (Table 1). It is also possible that this binding represents E2R being synthesized de novo on polyribosomes (16).

**Effect of Purification on Amount of E2NR.** Clark and Peck (7) and Clark et al. (6) have reported that purified nuclei from rat uterus behaved in a fashion similar to that of a crude nuclear preparation insofar as nuclear estrogen binding was concerned. However, Hansen and Brooks (11) and Edwards et al. (8) have indicated a significant decrease in both the 0.6 M KCI-soluble and insoluble (ethanol-extractable) "nuclear" estrogens from MCF-7 cells when nuclei are freed of membranes by detergent treatment and/or sedimentation through sucrose.

Different preparations of nuclei (as detailed in Table 2) were used in the determination of E2NR in order to clarify whether "purification" of nuclei with detergent and/or 0.25 M sucrose changes E2NR concentrations or affinity. The experiment described in Table 2 showed that treatment with 0.1% saponin or 0.25 M sucrose decreased E2NR to about 70% of that obtained in a crude nuclear preparation. Use of both 0.1% saponin and 0.25 M sucrose decreased E2NR to 54% of that obtained in a crude nuclear preparation.

A glucose-6-phosphatase assay was carried out on the various nuclear preparations in order to determine the extent of contamination of these preparations with cytoplasmic membranes. Approximately 17% of the total glucose-6-phosphatase activity (Table 1) remains in a crude nuclear preparation whereas "purified" nuclei contain only 8% of the total activity (Table 2). Thus, it can be seen that the use of 0.1% saponin + 0.25 M sucrose frees the nuclei considerably of cytoplasmic membranes although nuclei thus prepared cannot be considered completely pure.

Utilizing the membranes removed from nuclei during 3 washings with 0.25 M sucrose buffer, it was possible to examine certain characteristics of the receptor thus removed from the crude nuclear pellet. An exchange assay carried out on these membranes and the nuclear residue showed a nearly complete recovery of the total binding capacity found in the crude nuclei (Table 2). Furthermore, the dissociation constants of the estrogen binding in the crude and semipurified nuclear preparation as well as that of the receptor in the separated membranes were virtually identical and unlike that of the E2R in the cytoplasm.

**Variations in E2R with Growth.** Three days following passage of 2-week-old cultures, the partially purified nuclei from MCF-7 cells contained 0.8 pmol/mg DNA (Chart 1). When the cells were fed the regular medium, this level of E2NR was maintained until just before the culture reached confluence. The presence of nuclear receptor in these cells could be explained by the 17β-estradiol which was contained in calf serum added to the medium (final concentration, approximately 10⁻¹¹ M). However, even in the presence of this medium estrogen, the E2NR was seen to diminish rapidly following confluence of the culture. After 2 weeks of growth (or 1 week after the culture reached confluence), the nuclear concentration of exchangeable ligand on receptor appeared to assume a level near 0.2 pmol/mg DNA.

E2NR in cells grown in the absence of serum estrogen (in CDM) decreased at a linear rate, beginning after the medium change and continuing until the concentration of this nuclear receptor reached a level of 0.2 pmol/mg DNA (Chart 1). This final concentration was similar to the level of E2NR in postconfluent cells grown in the usual medium containing 10% calf serum. The...
absence of estrogen in the CDM did not alter the growth rate of MCF-7 cells (Chart 1).

The cytoplasmic receptor showed a pattern of cellular fluctuation different from that of nuclear receptors in MCF-7 cultures grown in regular medium. Cells passed 1 week after reaching confluence displayed amounts of estrogen receptor in the cytosol similar to that found in the nuclear compartment [4 days after passage (Chart 2); other experiments have shown similar E2CR concentration 3 days following passage]. The cytosol receptor increased steadily from Day 4 until just before the culture reached confluence (Day 10). At this time, the soluble E2CR concentration had peaked at 2.5 pmol/mg DNA. After reaching confluence, the MCF-7 culture displayed a declining concentration of soluble estrogen receptor which reached values as low as those initially observed (i.e., 0.6 pmol/mg DNA).

It has previously been shown that a large fraction of the nuclear receptor may be extracted by 0.6 M KCl solutions; however, a persistent amount of high-affinity, limited-capacity 17ß-estradiol binding remained in the nuclear residue (11). The data in Table 3 show the total E2NR concentration (exchange assay) decreased in older cultures similar to that depicted in Chart 1. Likewise, the salt-extractable E2NR became progressively less as the culture passed from logarithmic growth to a confluent culture and then to postconfluent.

Throughout the growth stages, protamine sulfate-precipitated E2RN extracted from the partially purified nuclei exchanged completely with 17ß-[3H]-estradiol at 37° and 0° (Table 3). A similar amount of receptor in intact nuclei also exchanged with labeled 17ß-estradiol at 0°. Slightly more (15%) binding was detected in these nuclei when the exchange reaction was carried out at 23°. In each case, these nuclear binding components displayed the same K_d (0.79 ± 0.3 nm). The possibility that the low-temperature-exchangeable nuclear receptor might represent uncharged receptor is weakened by the fact that [3H]estrone will not bind to these sites during equilibrium conditions at 0° (data not shown).

Functional Capacity of E2R during Growth. It has been shown above that the E2R levels in the cytosolic and nuclear compartments of MCF-7 undergo wide fluctuations during growth of the culture. Pulsing these cells with 10^{-8} M 17ß-estradiol for 1 hr at 37° gives an indication of the ability of cytosolic receptors in logarithmic growth or postconfluent cells to translocate to the nucleus. MCF-7 cells which are in the logarithmic growth phase and are accumulating estrogen receptor in the cytosol are capable of displaying the translocation of receptor complex into the nucleus when pulsed with 17ß-estradiol [10^{-8} M (Table 4)]. The response to this pulse was measured by following the reappearance of E2CR or by determining the induction of PGCR in MCF-7 cells. Three days after translocating E2CR to the nucleus with a 1-hr pulse of 17ß-estradiol (10^{-8} M), the cytosolic receptor has returned to its normally high level for preconfluent cultures (Table 4). PGCR levels peaked after 1 day and declined rapidly following the 1-hr pulse. In postconfluent cultures, the replenishment of E2CR following a pulse of 17ß-estradiol required more than 6 days. This same postconfluent culture of MCF-7 cells displayed minimal synthesis (within 1 day of pulse) of PGCR in response to the pulse with 17ß-estradiol (Table 4).

Experiments have demonstrated that the receptors in the

### Table 3

Exchange versus protamine sulfate assay of 17ß-estradiol throughout growth of MCF-7 cells

<table>
<thead>
<tr>
<th>Days (log phase)</th>
<th>K_d (nM)</th>
<th>pmol/mg DNA</th>
<th>4 days</th>
<th>11 days</th>
<th>21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°</td>
<td>0.80</td>
<td>0.80</td>
<td>0.82</td>
<td>0.44</td>
<td>0.78</td>
</tr>
<tr>
<td>23°</td>
<td>0.78</td>
<td>0.64</td>
<td>0.77</td>
<td>0.35</td>
<td>0.80</td>
</tr>
</tbody>
</table>

### Table 4

Response of log growth and postconfluent MCF-7 cells to a pulse with 17ß-estradiol

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>E2CR</th>
<th>E2RN</th>
<th>E2CR replenishment</th>
<th>PGCR induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log + 10^{-8} M 17ß-estradiol</td>
<td>0.81</td>
<td>0.50</td>
<td>1.18 (8)(b)</td>
<td>0.11 (1)(c)</td>
</tr>
<tr>
<td>Postconfluent</td>
<td>0.64</td>
<td>0.20</td>
<td>0.83 (8)(b)</td>
<td>0.09 (1)(c)</td>
</tr>
</tbody>
</table>

Numbers in parentheses, days to complete replenishment or maximal induction.

During log growth, E2CR had reached pretreatment levels 3 days following 17ß-estradiol pulse.

Maximum PGCR induction was seen 1 day following 17ß-estradiol pulse. Thereafter, PGCR declined to pretreatment levels by the next daily assay.

In postconfluent cells, E2CR had not reached pretreatment levels after 6 days.
S. C. Brooks et al.

Chart 3. Cytosolic receptor concentrations in MCF-7 cells during the administration and withdrawal of 17β-estradiol. Cells were passed into and grown in regular medium until 2 days prior to confluence (arrow) after which the cultures were fed COM ± 10⁻⁸ M 17β-estradiol. At the indicated times, cultures were harvested (2 Tₚ₀ flasks/point) and the cytosolic receptors were analyzed using procedures outlined in “Materials and Methods.” Each point represents the binding capacity as determined from Scatchard plots. 170-Estradiol was withdrawn from certain cultures (point) and the cytosolic receptors were analyzed using procedures outlined in “Materials and Methods.” Each point represents the binding capacity as determined from Scatchard plots.

DISCUSSION

When assayed in suspension, the MCF-7 cells utilized in these studies contained 1.66 pmol/mg DNA 17β-estradiol-binding capacity. This level is similar to that reported by others (30), although some passages have been shown to contain higher concentrations of estrogen receptor (8).

Utilizing standard cell fractionation procedures, these experiments have demonstrated that the estrogen receptor in MCF-7 cells may be found in cytosolic, microsomal, and nuclear preparations. As reported previously by Edwards et al. (8), crude nuclear preparations can be washed by detergent and/or sucrose buffers to remove contaminating or loosely held unoccupied binding proteins (Tables 2 and 3). Furthermore, the receptor which was removed from nuclear pellets by low-salt washes was cytosolic in its density gradient sedimentation characteristics (8). In these studies, we have compared the receptors in the various compartments by their dissociation constants. For example, within the cytosol, the estrogen receptor extracted from microsomes displayed a Kᵢ of 0.48 nm, which represents an affinity not unlike the mean value found for the cytosolic receptor (Kᵢ 0.33 nm) when one considers the standard deviation of 9 determinations (S.D. 0.10). Both of these binding assays were carried out on protamine sulfate-precipitated receptor at 0° for 15 hr. The low temperature conditions of this assay have been defined as indicating uncharged receptors [the unoccupied receptor without estrogen ligand (30)]. We have previously confirmed this premise by demonstrating that the E₂RC will bind estrone (Kᵢ 3 nm) in the absence of nm concentrations of 17β-estradiol (11).

The crude nuclear compartment contained receptors which bound 17β-estradiol less tightly (Kᵢ 0.77 ± 0.01 nm). Throughout the washing of the nuclear pellet with detergent and/or sucrose buffer, the Kᵢ of the residual estrogen binding remained unchanged. Similar Kᵢ values were obtained whether the assay utilized was of the exchange type or was carried out on the protamine sulfate precipitate of the 0.6 m KCl nuclear extract [which yielded the 4S receptor on density gradient centrifugation (8)]. Interestingly, the membranes which were sheared from the crude nuclei by centrifugation through sucrose buffer also displayed an estrogen-binding activity with a Kᵢ of 0.88 nm (exchange assay), a disassociation constant different from that found in the cytosol.

After detergent and buffered sucrose washing, the nuclei contained a salt-extractable binding component which in our studies was capable of exchanging with tritiated 17β-estradiol equally well at 37° or 0° (Table 3). According to others (30), this would denote uncharged receptor which at confluence comprised nearly one-fifth of the cellular receptor. Experiments (10) have demonstrated that the 0.6 m KCl extract of these cells, although it exchanges with 17β-[³H]estradiol at 0° [15 hr (Table 3)], is indeed charged. Basically, these studies showed that, when the salt-extractable E₂NR was incubated with 17β-estradiol (10⁻⁸ m) for 1 hr prior to precipitation with protamine sulfate, this ligand could be exchanged with 17β-[³H]estradiol at 0° for 15 hr. This was not the case when the protamine precipitate was exposed to estrogen after precipitation [also reported by Zava and McGuire (30)]. Thus, a difference in ligand exchangeability occurs because of a simple alteration in the technical procedures, indicating that the exchange with 17β-[³H]estradiol at 0° by the salt-extractable nuclear receptor does not necessarily prove that this receptor is uncharged. Following a 1-hr 37° pulse of MCF-7 cells with 10⁻⁸ m 17β-estradiol, the exchange characteristics of the salt-extractable nuclear receptor are again different. Under these circumstances, the ligand cannot be replaced with 17β-[³H]estradiol at 0°, even after a 20-hr incubation (11). This level of 17β-estradiol (which is optimal for processing) has brought about significant alterations in the exchangeability of the ligand on the nuclear estrogen receptor.

Just after passage of MCF-7 cells, the low E₂CR observed (Chart 1) would be expected in the presence of high nuclear E₂NR, especially in an estrogen-containing medium (although low, ~10⁻¹¹ m) and in cultures passed after 2 weeks growth (see Chart 2). The increasing concentration of E₂CR which occurred prior to confluence might be explained by de novo synthesis in the presence of low 17β-estradiol concentrations. This is quite possible in rapidly dividing cells since receptor is synthesized in the prereplicative stage (25). After reaching confluence, the mitotic rate of these cells slows and is accompanied by a decreasing level of both E₂CR and E₂NR (Charts 1 and 2). Other experiments (not shown) have demonstrated that E₂CR in MCF-7 cells maintained in CDM increased and decreased in a pattern identical to that depicted in Chart 2. It is also true that, when
expressed per mg cytosolic protein, the growth-related concentration pattern of soluble 17β-estradiol binding capacity is similar to that related to DNA (23), thereby diminishing the possible effects of cell size (which becomes smaller following confluence) on the cytosolic receptor growth-related pattern.

During logarithmic growth, there was apparently adequate E2CR in these cells to translocate the medium 17β-estradiol to the nucleus. In fact, 3 days following passage, the nuclear receptor level was highest and approximately equaled that in the cytosol. As the E2CR levels climbed in growing cultures, the nuclear receptor complex concentration remained unchanged. The decreasing level of E2NR which begins just prior to confluence and occurs in the presence of high E2CR concentrations and medium estrogen in postconfluent cultures is not understood but is related to the confluent state of the culture.

While undergoing logarithmic growth, these cells divided every 2 days (Chart 1, inset). Since there were no additional estrogens in the medium during growth in CDM, it might be expected that the nuclear binding capacity (per mg DNA) would display one-half the level with each cell division. The information in Chart 1 indicates that the nuclear concentration of estrogen receptor which was initially (Day 3) 0.8 pmol/mg DNA had decreased to 0.6 at Day 5, 0.4 at Day 7, and 0.3 after 3 cell divisions of the culture. Interestingly, by 4 divisions and thereafter, the E2NR remained at its minimum level of 0.2 pmol/mg DNA. The remaining E2NR was completely extracted by 0.6 M KCl (Table 3; Ref. 11), displayed a K0.5 of 0.78 nm, and would not bind estrone (10). This lowest nuclear concentration represents a class of E2NR with a greatly extended nuclear residence, particularly in postconfluent cultures. In fact, if one subtracts this level (0.2 pmol/mg DNA) from that determined initially and after each cellular division in CDM, a straight line is generated which shows the nuclear occupancy of the remaining E2NR in actively dividing MCF-7 cells to have a half-life of 3.5 days. Noteworthy is the observation that the slope of disappearing E2NR in cells grown in regular media (Chart 1) is similar to that seen in cultures fed CDM, suggesting the lack of nuclear translocation of E2CR in postconfluent cells grown in media low in 17β-estradiol (i.e., containing only that 17β-estradiol in the serum, approximately 10⁻¹¹ M). These overgrown cells, however, can be made to translocate the receptor complex if pulsed with, or grown in, higher concentrations of 17β-estradiol [10⁻⁸ M (Table 4); 10⁻⁹ M (Chart 3)].

Chart 1, inset, shows that the rate of growth of cells in CDM was the same up to Day 12 as it was for cells grown in regular serum-containing medium, indicating that the decrease in E2NR in the absence of estrogens is not due to a decreased mitotic rate of these cells. The functional activity of estrogen receptor is retained throughout the phases of the growth of the culture. This was demonstrated by measuring the replenishment of E2CR and the induction of PGCR. However, the cytoplasmic appearances of these 2 proteins following a pulse with 17β-estradiol were different in cells during logarithmic growth and after reaching confluence. Specifically, E2CR reappeared more rapidly during logarithmic growth (3 days) than it did in postconfluent cultures (>6 days). Continuous exposure of postconfluent culture of MCF-7 cells to 17β-estradiol resulted in the continued elevation in PGCR over a period of at least 5 days. Removal of the estrogen caused the concentration PGCR to decrease within 1 day accompanied by an elevation of E2CR to levels (1.5 fmol/mg DNA) far above that normally seen in postconfluent cultures.
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