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Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells

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ABSTRACT

We have developed a transient transfection system using the Cytomegalovirus (CMV) promoter to express the human estrogen receptor (ER) at very high levels in COS-1 cells and have used it to study the interaction of agonist and antagonist receptor complexes with estrogen response element (ERE) DNA. ER can be expressed to levels of 20–40 pmol/mg or 0.2–0.3% of total soluble protein and all of the soluble receptor is capable of binding hormone. The ER binds estradiol with high affinity (Kd 0.2 nM), and is indistinguishable from native ER in that the receptor is capable of recognizing its cognate DNA response element with high affinity, and of transactivating a transgene in an estradiol-dependent manner. Gel mobility shift assays reveal interesting ligand-dependent differences in the binding of receptor complexes to ERE DNA. Receptors occupied by estradiol or the type I antiestrogen trans-hydroxytamoxifen bind to DNA response elements when exposed to the ligand *in vitro* or *in vivo*. Likewise, receptors exposed to the type II antiestrogen ICI 164,384 *in vitro* bind to ERE DNA. However, when receptor exposure to ICI 164,384 is carried out *in vivo*, the ER-ICI 164,384 complexes do not bind to ERE DNA, or do so only weakly. This effect is not reversed by subsequent incubation with estradiol *in vitro*, but is rapidly reversible by *in vivo* estradiol exposure of intact COS-1 cells. This suggests there may be some cellular process involved in the mechanism of antagonism by the pure antiestrogen ICI 164,384, which is not observed in cell-free extracts.

INTRODUCTION

The actions of estrogens are mediated by their nuclear receptor. The estrogen receptor is a member of the conserved family of nuclear receptors characterized by a ligand binding region (carboxyl terminus), a variable region (amino terminus), and a well conserved central DNA binding region that shares homology with the transcription factor TFIIIA. Upon binding ligand, estrogen receptor dimers recognize their cognate response element on DNA. Both hormone and antihormone receptor complexes bind to DNA with only the former being able to effectively modulate gene expression (1–4). Data suggest that the hormone and antihormone complexes display different conformations which are dependent on the nature of the ligand (1,4). Presumably, the transcription apparatus 'reads' an antiestrogen-receptor complex differently from an estrogen-receptor complex, possibly involving the interaction of factors exclusive for one complex or the other. To study these possibilities, expression of high levels of biologically active receptor is a prelude to the development of an *in vitro* transcription system for studying the actions of estrogens and antiestrogens on a transcriptionally active promoter. Unfortunately, attempts to express active full-length steroid hormone receptors at high levels in bacteria and yeast have met with limited success (5–10). Receptors expressed in these organisms also show some differences in bioactivities, leading to speculation that the receptor may be modified by the host (8,9). It appears that a mammalian expression system may be necessary to produce receptor that is most ideal for these types of studies.

The nature of the interaction of antiestrogen-occupied receptor complexes with chromatin may be influenced by which of the two classes of antiestrogens occupies the receptor. AEs such as tamoxifen and LY117018 (11,12) are considered partial agonists/antagonists and are designated as type I antagonists, and compounds such as ICI 164,384 (13) that are complete/pure antagonists are designated as type II AEs. The differences in the biocharacter of these ligands suggest that they may exert their actions through different mechanisms.

Here we report high level transient expression of human estrogen receptor in COS-1 cells and its use in examining the interactions of type I (partial agonist/antagonist) and type II (pure

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antagonist) antiestrogen receptor complexes with estrogen response element DNA. Studies using the pure (type II) estrogen antagonist ICI 164,384 reveal differences in DNA-binding ability of these ligand-occupied receptor complexes when the receptor is exposed to the antiestrogen *in vitro* versus *in vivo*, suggesting that a cellular process or factor is involved in the actions of this antagonist. In contrast, this phenomenon is not observed with the type I estrogen antagonist tamoxifen, therefore indicating differences in the mechanism of action of type I and type II estrogen antagonists.

MATERIALS AND METHODS

Plasmids

All cloning was performed by standard methods (14). The full-length hER clone λOR8 (15) was provided by Dr. Geoffrey Greene, University of Chicago, and was modified by us as described (16) to the wild-type receptor (Gly 400). The cDNA was digested with Sal I and blunted with Klenow. The insert was then placed into the Sma I site of pCMV-4 (17) provided by Dr. David Russell of the University of Texas Southwestern Medical Center. The estrogen responsive plasmids pATC-2 (ERE-TATA-CAT) and ERE-vit-CAT (18) were a gift from Dr. David Shapiro of the University of Illinois at Champaign-Urbana and are described elsewhere (16,18). The oligonucleotide pCCAGGT-CACAGTGACCTGAGCTAAAATAACACATTCAG-OH was annealed to its complement, gel purified, end labeled with ATP-γ-^{32}P (sp. activity 6000 Ci/mmol) and diluted with radioinert oligonucleotide to 25,000 cpm/0.2ng. All enzymes were from BRL (Gaithersberg, MD) or United States Biochemical (Cleveland, OH).

Hormones and antihormones

The estrogens 17-estradiol (E_2), estrone, estriol, diethylstilbestrol and hexesterol were from Sigma. The antiestrogens trans-hydroxytamoxifen (OHT), 1-[4-(2-dimethylaminoethoxy) phenyl]-1-(4-hydroxyphenyl)-2-phenyl-but-1(Z)-ene and ICI 164,384, [N-n-butyl-11-(3,17-dihydroxy-oestra-1,3,5(10)-trien-7α-yl)N-methyl-undecamide] were kindly supplied by Dr. Alan Wakeling and ICI Pharmaceuticals, Macclesfield, U.K.

Cell culture and transfection

COS-1 cells were maintained in DME/F12 medium minus phenol red (Sigma, St. Louis, MO) supplemented with 10% charcoal-dextran treated fetal calf serum (CDFCS) (Sigma) and penicillin and streptomycin. For transfection, cells were seeded in 100mm dishes at a density of $2-2.5\times10^5$ cells per plate. Forty hours later, the cells were transfected by the DEAE-dextran (Pharmacia, Piscataway, NJ) method (19) or by the calcium phosphate method (16) at 40−60% confluency. Although both methods gave equivalent levels of expressed receptor, the DEAE-dextran method was used more frequently because of its simplicity. Prior to transfection, cells were washed twice with pre-warmed phosphate buffered saline (PBS). Three ml of PBS containing 500μg/ml DEAE-dextran plus 5μg vector DNA (unless noted otherwise) was added to the plates. The plates were incubated for 30 min at 37°C, the solution was aspirated and media containing 50μM chloroquine (Sigma, St. Louis, MO) was added. The cells were treated with chloroquine for 4−5 h, followed by a 3 min DMSO shock (10% DMSO in serum-free media). Plates were rinsed in Hanks Balanced Salt solution (HBSS) and 10 ml of fresh media were added. After 40 h, cells were harvested in cold PBS. The cell pellet was homogenized in a volume of 100μl per plate in whole-cell extract buffer (50mM Tris-HCL pH 7.5, 1.5 mM EDTA, 0.5 mM β-mercaptoethanol, 10 mM Na_2MoO_4, 0.5 M NaCl, 10% (v/v) glycerol) plus leupeptin 10 μg/ml, aprotinin 10μg/ml, 0.2 mM PMSF, and 2.5 μg/ml pepstatin-A. The homogenate was incubated on ice for 30−45 min and centrifuged at 100,000×g to yield a whole-cell extract. Cytosols were prepared similarly except the homogenization buffer contained 50 mM NaCl. The salt-extracted pellet was then resuspended in SDS-loading buffer, heated at 90°C for 10 min and spun at 100,000×g to yield the detergent soluble material.

For the gene transfer studies, 1.5×10^5 COS cells were plated in 60mm dishes in DME/F12 supplemented with 5% charcoal-dextran treated calf serum (CDCS). Forty-eight hours later cells were transfected by the $CaPO_4$ co-precipitation technique as described previously (16). 0.4 ml of precipitate containing 5ng CMV-ER, 2μg reporter plasmid (ERE-vit-CAT or pATC-2), 0.2μg pCH110 (β-galactosidase internal control plasmid), and 5.8μg carrier DNA was added per plate. Cells remained in contact with the precipitate for 4−6 h and were shocked for 2.5 min with 10% (v/v) DMSO in serum-free media. Fresh media and hormones were added and the cells harvested 36 h later. Much less ER expression vector was used in these CAT reporter experiments than those described above, where production of large quantities of receptor were desired. Because studies have shown that steroid receptors undergo a phenomenon similar to 'squelching' (21), much lower amounts of expression vector must be used in gene transfer experiments in order to avoid sequestering of necessary transcription factors by the excess receptor which would result in squelching of gene transcription (16,21,22). Hence, when larger quantities of expression vector are used, we find that the overexpression of receptor causes marked suppression of both the basal and estradiol-dependent CAT activity, as previously documented (2,22). Calcium phosphate co-precipitation was used to transfect the cells in these CAT reporter gene experiments, because we have found that this is a much more reliable method than is the DEAE-dextran procedure for delivering small quantities of a mixture of plasmid DNAs used in these experiments (22). Chloramphenicol acetyltransferase assays were performed as described (23) and were standardized to β-galactosidase activity in each sample.

Hormone binding assays

Whole cell extracts were diluted to 100−150 mM NaCl with homogenization buffer containing 10 mM NaCl. Cytosol from untransfected COS cells was added, when necessary, to maintain the protein concentration between 0.2−0.5 mg/ml. Forty-five microliters of diluted extract was incubated for 2 h at 22°C in the presence of 10 nM ^{3}H-estradiol (SA 90 Ci/mmol, NEN Boston, MA) ± 300-fold radioinert estradiol. Free steroid was removed by dextran-coated charcoal assay (16). Binding was analysed by the method of Scatchard (24). Whole cell extracts were also labeled with the ER affinity label ^{3}H-Tamoxifen Aziridine (TAZ) (SA 23.9 Ci/mmol, Amersham, Arlington Heights, IL) as described previously (25).

Hormone binding was also measured by whole cell assay (26). Briefly, cells were released in trypsin-EDTA and incubated in DME/F12 supplemented with 0.5% CDFCS containing 10 nM ^{3}H-E_2 ± 300-fold radioinert E_2 for 30 min at 37°C. Free steroid was removed by washing the cell pellet three times with ice-cold PBS plus 1% Tween 80 (Sigma, St. Louis, MO). The cell pellet was added directly to scintillation fluid and counted.

Cells were labeled with 25 nM ^{3}H-Tamoxifen Aziridine. Cells were incubated with 25 nM ^{3}H-TAZ ± 500-fold E_2 in media supplemented with 0.5% CDFCS at 37°C for 40 min, medium was removed and 10 ml fresh medium lacking hormones was added and incubated for 10 min at 37°C. Media were removed and the cells were washed twice with cold PBS and harvested. Extracts were prepared as described above. Samples were then fractionated on an 11% SDS-PAGE gel as described (27). The gel was fixed in 10% methanol, 8% acetic acid in water (v/v) and stained with Coomassie blue. The gel was destained and treated with ENHANCE (NEN, Boston, MA) as recommended by the manufacturer. The gel was dried onto filter paper and exposed at −80°C for approximately 1−3 days.

Western blotting

Cell extracts were added to SDS-PAGE loading solution and boiled for 3−5 min. Samples were then loaded onto a denaturing gel consisting of a 3.8% stacking gel and an 11% separating gel according to Laemmli (27). Proteins were transfered to nitrocellulose by electrophoresis and immunoblotting performed as described using the ER specific monoclonal antibody H222 (28).

Gel shift assays

Transfected cells were treated with hormones for 2 h at 37°C in their media, washed twice in ice-cold PBS, and harvested. The cell pellet was resuspended in 75μl of gel shift buffer (20 mM Tris-HCl pH 7.4, 1 mM DTT, 0.5 M NaCl, 10% (v/v) glycerol plus 50μg/ml leupeptin, 50μg/ml aprotinin, 2.5μg/ml pepstatin, 5μg/ml antipain,and 0.2 mM PMSF) and frozen on dry ice. The cells were lysed by two cycles of freeze/thaw (dry ice/ice) and the debris pelleted in a microfuge at 4°C. The extracts were frozen away in aliquots at −70°C.

One-two microliters of extract (400−800ng protein) were pre-incubated on ice for 15 min in a reaction containing: 20 mM Tris-HCl pH 7.4, 1 mM DTT, 100 mM NaCl, 10% glycerol (v/v), 100μg/ml BSA, 1μg poly dI-dC in 15μl. Radiolabeled ERE (0.2 ng) in one microliter of TE was added and incubated for 20 min at room temperature. The samples were directly loaded onto a pre-run 4.5% acrylamide (30% acrylamide/0.9% bis-acylamide) nondenaturing gel, using 0.5×TBE as a buffer. The gel was run at 200V until the free probe approached the bottom of the gel. The gel was fixed in 20% methanol-10% acetic acid (v/v) in water and dried onto filter paper.

In Situ histochemistry

DNA uptake by the cells was estimated by incubating cells transfected with pCH110, the β-galactosidase expression vector, with X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside). Transfected cells were washed with PBS and fixed for 15 min at 22°C in a solution containing 100 mM sodium phosphate pH 7.0, 1 mM $MgCl_2$, and 1% glutaraldehyde. The plates were rinsed with PBS and then 3 ml of incubation solution was added which consisted of: 10 mM phosphate pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_63H_2O$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-gal, and 20% DMSO. Plates were incubated at 37°C for 30−60 min. The blue cells were identified under a phase-contrast microscope.

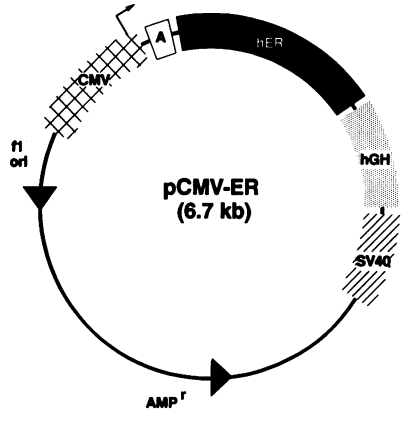

Figure 1. Plasmid map of pCMV-ER. Expression of the wild-type estrogen receptor cDNA (hER) is driven by the Cytomegalovirus promoter (CMV). This plasmid also contains the tobacco mosaic virus translational enhancer signals 5′ to the polycloning site (A) and the human growth hormone polyadenylation and RNA processing sequences (hGH). The Simian virus 40 origin of replication (SV40) allows the plasmid to achieve multiple copies within cells expressing the SV 40 T antigen.

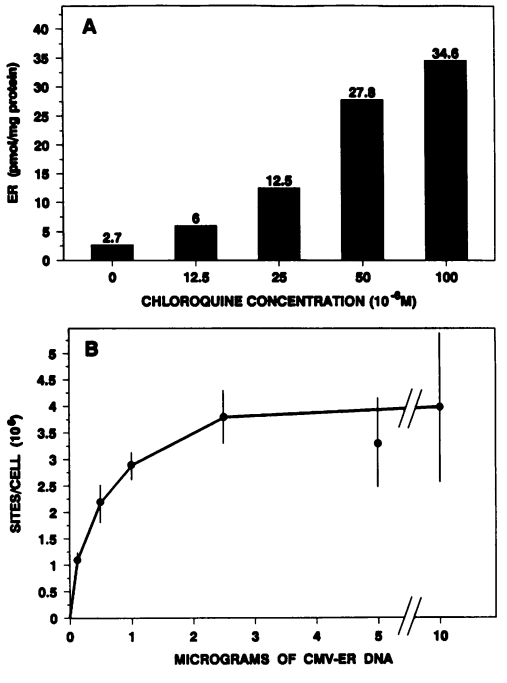

Figure 2. (A) Regulation of ER expression in COS-1 cells by chloroquine treatment. COS-1 cells were transfected with 5μg of CMV-ER by the DEAE-dextran method as described in the Methods section. Following DEAE-dextran treatment, the cells were treated with increasing concentrations of chloroquine in their culture medium for 5 h, followed by DMSO shock. Cells were harvested 42 h later. ER content in whole cell extracts was measured by dextran-coated charcoal assay as described in Methods and is expressed as pmol/mg protein. Data shown represents typical results from one experiment. The mean and standard deviation of ER expression from eight independent transfections when the cells were treated with 50μM chloroquine was 27.3 ± 2.2 pmol/mg protein when the same CMV-ER plasmid preparation was used. *(B) ER expression in COS-1 cells in response to increasing plasmid concentrations.* COS-1 cells were transfected with increasing amounts of CMV-ER, followed by chloroquine treatment (50μM) as described in Methods. ER content was measured by a whole cell binding assay (26), and is expressed as sites/cell on a mixed population of transfected and untransfected cells. Data is not corrected for the percentage of cells actually taking up the plasmid (*ca.* 10−20%, see text). Points represent the mean and standard deviation of 2−5 independent transfections.

RESULTS

Expression of ER in COS-1 cells

The plasmid used in this study to express ER in COS-1 cells, pCMV-ER (Fig. 1), was constructed by placing the human ER cDNA (containing Gly 400) into the Sma I site of pCMV-4 (17). This plasmid contains the powerful Cytomegalovirus promoter, the tobacco mosaic virus translational enhancer sequences, the human growth hormone (hGH) 3′ sequences, and the SV 40 origin of replication (29).

The level of expression of ER in these cells can be controlled by two means: by varying the amount of DNA transfected or by the addition of chloroquine to the culture media. In the absence of chloroquine these cells can express ER to levels around 3 pmol/mg protein (Fig. 2A). Increasing the chloroquine concentration up to 100μM raises the receptor levels to 30−40 pmol/mg protein. This represents 0.2−0.3% of total soluble protein. The expressed receptor bound estradiol with high affinity, displaying an equilibrium dissociation constant (Kd) of 0.2 nM (data not shown). At chloroquine concentrations above 50μM cell death increases as a function of the chloroquine concentration, so for most studies the chloroquine concentration used is 50μM.

The ER levels per cell can also be controlled by increasing the amount of plasmid transfected into the cells. As shown in Figure 2B, even relatively small amounts of plasmid DNA give high levels of ER expression. As little as 125 ng of plasmid per 100mm plate of cells achieves ER levels of approximately 1 million sites per cell as measured by a whole cell binding assay. ER levels plateau at approximately 2.5 μg of plasmid DNA when 50μM chloroquine is used, achieving levels of 3−5 million sites per cell (Fig. 2B). These same trends are also seen when receptor levels are measured in whole cell extracts rather than by whole cell assay (data not shown).

It should be noted that the receptor levels expressed as sites per cell represent an estimation on a mixed population of cells, most of which do not take up the plasmid. So these values do not give an accurate estimation of the number of receptor molecules in any given cell. To estimate the percentage of cells that take up DNA, cells were cotransfected with 4 μg of pCMV-ER and 1 μg of the β-galactosidase expression vector pCH110. These cells were then subjected to *in situ* histochemistry using the β-galatosidase substrate X-gal. By observing the number of blue cells, it was estimated that 10−20% of the cells take up the plasmid. It is conceivable, therefore, that ER content in the transfected cells could exceed 40 million sites per cell.

Appearance of the expressed ER was observed as early as 12 hours, reached a plateau at 36 hours and was stable up to 60 hours following transfection (data not shown).

Intracellular distribution and activity of COS expressed ER

The intracellular distribution of the expressed ER was examined by Western blot analysis using the ER specific monoclonal antibody H222. Cytosols, nuclear extracts and detergent soluble fractions were prepared from cells treated with or without estradiol and were subjected to analysis (Fig. 3). In the presence or absence of estradiol, most of the soluble ER was in the salt-extractable form. A significant fraction of the ER is in the detergent-soluble form. The amounts of ER found in the detergent-soluble fraction varied between transfections, typically ranging between 2 and 5 times that of the salt soluble component. To determine if the ER contained in the detergent soluble fraction was capable of binding hormone, cells were labeled with the ER specific affinity label Tamoxifen Aziridine (TAZ) in culture. Figure 4 reveals that both the salt-extractable and the detergent-extractable ER were labeled by ^{3}H-TAZ in an estradiol competable fashion, indicating that the detergent soluble material is capable of binding hormone.

Western blot analysis (Fig. 3) and fluorography of TAZ labeled receptors (Fig. 4) reveal that the ER exists predominantly as one form with a migration similar to that of ER from the receptor-rich breast cancer cell line MCF-7. It is of note that three additional forms are observed by both methods of receptor detection. A larger component that migrates at approximately 75 kD and two smaller forms at 55 and 50 kD were observed.

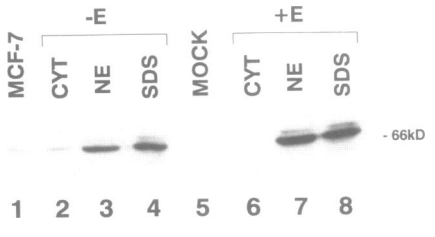

Figure 3. Intracellular distribution of ER in COS-1 cells. Transfected cells treated with (lanes 4−7) or without (lanes 2−3) 10^{-8} M estradiol for 40 min were fractionated to yield a low-salt (50mM NaCl), a high salt (0.6M NaCl), and a detergent (SDS) soluble fraction as described in the Methods section. Approximately one-forth of the total cytosol (approximately 30μg of protein; lanes 2 and 6), nuclear extract (approximately 30μg of protein; lanes 3 and 7) and detergent soluble material (lanes 4 and 8) recovered from one 100 mm plate of transfected cells was fractionated on an 11% SDS-PAGE gel. Seventy-five micrograms of protein from a whole cell extract of mock transfected COS-1 cells (lane 5) and 100μg of protein from a whole cell extract of MCF-7 cells were added as negative and positive controls, respectively. Proteins were transferred to nitrocellulose and ER was detected by Western blotting using the ER-specific monoclonal antibody H222.

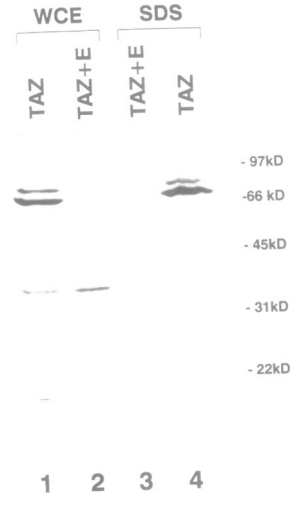

Figure 4. Covalent labeling of ER in intact cells. Transfected cells were incubated with 25 nM ^{3}H-Tamoxifen Aziridine plus (lanes 2 and 3) and minus (lanes 1 and 4) 500-fold excess radioinert E_2 for 40 min in their culture media. Cells were harvested and salt (WCE, whole cell extract) and detergent (SDS) soluble fractions were prepared as described in Methods. One-forth of the salt (lanes 1 and 2) and detergent soluble (lanes 3 and 4) material recovered from one 100 mm plate of transfected cells was fractionated on an 11% SDS-PAGE gel. Bands were visualized by fluorography. Mobilities of the molecular weight protein markers are shown at the right

The smaller forms are observed in MCF-7 cell extracts and may represent either alternate translation or proteolytic fragments of the receptor (30). The larger form has not been observed in MCF-7 cells, although a similar form has been seen with ER expressed in the insect Baculovirus system (4). This form most likely represents spurious translation from a start codon in-frame with the ER cDNA and could be eliminated, we found, by cloning into a different site in the vector (see Fig. 8). These extra bands usually represented less than 10% of the total ER produced in these cells.

The hormone binding activity of the soluble ER was compared with that of ER isolated from MCF-7 cells. ER content in whole cell extracts from both transfected COS cells and MCF-7 cells was measured by hormone binding assay, and then amounts of extract corresponding to 50, 100, 200 and 400 fmol ER were subjected to Western blot analysis. Comparison of the immunoreactivity of the COS and MCF-7 ER revealed that the COS ER and the MCF-7 cell ER contained the same levels of ER protein, indicating that the COS expressed ER was as active as native receptor in binding hormone (data not shown).

COS-expressed ER can stimulate CAT activity from an estrogen-responsive promoter

The ability of COS-ER to transactivate was examined by co-transfecting into COS-1 cells pCMV-ER and one of two estrogen-responsive reporter plasmids. COS-ER was able to induce CAT activity in cells transfected with pATC-2, a minimal promoter containing two EREs and a TATA box, or ERE-vit-CAT, a complex promoter which contains an exogenous ERE inserted into the vitellogenin promoter linked to the CAT gene (Fig. 5). As shown in this experiment, and in additional experiments not shown, the increase in CAT activity was estradiol-dependent and could be abolished by treating the cells with a 500-fold excess of the antiestrogen ICI 164,386. Cells treated with ICI alone showed no agonist activity, similar to results that have been reported elsewhere (3,31). The specificity of the transcriptional response was examined by treating cells with various steroids. The estrogenic compounds estradiol, estrone, estriol, diethylstilbestrol, and hexestrol were able to stimulate CAT activity, while the steroids progesterone, testosterone, and dexamethasone failed to elicit CAT activity (data not shown).

Receptor DNA-binding: differences between *in vitro* versus *in vivo* hormone exposure

The ability of the COS-expressed ER to recognize its cognate DNA response element *in vitro* was examined by gel mobility shift assay. Gel mobility shift patterns show a major retarded complex that can be displaced by 100-fold excess radioinert oligonucleotide and can be 'super shifted' by pre-incubation with the ER monoclonal antibody H222 (Fig. 6). The affinity (K_d) of the expressed ER for the ERE probe *in vitro*, estimated by gel shift assay, was approximately 1 nM (Data not shown). A slower and a faster-migrating band are also observed in the gel shift patterns. These bands are also super shifted up by H222, indicating that ER is present in the complexes. Interestingly, a similar mobility pattern was observed for Baculovirus-expressed ER (4). The COS-expressed ER demonstrates mobility patterns, when bound to various ligands, similar to those observed by others (1−4); that is, the ER binds to the ERE in the presence or absence of estradiol, and ER-estradiol complexes move more rapidly than ER-antiestrogen complexes (Fig. 7). These distinct electrophoretic mobilities of estrogen- and antiestrogen-occupied ER-ERE complexes may reflect differences in the structures of the hormone and antihormone receptor complexes.

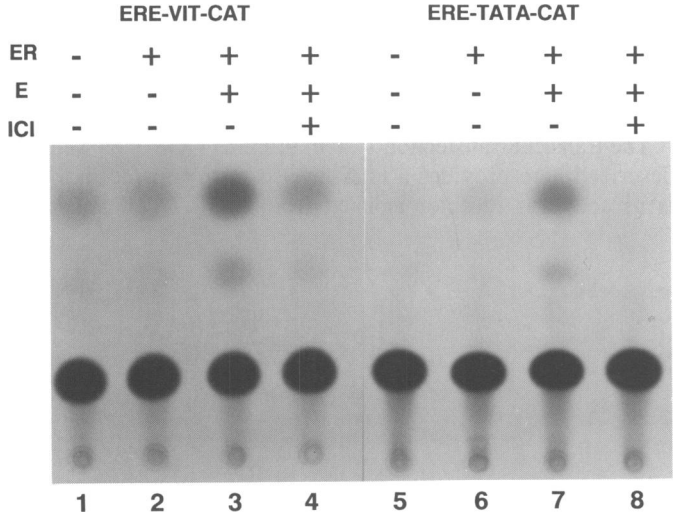

Figure 5. COS expressed ER is able to induce activation of transgenes in response to estradiol. COS cells were transfected with ERE-vit-CAT (lanes 1−4) or pATC-2 containing ERE-TATA-CAT (lanes 5−8) plus an internal control plasmid which expresses β-galactosidase (all lanes), with (lanes 2−4, and 6−8) or without (lanes 1 and 5) CMV-ER by the calcium phosphate coprecipitation technique as described in the Methods section. Cells were treated with 10^{-9} M E_2 (lanes 1,3,5 and 7), E_2 plus a 500-fold excess of ICI (lanes 4 and 8) or control ethanol vehicle (lanes 2 and 6) and were harvested 36 h later and assayed for CAT activity. CAT assays were performed on extracts containing equal amounts of β-galactosidase activity.

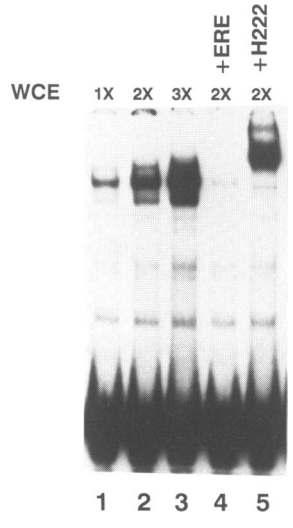

Figure 6. DNA binding activity of COS expressed ER. The DNA binding activity of ER in whole cell extracts (WCE) prepared from COS cells exposed to 10^{-8} M estradiol was examined by gel mobility shift assay. Volumes of extract corresponding to 0.17 μg (1×), 0.34 μg (2×), and 0.68 μg protein (3×) were incubated with 0.2 ng ^{32}P-labeled ERE probe alone (lanes 1, 2, and 3, respectively); or 0.34 μg protein was incubated with ^{32}P-labeled ERE probe plus a 100-fold excess of radioinert probe (lane 4), or plus 1 μg of the anti-ER antibody H222 (lane 5). DNA-protein complexes were resolved from the free probe on a 4.5% nondenaturing gel.

Surprisingly, marked differences in DNA-binding activity are seen with the antiestrogen ICI 164,384 when the receptor is exposed to the ligand *in vitro* versus in intact cells *in vivo*. Receptor isolated from transfected cells that had been treated with ICI in the culture media for 2 h is unable to bind DNA or does so only weakly, while receptor preparations from untreated cells can bind to DNA if the receptor preparations are exposed to the ligand *in vitro* (Fig. 7, compare lanes 4 and 7). The reduced DNA-binding of receptor exposed to ICI *in vivo* cannot be compensated for by increasing the amount of the ER-containing extract in the binding reaction by two, three, or four-fold (data not shown), indicating that the residual DNA-binding observed in some preparations does not follow traditional kinetic mechanisms. This effect is specific for ICI, because receptors incubated *in vitro* or in intact cells with estradiol or the antiestrogen trans-hydroxytamoxifen (OHT) do bind to DNA (Fig. 7, lanes 3 and 6). Western blot analysis of the whole cell extracts confirms that the extracts from cells treated with ICI *in vivo* contain similar amounts of receptor protein (Fig. 8), indicating that this effect is not due to a rapid down-regulation of ER which has been observed in the mouse uterus (34) and in some breast cancer cells treated with ICI (22). The ICI-dependent loss of DNA-binding can be antagonised when cells are treated with ICI plus a ten-fold excess of estradiol (Fig. 7, lane 8). This effect could not be reversed by subsequent incubation with an excess of E_2 or ICI under exchange conditions *in vitro* (fig. 7, lane 9); however it is rapidly reversible *in vivo*. Receptor isolated from cells that had been treated with 10^{-7} M ICI for 2 h, followed by a 30 min exposure of the cells to 10^{-6} M estradiol alone, was capable of binding to DNA, indicating that the effect of ICI is rapidly reversible by estradiol *in vivo* (Fig. 7, lane 10).

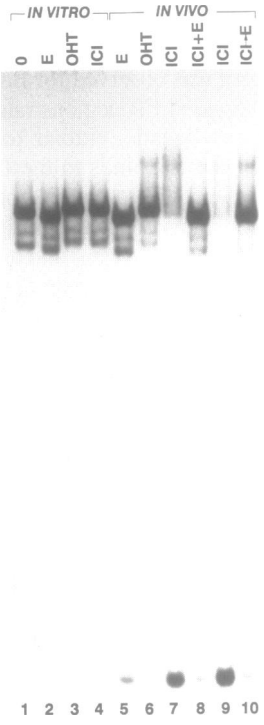

Figure 7. DNA binding activity of COS expressed ER exposed to estrogen and antiestrogen ligands *in vivo* or *in vitro*. Whole cell extracts were prepared from control cells which have been transfected under identical conditions and treated with control ethanol vehicle only (lanes 1–4) or from cells treated with 10^{-8} M E_2 (lane 5), 10^{-8} M trans-hydroxytamoxifen (OHT) (lane 6), or 10^{-7} M ICI 164,384 (lane 7 and 9) in their culture media. A separate plate of cells was used for each treatment. Receptor isolated from treated cells were incubated directly with 0.25 ng of ^{32}P-labeled probe (lanes 5–8) without further treatment. Extracts from control cells were incubated with ligands *in vitro* for 30 min at 37°C (lane 1, control, no hormone; lane 2, 10^{-8} M E_2; lane 3, 10^{-8} M OHT; and lane 4, 10^{-7} M ICI). The DNA binding abilities of receptor isolated from cells treated with ICI 164,384 in their culture media followed by no treatment (lane 7), or followed by incubation with 10^{-6} M estradiol *in vitro* under exchange conditions (30 min at 37°C; lane 9) were examined. Lane 8 shows the DNA binding of receptor exposed to ICI plus a 10-fold excess of estradiol *in vivo*. DNA-binding activity was also examined in extracts from cells treated with 10^{-7} M ICI in their culture media for 2 h, followed by removal of the media and replacement with media containing 10^{-6} M E_2 alone for 30 min (lane 10).

DISCUSSION

A potentially informative stategy for examining the mechanism by which antihormones manifest their activities involves the use of *in vitro* techniques such as DNA-binding assays and *in vitro* transcription systems. Since the estrogen receptor (ER) is a very rare protein and these techniques require significant quantities of receptor, it is important to have an expression system that yields a high level of active material. Although classical overexpression systems have frequently involved the use of bacteria, attempts at expressing a full-length steroid receptor in bacteria have failed. While steroid receptors have been expressed more successfully in yeast and insect cells than in bacteria, isolation of large quantities of active material capable of binding hormone has been troublesome (4,8–10). These problems have forced researchers to establish permanently transfected mammalian cell lines. Establishing these cell lines is a very labor intensive process and results have sometimes been disappointing since the receptor levels achieved have often been only an order of magnitude above physiological tissues (32,33). Surely there is a need for a transient transfection system that allows expression of high levels of active receptor without the tedious tasks of cell selection/cloning or viral plaque purification, which preclude the possibility of expressing a large number of different mutant proteins for structure-activity studies on receptor.

The transient transfection system utilizing COS cells we have described here provides a high level of estrogen receptor that is as active as receptor isolated from MCF-7 cells: it binds

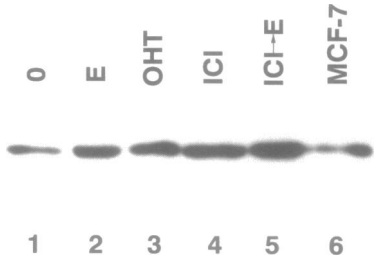

Figure 8. Western blot analysis of ER in whole cell extracts used in the DNA-binding studies. Receptor content in 5 μg of protein from the whole cell extracts used in Figure 7 (ten times the protein used in the DNA binding reactions) was examined using the ER-specific monoclonal antibody H222 and was found to be similar following the different hormonal treatments. Cells were treated with control vehicle (lane 1), E_2 (lane 2), OHT (lane 3), ICI (lane 4), and ICI followed by treatment with estradiol alone for 30 min (lane 5), as in Fig. 7. One-hundred micrograms of whole cell extract protein prepared from MCF-7 cells was used as a positive control (lane 6).

estradiol with similar affinity, and binds to its cognate DNA response element specifically and with high affinity and it is capable of driving transcription from an estrogen-responsive promoter with the expected ligand specificity. This ER has enabled us to examine in detail the interaction of estrogen and antiestrogen ligand-receptor complexes with DNA, as discussed below.

Overexpression of a biologically active estrogen receptor

The estrogen receptor can be expressed in COS cells to levels of 20−40 pmol/mg protein, which are approximately 50 to 100 times that found in normal estrogen target tissues such as rat uterus or pituitary, and human breast cancer cells that contain high levels of ER (35,36). Unlike many of the yeast systems (8−10) and ER produced in Baculovirus (4), the expressed receptor is as active in hormone binding studies as MCF-7 cell ER. This system also has the important feature of expression control. By simply varying the chloroquine concentration, one can achieve receptor levels ranging between 2 and 35 pmol/mg protein (Fig. 2A). Using a whole cell receptor assay, we measure 3−5 million sites per cell (Fig. 2B), an astonishing number considering that only 10−20% of the cells are transfected.

The soluble receptor exists predominantly in a salt-extractable form in the absence or presence of estradiol. There appears to be a large fraction of receptor that remains in the nuclear pellet following salt extraction, and this can be solubilized by detergent (Fig. 3). The detergent soluble material is capable of binding hormone, because tamoxifen aziridine (TAZ) labels this fraction and the labeling is competed for by estradiol (Fig. 4). The appearance of detergent soluble material is likely a consequence of overexpression and not an artifact of this particular system. This hypothesis is supported by the fact that the stably transfected Chinese Hamster Ovary cell line ER409 (37), that expresses ER to levels of 3 million sites/cell, also contains a significant amount of ER in the detergent soluble fraction. It is possible that this phenomenon may be a cell's defense against the problems associated with very high level expression of proteins that may become deleterious to cellular functions.

The system described here may be useful for the expression of a wide range of proteins, because it is also capable of expressing progesterone receptor to high levels as well. Using the cDNA which encodes for either the A or the B form of the receptor, we find that progesterone receptor can be expressed to levels of 20−30 pmol/mg protein, or 3−4 million sites per cell (data not shown).

DNA-binding assays reveal different mechanisms for the two classes of antiestrogens

The mechanisms by which antiestrogens exert their biological effects are unclear (1−4,11,12). One class of antiestrogens (type I antiestrogens), such as tamoxifen, display mixed agonist/antagonist properties. Recent studies suggest that the partial agonist activity of type I antiestrogens may be a consequence of the hormone-independent transactivation function of the estrogen receptor localized in the amino terminal of the receptor, while their antagonistic action is attributable to the fact that they block the hormone-dependent transcription activation mediated by the hormone binding domain of the receptor (3). The other class of antiestrogens (class II antiestrogens) represented by ICI 164,384, displays no agonist activity and is therefore considered to comprise 'pure' antiestrogens. It is reasonable to believe that these two classes of antiestrogens manifest their bioactivities by a dissimilar mechanism.

Gel mobility shift assays reveal that the human ER binds to an ERE *in vitro* in the presence or absence of hormone (Fig. 7). This is in good agreement with several previous reports (2,4). In addition we find that the human ER is capable of binding to an ERE when occupied by the antiestrogen ICI 164,384 when ligand exposure is carried out *in vitro*. This contrasts with a recent study which suggests that ICI 164,384 exerts its antagonistic effects by impairing the receptor's ability to bind to DNA (31). However, this latter view is not universially accepted and other studies also indicate that ER-ICI receptor complexes do bind to DNA (2,38−40).

This study is unique in that it describes differences in the DNA binding abilities of receptors exposed to ligand *in vitro* or *in vivo*, therefore suggesting a role for a cellular process or factor in the actions of the antiestrogen ICI 164,384. The differences reported in the literature in different studies may be explained by similar phenomena. Most studies have examined the ability of receptor exposed to ICI and other ligands *in vitro* to bind to EREs (2,4,31,39), and some aspects of ligand-receptor interaction in intact cells could have been overlooked.

It is of interest that we observe differences in ERE DNA binding when the receptors are exposed to ICI *in vivo* versus *in vitro*. This suggests there may be some modification of the receptor inside the cell that prevents the transition of ER to a state that can interact effectively and stably with EREs. This modification is not reversed by estradiol *in vitro*, but is rapidily reversible *in vivo*, suggesting that a cellular mechanism is involved. The modification of DNA binding proteins by cellular factors is not unprecedented. Studies on the fos and jun proteins reveal that DNA binding is modulated through a redox of a single cysteine in the DNA-binding regions of the protein (41). The authors identified a nuclear protein that reduced fos and jun and this reduction was necessary for DNA binding. We do not propose that the phenomenon seen here involves a redox reaction, but certainly some intracellular event is occurring that influences the binding of the ER-ICI complex to DNA; and it could possibly involve the modification of the receptor by cellular factors. Cellular extracts have been shown to influence the DNA binding abilities of progesterone receptor (42), retinoic acid receptor (43) and the thyroid hormone receptor (44,45). Another possible explanation for this phenomenon may be that the binding of ICI to the receptor inside the cell may cause dissociation of factors that are necessary for receptor-DNA interaction. Non-receptor proteins do play a role in receptor-DNA binding, and it is of note that a previous study identified a single-stranded DNA binding protein that is obligatory for ER-DNA interaction (46). The results we have observed are specific for the type II antiestrogen ICI, because no differences in ER-ERE binding are observed between *in vivo* and *in vitro* incubations with the type I antiestrogens OHT (Fig. 7) or LY117018 (data not shown). It is possible that the disparity seen between ICI and the other antiestrogens could reflect the dissimilarity in their mechanisms of action. Whereas both OHT and LY117018 (type I antiestrogens) display partial agonist activity, the relatively pure (type II) antiestrogen ICI does not (3,31).

Like the ER, the progesterone receptor (PR) also binds two classes of antihormones, type I and type II antiprogestins. A recent study revealed, through DNA-binding assays, that PR bound by a type II antiprogestin (a pure antagonist) failed to bind to DNA (47), and thus was unable to drive transcription from

a reporter template *in vitro*. This result suggests that there are similarities in the molecular actions of type II antihormones of different steroid classes.

Further examination of this system will be useful in elucidating the mechanism of antihormone action by revealing the means by which the cell alters the receptor-ICI complexes so as to preclude its interaction with DNA.

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