Isolation and Characterization of a Tamoxifen-resistant Cell Line Derived from MCF-7 Human Breast Cancer Cells*

(Received for publication, October 24, 1980, and in revised form, February 3, 1981)

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A tamoxifen-resistant variant (R27) was selected by cloning wild type MCF-7 cells. R27 has the same growth rate as MCF-7 under optimal conditions. But tamoxifen has no effect on the growth rate (growth constant of R27 under control condition, 0.071 ± 0.007 day⁻¹; tamoxifen-treated cells, 0.073 ± 0.011 day⁻¹) and minimal effects on thymidine incorporation in R27, whereas tamoxifen has a strongly suppressive effect on the growth rate and thymidine incorporation in MCF-7. The estradiol-mediated stimulation of growth in R27 is somewhat less than in MCF-7 (growth constants of R27 and MCF-7 are 0.112 ± 0.015 and 0.125 ± 0.009 day⁻¹, respectively. R27 contains unoccupied receptors in both cytoplasmic and crude nuclear extract fractions with slightly higher numbers of cytosol receptors than wild type MCF-7. The same dissociation constant and the same molecular weight estimated by Sephadex chromatography and the same sucrose density behavior were observed in R27 and MCF-7. Extent of competition with estradiol and tamoxifen for cytoplasmic estrogen receptor is the same for R27 and MCF-7. Induction of progesterone receptor following treatment with estradiol is the same for R27 and MCF-7. Estrogen receptor is activated by salt and nucleotide in both MCF-7 and R27; however, the extent of activation is much higher in MCF-7 than R27.

Antiestrogens have been used in clinical trials in the treatment of breast cancer and have been found to be highly effective. The response rate is similar to that of other endocrine manipulations. About 50 to 70% of those patients whose tumors contain estrogen receptors have a beneficial response to antiestrogen therapy (1). Following treatment failure with antiestrogens, some patients will go on to have responses to further ablative endocrine therapies. In the hope of gaining further insight into such paradoxical results we attempted to select antiestrogen-resistant variants derived from a parental hormone responsive population. The MCF-7 cell line is an established cell line derived from a patient with metastatic breast cancer (2), which contains estrogen receptor (3) and is estrogen-responsive (4). Antiestrogens have been shown to compete with estradiol for specific estrogen receptor sites in the cells (4, 5), but competition experiments suggest that they bind to receptor with approximately 100-fold lower affinity than estradiol (5). Antiestrogens translocate the MCF-7 cytoplasmic estrogen receptor to the nucleus (6) and strongly inhibit both macromolecular synthesis and growth in the absence of estrogen (5). Although antiestrogen effects appear to be mediated by the estrogen receptor, evidence for additional saturable antiestrogen specific binding sites distinct from the classical estrogen receptor have been reported in rat uterus (7), chick oviduct (8), and human breast cancer samples (9). In addition, extraction experiments with various ionic strength salt solutions suggest that estrogen receptor complexes are more tightly bound to nuclear components than antiestrogen receptor complexes leading to the suggestion that antiestrogen receptor complexes and estrogen receptor complexes bind to different chromatin loci (10). The facts that not all breast cancers which are estrogen receptor-positive respond to antiestrogen therapy and that distinct cytosol binding sites for estradiol and tamoxifen and different chromatin loci of estrogen receptor complexes and tamoxifen receptor complexes may exist suggest that parallel but separate pathways for estrogen and antiestrogen action may exist. In this study we describe a variant clone derived from MCF-7 human breast cancer cells which retains substantial estrogen responsiveness while having lost inhibitory responses to antiestrogen.

MATERIALS AND METHODS

Chemicals—[2,4,6,7-³H]Estradiol-17β (100 Ci/mmol), R5020, [6,7-³H]17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-diene (87 Ci/mmol), and methyl [³H]thymidine (46 Ci/mmol) were purchased from New England Nuclear. Tamoxifen (trans-[(p-dimethylaminooxy)phenyl]-1,2-diphenyl-but-1-ene; ICI, 46474) was obtained from ICI-US (Wilmingon, DE).

Cells and Tissue Culture—The MCF-7 cell line (2) was the generous gift of Dr. Marvin Rich, Michigan Cancer Foundation (Detroit, MI). The estrogen receptor-deficient MDA-MB-231 human breast cancer cell line derived by Cailleau et al. (11) was provided by Dr. Ronald Herberman, National Cancer Institute (Bethesda, MD). All cell lines were grown in Falcon plastic flasks (75 cm²) or in plastic roller bottles (692 cm²) in improved minimal essential medium developed by Richter et al. (12) (National Institute of Health Media Unit, Bethesda, MD), supplemented with glutamine (0.6 g/l), gentamicin (25 mg/ml), and 5% fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cells were grown in a humidified incubator in 5% CO₂ at 37° C.

Experimental Medium—One day after plating, growth medium was routinely replaced by medium consisting of IMEM® supplemented with glutamine (0.6 g/l), 0.1 unit/ml of porcine insulin (Eli-Lilly), and 5% calf serum stripped of endogenous hormones by 48 min incubation at 50°C twice with a dextran-coated charcoal pellet (0.25% Norit A, 0.0025% dextran in 0.01 M Tris-HCl, pH 7.4, at 4° C) as described previously (4).

Cloning—Cells were cloned in soft agar using a modification of the methods of Sibley and Tomkins (13) and Pfahl et al. (14). MCF-7 cells were grown in IMEM, insulin (0.1 unit/ml), and 5% charcoal-treated calf serum in T-75 flasks as monolayer cultures for 2 weeks.

* This work was presented in part at the 77th Annual Meeting of American Society for Clinical Research, May 1980, Washington D. C. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviation used is: IMEM, improved minimal essential medium.
Medium was changed to serum-free IMEM for 1 day and then MCF-7 cells were treated with 10⁻⁸ M tamoxifen for 1 day. The agar mixture contained 25 ml of 2.5% soft agar (Bacto-agar, Difco), which was autoclaved and, after cooling to 40 to 50 °C, 25 ml of double strength IMEM. 5 ml of charcoal-treated calf serum and 1 ml of insulin in the presence and absence of 0.1 ml of 10⁻⁸ M tamoxifen were added. The final agar concentration was 0.62%. The bottom layer of agar contained 7 ml of agar mixture and the top layer of agar contained 2 ml of agar mixture and 1 ml of cell suspension, yielding a final agar concentration of 0.14%. Both ml of soft agar mixture were transferred to plastic 4-well plates. Agar plates were then covered to stand at room temperature until solidified. The top layer of soft agar and cells (0.7 to 1 x 10⁵ cells/ml of plate) of MCF-7 cells pretreated with tamoxifen) were plated on the bottom layer of soft agar and incubated at 37 °C in the incubator for 1 day. Then 1 ml of IMEM supplemented with 5% charcoal-treated calf serum with 10⁻⁶ M tamoxifen was added on the top layer of soft agar. Once a week this medium was changed. MCF-7 cells were allowed to grow for 6 to 8 weeks to become 2- to 3-mm diameter colonies. Then colonies were isolated with a sterile Pasteur pipette and grown up sequentially in 24-well dishes, progressing to larger flasks with IMEM and 5% fetal calf serum. The efficiency of colony formation for wild type cells was reduced from 10⁻¹ to 10⁻⁴ by the addition of 10⁻⁶ M tamoxifen. One variant, R27, selected in tamoxifen which has remained stably resistant to tamoxifen for 6 months, is characterized in this study.

**Chromosomes Analysis**—Logarithmically growing cells were treated with colchicine (Sigma) and karyotype of R27 and MCF-7 was determined as described (14).

Cells were harvested by washing the dishes once in ice-cold calcium- and magnesium-free phosphate-buffered saline, pH 7.4, and then either suspending the cells with 0.02% EDTA or scraping with a rubber policeman.

**Cell Growth Experiments**—To examine the effects of estradiol and tamoxifen on cell growth, cells growing in log phase plated in 6-well dishes (Costar, Data Packaging, Cambridge, MA) at a density of 2 x 10⁴ cells/dish in IMEM supplemented with 5% charcoal-treated calf serum without insulin. On the following day (day 0) the medium was changed and estradiol (final concentration, 10⁻⁸ M) and tamoxifen (final concentration, 10⁻⁶ M) in ethanol (final concentration, below 0.1%) were added as described (4). Then 0.1% ethanol was added to the control wells. Every 3 days medium was changed and dishes were harvested for cell counts. Cell numbers were determined using an electronic particle counter model B (Coulter Electronics Inc. Hialeah, FL).

**Thymidine Incorporation**—Cells, which had been grown in IMEM supplemented with 5% charcoal-treated calf serum and insulin for 2 weeks, were suspended and plated in 6-well dishes in the same medium. 45°-radiolabelled thymidine was added in suspension of 4 x 10⁴ cells/dish in IMEM supplemented with 5% charcoal-treated calf serum without insulin. In the following day (day 0) the medium was changed and estradiol (final concentration, 10⁻⁸ M) and tamoxifen (final concentration, 10⁻⁶ M) in ethanol (final concentration, below 0.1%) were added as described (4). Then 0.1% ethanol was added to the control wells. Every 3 days medium was changed and dishes were harvested for cell counts. Cell numbers were determined using an electronic particle counter model B (Coulter Electronics Inc. Hialeah, FL)

**Cytosol and Nuclear Extracts**—Cells (1 ml of packed cells/ml of buffer) were resuspended in Tris buffer (10 mm Tris-HCl (pH 7.4), 1.5 mm EDTA, and 0.5 mm dithiothreitol) (TED buffer) for estrogen receptor preparations or in phosphate buffer (5 mm sodium phosphate (pH 7.4), 0.5 mm dithiothreitol, and 10% glycerol) for progesterone receptor preparations and homogenized with a glass Dounce homogenizer ( Kontes) using the B pestle until they were more than 90% disrupted (approximately 50 strokes) as seen by phase microscopy. The homogenate was centrifuged at 800 x g for 5 min and the supernatant was centrifuged at 105,000 x g for 60 min and the supernatant was used as cytosol. The nuclear pellet was washed twice with TED buffer, then resuspended in 1 to 3 ml of Tris buffer (10 mm Tris-HCl (pH 8.5), at 4 °C), 1.5 mm EDTA, 0.5 mm dithiothreitol, and 10% glycerol) containing 0.6 M KCl (TEDK buffer) and incubated for 30 min. Every 10 min the pellet was homogenized with a glass Dounce homogenizer. Solubilized protein was then obtained by centrifugation at 100,000 x g for 60 min. DNA was determined from the high speed nuclear pellet by a ethidium bromide method (15).

**Dextran-coated charcoal assay for cytosol estrogen receptor**—Concentrated cytosol protein content was measured by absorbance ratio at A₂₅₀ nm/A₃₄₀ nm (16), then adjusted to a concentration of 2 mg/ml with TED buffer. Protein values were determined later by the method of Lowry et al. (17). Overnight, 0.1 ml of cytosol with 0.1 ml of increasing concentrations of [³H]estradiol with or without a 100-fold excess of diethylstilbestrol were incubated at 0 °C. Then 0.1 ml of dextran-coated charcoal (0.25% Norit A and 0.0025% dextran in TED buffer) was added. The tubes were vortexed and kept for 2 min at 0 °C and then centrifuged at 800 x g for 10 min. As described (18), 0.8 ml of supernatant was counted in 8 ml of aquasol. Binding data were prepared for Scatchard analysis (19) by computer-assisted methods (20).

**Hydroxypatite Assay for Nuclear Estrogen Receptor**—A hydroxypatite slurry (200 ml) in 50 mm Tris-HCl and 10 mm KH₂PO₄, pH 7.2, in a packed/volume ratio of approximately 0.7 was added to the tubes as described (21) and then centrifuged at 800 x g for 5 min and the supernatant was removed. Then the nuclear extract (200 µl) previously diluted with phosphate buffer to produce a protein concentration of 1.0 mg/ml was added to the tubes. The mixture was incubated on ice for 30 min, vortexed every 10 min, and centrifuged at 800 x g for 3 min and the supernatant was discarded. Then 0.1 ml of increasing concentrations of [³H]estradiol with or without a 100-fold excess of diethylstilbestrol was added to the tubes and incubated at 0 °C overnight. Following incubation the pellets were washed three times with phosphate buffer plus 1% Tween 80 (Sigma) and then extracted overnight at room temperature with 1 ml of ethanol. The extracts were counted in 10 ml of Aquasol as described (22). Binding data were analysed by Scatchard plot (19).

**Sucrose Gradients for Progesterone Receptor and Estrogen Receptor**—Ten nm [³H]R5020 (final concentration) and 100 nm nonradioactive R5020 were added in 5 µl of ethanol to 500 µl of cytosol (2 to 5 mg/ml of protein) and incubated at 0 °C for 4 h. After incubation the complex was centrifuged at 100,000 x g and 0.1 ml of the supernatant was added to 200 µl of 200-fold excess of diethylstilbestrol. The cytosol was then extracted with 0.1 ml of 100-fold excess of diethylstilbestrol. [³H]Estradiol charged receptor was sedimented through a 5 to 20% sucrose gradient prepared with TED buffer or TED buffer containing 0.4 M KCl. The procedure for gradients was the same as that for progesterone receptor.

**DNA-Cellulose**—DNA-cellulose was prepared according to Litman (23), with Whatman CF-11 cellulose and calf thymus DNA (Worthington). Then 0.1 ml cytosol (2 to 3 mg/ml) prepared in TED buffer was incubated with 0.1 ml of 5 nm [³H]Estradiol for 4 h at 0 °C with either 100-fold excess of diethylstilbestrol. The cytosol complexes were "activated" by incubating with different concentration of KCl or with 10 mM ATP, CTP, and GTP (final concentration) at 0 °C for 1 h. Then these charged cytosols (0.5 ml) were incubated with DNA-cellulose (1 ml packed volume) for 1 h with shaking at 0 °C. At the end of the incubation, 10 ml of cold TED buffer were added and then the contents were centrifuged. This washing was repeated 5 times. Then [³H]-labeled estrogen receptor complexes bound to DNA-cellulose were extracted with 1 ml of ethanol at room temperature overnight. The 2,500 x g supernatant was assayed for radioactivity.

**Single saturating dose hydroxypatite exchange assay**—Unoccupied cytoplasmic and nuclear receptors were determined by incubation of hydroxylapatite-receptor complexes at 0 °C for 15 h with 5 x 10⁻⁸ M [³H]Estradiol. Total (tamoxifen-occupied plus unoccupied) sites were determined by incubation of cytosol at 30 °C for 2 h and nuclear extracts at 37 °C for 2 h with 1 x 10⁻⁸ M [³H]Estradiol, when maximal saturation was obtained. Nonspecific binding was determined by a parallel incubation with [³H]Estradiol plus a 100-fold excess of diethylstilbestrol. The difference between total and unoccupied sites yields the values for tamoxifen-occupied sites.

**RESULTS**

The karyotype of R27 is the same as that of MCF-7 by chromosome analysis. R27 contains the marker chromosomes of MCF-7 cells.

**Effect of Hormones on Cell Proliferation**—Effects of 10⁻⁸ M estradiol and 10⁻⁷ M tamoxifen on cell growth of MCF-7 and R27 are shown in Fig. 1. Physiological concentrations of
estradiol (10^{-8} M) clearly stimulate cell division, while the antiestrogen, tamoxifen, is strongly inhibitory in MCF-7 cells. Growth constants (K) under optimal growth conditions for MCF-7 and R27, which were calculated from the slope of linear regression lines, are 0.071 ± 0.005 and 0.070 ± 0.007 day^{-1}, respectively. MCF-7 and R27 have the same growth rate. Addition of 10^{-8} M tamoxifen to MCF-7 cells results in strong growth inhibition. R27 shows no growth inhibition by tamoxifen treatment. The growth constant of R27 with tamoxifen is 0.073 ± 0.011, almost the same as that of R27 under control conditions. In fact, the dose-response curve (Fig. 2A) demonstrates that 50% growth inhibition by tamoxifen is achieved at 2 × 10^{-7} M in MCF-7. However, R27 shows the same dose-response curve to tamoxifen as MDA-231, which does not contain any estrogen receptor. No growth inhibition was seen even at 2 × 10^{-7} M tamoxifen. The growth inhibition at 5 to 10 × 10^{-6} M tamoxifen is thought to be a nonspecific toxic effect of tamoxifen both in MDA-231 and R27. Thus, R27 is completely resistant to the specific inhibitory effects of tamoxifen. With 10^{-6} M estradiol stimulation, the growth constant increased from 0.071 ± 0.005 to 0.125 ± 0.009 day^{-1} in MCF-7 (P < 0.05) and from 0.071 ± 0.007 to 0.112 ± 0.015 day^{-1} (P < 0.05) (Fig. 1). Thus, both MCF-7 and R27 show the stimulatory effect of estradiol on cell growth. But the effect of estradiol on cell growth is slightly weaker in R27 than MCF-7 (P < 0.1). MCF-7, R27, and MDA-231 have 5-, 2.5-, and 0-fold increases in cell numbers after 15 days of incubation with estradiol, respectively. Thus, R27 is slightly less responsive to estradiol with respect to cell growth than MCF-7 (Fig. 2B).

**Effect of Hormones on Thymidine Incorporation in MCF-7 and R27**—The effect of estradiol and tamoxifen alone and in combination on MCF-7 and R27 are shown in Fig. 3A. As we have previously shown (4, 5), 10^{-8} M estradiol stimulates thymidine incorporation by 50% over control. Combination of 10^{-8} M estradiol and 10^{-6} M tamoxifen reverses the tamoxifen suppressive effect in MCF-7 cells. On the other hand, in R27 there was no significant suppression of thymidine incorporation induced by tamoxifen. Stimulation by estradiol is seen. The dose-response curve for inhibition of thymidine incorporation by tamoxifen is shown in Fig. 3B. The thymidine incorporation of MCF-7 cells was inhibited by 60% at 5 × 10^{-6} M tamoxifen. However, R27 was inhibited only by 10%.

**Sucrose Density Gradients of Progesterone Receptor**—Progesterone receptor activity is known to be under estrogen control and progesterone receptor synthesis involves the estrogen receptor system (6). Progesterone receptor activity was determined by sucrose density gradient analysis to see whether the entire sequence of events involving the estrogen receptor system was functional. Cells growing in 5% charcoal-stripped calf serum and insulin have very low quantities of progesterone receptor both in MCF-7 and R27. Shows that this low basal level is increased 5- to 6-fold by a 4-day treatment of log phase cells with 10 nm estradiol in both MCF-7 and R27. Progesterone receptor sediments at 4.5 S in 0.4 M KCl. Similar inductive effects were obtained when progesterone receptor activity was examined in gradients prepared under low salt conditions except that binding peaks of R5020 sedimented at about 8 S (data not shown). Although these results suggest that the estrogen receptor system is functional in R27, estrogen receptor activity in R27 was determined in cytosol and nuclei and compared with that of MCF-7.

**Subcellular Distribution of Estrogen Receptor**—To determine any differences in estrogen receptor between MCF-7 and R27, saturation curves and Scatchard analysis (19) of estrogen receptor in nuclei and cytosol were prepared. Results are shown in Fig. 5. Cytosol estrogen receptor was determined by dextran-coated charcoal assay and nuclear estrogen receptor was determined by hydroxyapatite assay. R27 contains slightly more cytosol estrogen receptor than MCF-7 (R27, 142 ± 25 fmol/mg of protein, MCF-7, 106 ± 15 fmol/mg of protein). The nuclear receptor has a 2-fold higher binding affinity than cytosol receptor both in MCF-7 and R27 (Fig. 5). MCF-
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Fig. 4. Sucrose gradients of cytoplasmic progesterone receptor in MCF-7 and R27. Cells grown in T-75 flasks were treated 4 days with IMEM containing 5% charcoal-treated calf serum and insulin with (○) or without (□) 10 nM estradiol. Medium was changed every 2 days. Cytosol (3 to 5 mg of protein/ml) was incubated with 10 nM [3H]R5020 at 4°C for 4 h, treated with dextran-coated charcoal for 10 min, and then centrifuged through 5 to 20% sucrose gradients with phosphate buffer plus 10% glycerol. To determine nonspecific binding, cytosol from estradiol-treated cells was incubated with 10 nM [3H]R5020 and a 100-fold excess unlabeled R5020 (△). Arrow indicates 4.6 S sedimentation peak of [14C]bovine serum albumin (BSA).

7 and R27 each have a 3-fold higher content of nuclear associated receptor than cytosol receptor. Values for nuclear receptor in MCF-7 and R27 are: 310 ± 34 and 379 ± 45 fmol/mg of protein; values for cytosol receptor in MCF-7 and R27 are: 106 ± 15 and 142 ± 25 fmol/mg of protein.

Competition of Unlabeled Estradiol and Tamoxifen with [3H]Estradiol for Estrogen Receptor in MCF-7 and R27—In view of the fact that the failure to respond to tamoxifen could represent an affinity mutant of receptor for antiestrogens, the ability of increasing concentrations of unlabeled estradiol or tamoxifen to compete with [3H]estradiol for binding sites was compared. Both compete to the same extent. However, the tamoxifen competition curve was displaced 100-fold to the right of the estradiol competition curve as described previously (5). No difference in competition curves was observed between MCF-7 and R27.

Sucrose Gradients and Sephadex Chromatography on Cytoplasmic and Nuclear Estrone Receptor—To determine any differences in the molecular moiety of estrogen receptor in cytosol and nuclei between MCF-7 and R27, sucrose density gradients and Sephadex G-100 gel filtration of estrogen receptor were done. Fig. 6 shows that, in the presence of 0.4 M KCl salt, cytosol estrogen receptor of MCF-7 and R27 sediments at 4.5 and 4.6 S, respectively. Unoccupied receptor obtained from a crude nuclear estrogen receptor of MCF-7 and R27 sediments at 4.4 and 4.5 S, respectively. There is no significant difference in sedimentation coefficients for nuclear or cytosol estrogen receptor between MCF-7 and R27. Molecular weights of cytosol and nuclear estrogen receptor both in MCF-7 and R27 were calculated and compared from the elution position on Sephadex G-100 using bovine serum albumin, ovalbumin, and chymotrypsinogen as internal standard in the presence of 0.4 M KCl. All receptor forms from both cytosol and nuclei in MCF-7 and R27 have the same estimated molecular weight of 51,000 under these conditions.

Activation of Estrogen Receptor Complexes in MCF-7 and R27—Activation of estrogen receptor complex by heat and salt results in increased binding to nuclei purified from mammary tissue. DNA-cellulose was used as a substitute for purified nuclei in measuring the binding of hormone receptor complexes (24). No heat activation of estrogen receptor complex was observed using gentle temperature and short heating periods. Dose-dependent increases in activation of estrogen receptor complexes by salt (KCl) and nucleotide are shown in

Fig. 5. Saturation curves for estrogen receptor in cytosol and nuclei of MCF-7 (○) and R27 (□). The insets show the Scatchard analyses of [3H]estradiol binding to MCF-7 (○) and R27 (□) cytosol (A) and nuclear extract (B). Cytosol estrogen receptor was determined by dextran-coated charcoal assay and nuclear estrogen receptor was determined by hydroxyapatite assay as described under "Materials and Methods." All plots are corrected for nonspecific binding. E2, estradiol; B/F, bound/free.

Fig. 6. Sucrose gradients of cytoplasmic and nuclear estrogen receptor with 0.4 M KCl. Cytosol and nuclear extracts (3 to 5 mg of protein/ml) were incubated with 10 nM [3H]estradiol at 0°C for 4 h, treated with dextran-coated charcoal for 10 min, and then sedimented through 5 to 20% sucrose gradient with TED buffer containing 0.4 M KCl. Competition with 100-fold excess diethylstilbestrol was essentially the same for MCF-7 and R27. Arrow indicates the 4.6 S sedimenting peak of [14C]bovine serum albumin (BSA).

Fig. 7. Significant differences in activation as assessed by DNA cellular binding between MCF-7 and R27 were observed. The 3.3- and 2.5-fold increases in DNA-cellulose binding of estrogen receptor complexes were induced by KCl and nu-
cleotide in MCF-7 and R27, respectively. Similar differences in DNA-cellulose binding were detected in repeated experiments.

Effect of Treatment Time with Tamoxifen on Estrogen Receptor Content and Distribution in MCF-7 and R3 Treated with $10^{-6}$ M Tamoxifen—Fig. 8 shows the levels of total cytoplasmic and nuclear receptors in cells treated with $10^{-6}$ M tamoxifen for 5 h in both MCF-7 and R27. Untreated control cells have unoccupied estrogen receptor both in cytoplasm (Rc) and in a crude nuclear fraction (Rn). When $10^{-6}$ M tamoxifen is added, the cytoplasmic receptor binds tamoxifen and complexes are translocated to the nucleus as shown by the reciprocal increase in tamoxifen-occupied sites and decrease in unoccupied cytoplasmic receptor. Once a tamoxifen-occupied site appears in the nucleus, a step occurs as described by Horwitz and McGuire (6). The level of total detectable estrogen receptor is reduced by 50% from the level seen in untreated cells by the end of the 5-h incubation. On the other hand, total receptor in R27 is not changed during this incubation period. In two other experiments of a similar nature, processing of total receptor in R27 was always less than 20% while 50 to 70% of the receptor in MCF-7 was processed. Thus, processing of tamoxifen receptor complexes is much less in R27 as compared to wild type MCF-7 cells. In all experiments, the persistence of high levels of apparently unoccupied nuclear receptor and less amounts of unoccupied cytoplasmic receptor is presumably an artifact of the substantial exchange of occupied receptor sites which can occur even at 0°C with the less tightly bound tamoxifen.

**FIG. 7.** Binding of $[^3]$H]estradiol (E2) receptor complex to DNA-cellulose. DNA binding was determined as described under “Materials and Methods.” Per cent bound of $[^3]$H]estradiol (E2) receptor complex to DNA-cellulose is shown. Open bars, MCF-7; closed bars, R27.

**FIG. 8.** Time course of translocation of estrogen receptor (ER) and estrogen receptor distribution in MCF-7 and R27 exposed to 1 μM tamoxifen. Two T-150 flasks/point were incubated at 37°C with IMEM plus 5% stripped calf serum and insulin plus 1 μM tamoxifen. Total receptor complexes were assessed as described under “Materials and Methods.” Rc, unoccupied cytoplasmic receptor; Rn, unoccupied nuclear receptor; RnT, sites occupied by tamoxifen.

**DISCUSSION**

As already shown in glucocorticoid resistant lymphoma cell lines (14, 25, 26, 27), the isolation and characterization of steroid-resistant clones from cells normally inhibited by the hormone has aided in clarifying the molecular mechanism of hormone resistance. We used the same method to select tamoxifen-resistant clones in soft agar containing $10^{-6}$ M tamoxifen which kills the MCF-7 cells. R27, which was selected in soft agar with tamoxifen, is virtually completely resistant to tamoxifen as assessed by lack of suppression of cell growth with $10^{-6}$ M tamoxifen treatment and very little suppression of thymidine incorporation by tamoxifen. R27 shows a stimulatory effect of estradiol on cell growth and also has progesterone receptor inducible to about the same extent as MCF-7. Progesterone receptor is known to be controlled by the estrogen receptor system (6). Therefore, these results suggest that the sequence of events controlled by the estrogen receptor system in R27 is functional even in the antiestrogen resistant cell. In R27, antiestrogen and estrogen effects are clearly separable. The discrepancy between nearly complete resistance to tamoxifen and a functional estrogen receptor system in R27 suggests as one possibility that a different receptor mechanism for estrogen and tamoxifen may exist. Antiestrogens bind and translocate cytoplasmic estrogen receptor to the nucleus (4–6). In these respects antiestrogen effects appear to be mediated by estrogen receptor. However, the antiestrogen specific binding site may be different from the classical estrogen receptor (7, 8). Antiestrogen chromatin binding loci appear different from estrogen receptor complex binding (10). Different nuclear processing reactions for estrogen and antiestrogen-bound receptor (27) also suggest that estrogen and antiestrogen may have two different but functionally related binding sites and/or chromatin loci, one for the hormone, the other for antiestrogen.

The studies shown in Fig. 8 suggest that some event distal to the initial binding of tamoxifen to receptor may be defective in R27. This is shown by the substantial decrease in processing of antiestrogen-receptor complexes as compared to wild type cells.

The activation studies shown in Fig. 7 suggest that a single receptor (or its activating system) may be altered in R27. A more potent agonist such as estradiol may still be able to initiate estrogen mediated events whereas tamoxifen is ineffective. In any event further analysis of this and other unusual variants may permit a more exacting knowledge of the mechanisms by which estrogens and antiestrogens induce their diverse phenotypic effects.

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J. Biol. Chem. 1981, 256:5016-5021.

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