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Relationship between Estrogen Structure and Conformational Changes in Estrogen Receptor/DNA Complexes

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The effect of estrogen structure on the conformation of the complex formed with estrogen receptor (ER) and the consensus estrogen response element (ERE_c) has been examined with gel mobility shift assay. Proteins in MCF-7 cell extracts formed three distinct complexes with ERE. Only the slowest moving complex contained ER as indicated by binding with anti-ER antibodies H222 and D547. This ER–ERE complex displayed increased electrophoretic mobility when formed in the presence of estradiol (E_2) and bound radiolabeled 16α-iodoestradiol. The antiestrogen ICI 164,384 decreased the mobility of the ER–ERE complex and blocked the effect of E_2. The results reported here indicate that the position and location of hydroxyl groups on the estratriene nucleus is an important factor in determining the mobility of ER–ERE_c (or a variant ERE) in gel shift assays. The ability of E_2 analogs to cause conformational changes detectable as altered mobility was not directly related either to their binding affinity for ER or to their ability to activate E_2 responsive genes. Although several dihydroxyestrogens (estradiol-16α, 1- and 2-hydroxyestratrien-17β-ol) caused an increase in the mobility of the ER–ERE_c, other ligands (estradiol-17α, 4-hydroxyestratriene-17β-ol, 3-hydroxy estratriene, estratrien-17β-ol and 5-androsten-3β, 17β-diol) with a capacity for activating at least some E_2 responsive genes in MCF-7 cells had little or no effect. On the basis of these and previously published results, it can be concluded that specific structure features of estrogens are responsible for conformational changes of ER–ERE complexes detectable in gel-shift assays. Furthermore, the identified structural characteristics of the ligand which are required for gel-shift are not the same as those previously reported to be essential for stimulation of transcriptional activity of ER.

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INTRODUCTION

Steroid hormones regulate gene transcription via receptor binding [1]. Although the hormone–receptor complex is required for transactivation, the mechanisms governing this phenomenon are not fully understood. Available information indicates that an important aspect of activation resides in conformational changes induced in the receptor by ligand binding [2].

Recently, Allan *et al.* have distinguished between the conformational changes necessary for DNA binding of the steroid–receptor complex and those directly related to the creation of a transcriptionaly active form [3]. Ligand-free receptor is able to form a complex with the ERE in the absence of Mg^{2+} that is stable to electrophoresis through native polyacrylamide gels [4]. However, in presence of estradiol-17β (E_2), this complex takes on a conformation that moves more rapidly in gels than the ligand-free receptor–ERE complex. It has been postulated that the conformational change induced in the estrogen receptor (ER) by E_2 binding results in activation of the transactivation function of the ER [2, 4, 5]. A similar alteration in the ER–ERE complex is brought about when the non-steroid estrogen DES is the ligand [4, 6]. However, in the presence of antiestrogenic ligands, the mobility of the ER–ERE complex is actually decreased (ICI 164,384, [6]; tamoxifen, [7]). These findings indicate that even though antiestrogens do not interfere with the binding of ER

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to the ERE, they have markedly different effects on the conformation of the ER–ERE complex than estrogenic ligands. This suggests a relationship between conformation and the capacity of the ligand–ER–ERE complex to activate transcription [2, 4, 6].

In the studies reported here, we have assessed the effect of the ligand's structure on the mobility of ER–ERE complexes in native polyacrylamide gels and related this effect to our previously published results defining the capacity of these estrogen analogs to stimulate a series of estrogen responsive endogenous and transfected genes [8–10].

MATERIALS AND METHODS

Steroids and antibodies

The estrogens used in these investigations were either purchased (estratriene, estrone, estradiol-16α, estradiol-17α, estriol and E_2) from Research Plus, Inc. (Bayonne, NJ) or synthesized in this laboratory. The A-ring isomers of E_2 (1-, 2-, and 4-hydroxyestratriene-17β-ol) were synthesized according to published procedures [11]. Synthesis of monohydroxyestrogens (3-hydroxyestratriene and estratriene-17β-ol) has also been reported [12, 13]. Each estrogen analog was purified by thin layer chromatography and crystallization. The level of contaminants in each estrogen was shown to be less than 1 part in 10,000 [8]. 5-Androstene-3β,17β-diol was purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified as described by VanderKuur *et al.* [8]. The antiestrogen 4-hydroxytamoxifen was a gift from Stuart Pharmaceutical (Division of ICI Americas, Inc., Wilmington, DE) and ICI 164,384 was kindly supplied by D.A.E. Wakeling, Imperial Chemical Industries (Alderly Park, England). 3,17β-Estradiol-16α-[^{125}I]iodo (2200 Ci/mmol) was obtained from Dupont NEN (Wilmington, DE).

Monoclonal antibodies directed against ER, H222 and D547, were gifts from Abbott Diagnostics Division (Abbott Laboratories, Abbott Park, IL). The antibody to P53 (Ab-I) was obtained from Oncogene Science (Uniondale, NY).

Cell culture

MCF-7 human breast cancer cells (subclone E3, [14]) were maintained at 37°C in phenol red-free, HEPES buffered Eagles modified MEM supplemented with 0.05 μg/ml gentamicin sulfate and 5% donor calf serum. Cells were plated in 75 cm^2 tissue culture flasks as described previously [15] and routinely passaged prior to reaching confluency. All experiments utilized cells derived from passages 168–197.

ER extraction

MCF-7 cells were grown to near confluence ($\sim 1.8 \times 10^7$ cells/75 cm^2 flask). Cells were harvested by removing growth medium and then disrupting the monolayer with a stream of warm (37°C) culture medium rapidly expelled through a 5″-long, 14-gauge cannula attached to a 25 ml syringe. Suspensions of single cells were obtained by repeated (10–15 strokes) aspiration and expulsion through the cannula. All subsequent operations were carried out at 4°C. Cells were collected by centrifugation at 800 *g* and suspended in 2 ml of TED buffer [10 mM Tris–HCl, pH 7.4; 1.5 mM ethylene-diaminetetracetic acid (EDTA); 1 mM dithiothreitol (DTT), 5 μg/ml each of antipain dihydrochloride, leupeptin, chymostatin and pepstatin (Boehringer Mannheim, NY)]. The suspended cells were transferred to a 1 ml glass homogenization tube which was centrifuged at 600 *g* for 3 min. The pelleted cells were suspended in an equal volume of TED and lysed with 20 strokes of a teflon pestle. The homogenate was spun at 100,000 *g* for 1 h. The supernatant (cytosol), which contained a range of 3–5 pmol/ml of functional ER as determined by the dextran coated-charcoal binding assay [11] was stored at 4°C for use within 6 h.

Preparation of radiolabeled ERE

Complementary oligodeoxyribonucleotide strands containing a consensus ERE (GATCCAGGTCACAGTGACCTGGGCCCG-27 bp) in 0.4 M Tris (pH 7.5) were annealed by heating to 90°C for 10 min, followed by slow cooling (70°C, 1 h; 60°C, 1 h; 50°C, 0.5 h; 37°C, 0.5 h). For end labeling with ^{32}P, 900 ng of annealed ERE was incubated for 30 min at 37°C with 50 U T_4 polynucleotide kinase and 100 μCi γ^{32}P-ATP (sp. act. 3000 Ci/mmol) in a final volume of 50 μl reaction buffer (buffer and enzyme were supplied by New England Biolabs). After stopping the reaction by addition of 2 μl 0.5 M EDTA, pH 8.0, radiolabeled DNA was separated from unreacted ATP by filtration through Sephadex G-50 equilibrated with TE buffer (10 mM Tris–HCl, pH 7.4; 1.5 mM EDTA). Radiolabeled DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 200 μl TE.

Complex formation

Conditions for optimal complex formation between ERE and ER in MCF-7 cytosol were determined empirically. To maintain the highest possible concentration of ER, reaction components were added directly to the cytosol at concentrations which did not increase its volume by more than 10%. In the standard reaction, 1.25 μg poly dI-dC, 1.8 ng ($\sim 10^5$ cpm) ^{32}P-labeled ERE and the indicated concentrations of estrogens were added to 25 μl or ER extract, mixed and incubated at 4°C for 18 h. All stock solutions of estrogens were prepared in ethanol at concentrations that would not increase the volume of the reaction to >27.5 μl or the concentration of ethanol in the reaction to >2%. Control reactions without estrogens contained an equivalent amount of ethanol.

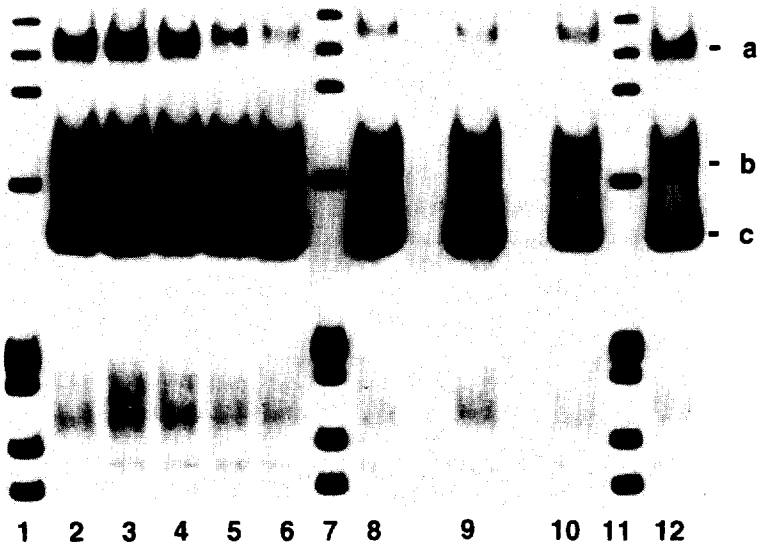

Fig. 1. Effect of E_2 and antiestrogen (ICI 164,384) on the mobility of MCF-7 protein–ERE_c complexes in a gel shift assay. Extracts of MCF-7 cells were incubated with ^{32}P-labeled ERE_c, E_2 and/or ICI 164,384 for 18 h at 4°C. The number and mobility of complexes formed was identical when ODNs were only added during the final 60 min of incubation. ^{32}P-radiolabeled ϕX174 HaeIII was loaded in lanes 1, 7 and 11. Additions to binding reactions were: 5×10^{-7} M E_2 (lane 2), 5×10^{-8} M E_2 (lane 3), 5×10^{-9} M E_2 (lane 4), 5×10^{-10} M E_2 (lane 5), ethanol (lane 6), 5×10^{-6} M ICI 164,384 (lane 8), 2.5×10^{-6} M ICI 164,384 + 5×10^{-8} M E_2 (lane 9), 2.5×10^{-6} M ICI 164,384 + 5×10^{-9} M E_2 (lane 10), 5×10^{-8} M E_2 (lane 12). Protein–ERE complexes are indicated (a, b and c). High levels of E_2 ($>10^{-10}$ M) are shown to increase the intensity of complex-a. This occurred in only 25% of the gels regardless of the ligand (see other figures).

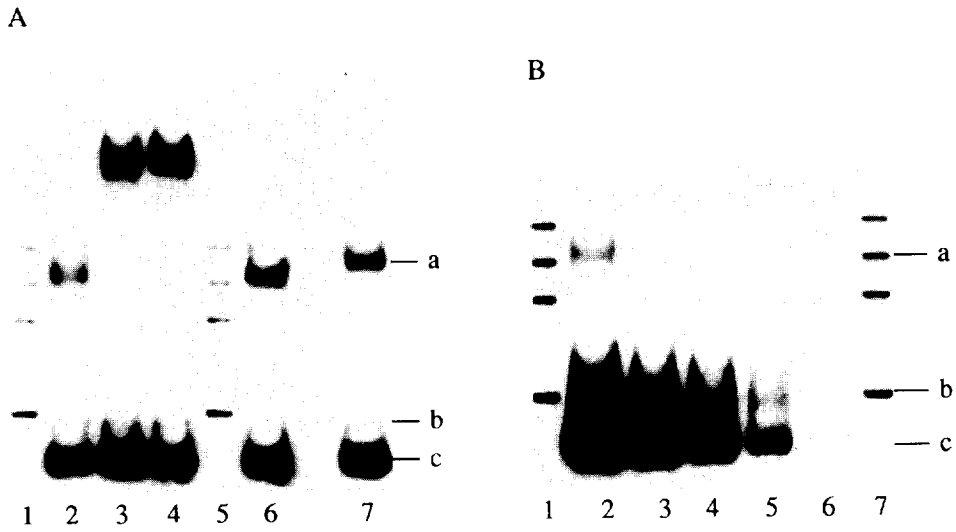

Fig. 2. (A) Effect of anti-ER antibodies on the mobility of MCF-7 cell protein–ERE_c complexes. ^{32}P-radiolabeled ϕX174 HaeIII fragments were loaded in lanes 1 and 5. Additions to the binding reactions were: 1.2×10^{-8} M E_2 + 0.6 μg p53 nonspecific monoclonal Ab (lane 2), 1.2×10^{-8} M E_2 + 1.3 μg H222 (lane 3), 1.2×10^{-8} M E_2 + 0.9 μg D547 (lane 4), 1.2×10^{-8} M E_2 (lane 6), ethanol (lane 7). The binding reaction in lane 2 contained half the level of ER as lanes 3, 4, 6 and 7. In these incubations the E_2 was equilibriated at 4°C for 15 h before the antibody was added to the cold mixture for 2 h. Similar results were obtained in experiments in which E_2 was equilibriated for 2 h and the antibody added to the mixture for 15 h. Binding of antibody to complex-a is shown in lanes 3 and 4. (B) Specificity of binding of MCF-7 cell extracts to ERE_c. Proteins in extracts of MCF-7 cells were allowed to bind to ^{32}P-radiolabeled ERE_c in the presence of 1.2×10^{-8} M E_2 in the absence (lane 2) or presence of unlabeled ERE_c. The molar excess of unlabeled competitor ERE_c was: 12.5-fold (lane 3); 50-fold (lane 4); 100-fold (lane 5); 500-fold (lane 6). ^{32}P-radiolabeled $\phi \times 174$ HaeIII fragments were loaded in lanes 1 and 7. Similar results were obtained in gel mobility shift assays of complexes formed in the absence of E_2.

Gel shift assays

Native polyacrylamide gels (4% polyacrylamide, 14 × 16 cm × 1.5 mm) were prepared and pre-run (running buffer; 6.7 mM Tris, pH 8.0, 3.3 mM sodium acetate, pH 5.2, 1 mM EDTA) as described by Carthew *et al.* [16]. Immediately before loading, 6 μl samples of each reaction mixture were brought to a final volume of 20 μl with a concentration of 5 mM Tris–HCl, pH 7.4; 0.5 mM DTT; 100 mM KCl and 1.5 mM EDTA, 5% v/v glycerol. ^{32}P-radiolabeled φX174 HaeIII markers were diluted in the same manner for use as internal standards for measuring migration distances. Following electrophoresis for 3 h at 15°C with a current of 25 mA, the gels were placed on Whatman 3 mm paper, covered with plastic wrap and dried at 80°C under vacuum for 1 h. Autoradiographs were prepared by exposing Kodak X-OMAT AR film to the dried gel at −70°C with Dupont Cronex Intensifying Screens.

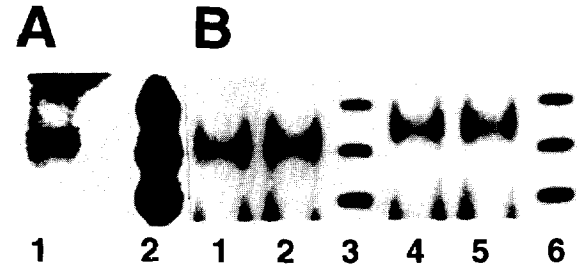

Fig. 3. Binding of 16α[^{125}I]iodo-3,17β-estradiol to MCF-7 cell extract protein–ERE$_c$ complexes. (A) Unlabeled ERE$_c$ (3.6 ng) incubated with 50 μl extract containing 0.13 pmol ER and 0.14 pmol 16α[^{125}I]iodo-3,17β-estradiol (2200 Ci/mmol). The radiolabeled 16α-iodoE$_2$–ER–ERE$_c$ complex is shown in lane 1. The three highest molecular weight φX174 HaeIII fragments can be seen in lane 2. (B) Aliquots of the same MCF-7 cell extract containing 0.13 pmol of ER and 3.5 ng ^{32}P-radiolabeled ERE$_c$ incubated in the presence of 1.2 × 10^{-8} M E$_2$ (lanes 1 and 2) or in the absence of E$_2$ (lanes 4 and 5). ^{32}P-radiolabeled φX174 HaeIII fragments were loaded in lanes 3 and 6. All details as in Fig. 1. Only complex-a is shown in this photograph. The photograph in panel A was made from a digitized image produced on a Molecular Dynamics Laser Densitometer with contrast enhanced by use of display function of the 1-D Gel Analysis software from Protein and DNA Imageware Systems (Huntington Station, NY).

RESULTS

Identification of ER–ERE complexes formed in the presence and absence of E$_2$

Whole cell extract of MCF-7 cells, prepared as described (Materials and Methods), contains a variety of proteins capable of binding to double-stranded oligodeoxyribonucleotides (ODNs) containing an ERE, even in the presence of a 700-fold excess (w/w) of non-specific competitor DNA (poly dI-dC). The three most abundant complexes formed with the consensus ERE (ERE$_c$) are readily separated by electrophoresis in native 4% polyacrylamide gels (Fig. 1). Only the slowest moving of these complexes (complex-a), which has an electrophoretic mobility midway between that of ds-DNA marker fragments 1353 and 1078 bp, displayed altered mobility when formed in the presence of E$_2$. At 10^{-10} M E$_2$, complex-a migrated almost as rapidly as the 1078 bp marker; at higher concentrations of E$_2$ (≥10^{-9} M), the mobility of complex-a slightly exceeded that of the 1078 bp DNA. An identical shift in mobility was observed when the ER–ERE$_c$ complex was formed in the presence of the non-steroidal estrogen, DES at 10^{-8} M (data not shown). The anti-estrogen ICI 164,384 caused a decrease in the mobility of complex-a. At 10^{-6} M, ICI 164,384 completely blocked the positive effect of 10^{-8} M E$_2$ on the mobility of complex-a. Since the migration of complexes-b and -c was unaffected by either E$_2$ or ICI 164,384, it is suggested that only complex-a was formed by binding of ER to ERE$_c$. This was confirmed by adding monoclonal antibodies (mAbs) to ER to the reaction mixture. Antibodies to

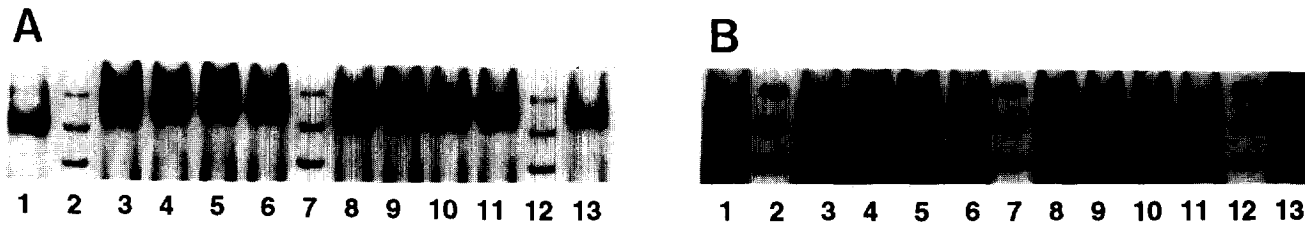

Fig. 4. (A) Effect of estratriene and estratrien-17β-ol on the mobility of the ER–ERE$_c$ complex in the gel shift assay. Estrogen–ER–ERE$_c$ complexes were formed as described in "Materials and Methods" with the indicated concentrations of estratriene, estratrien-17β-ol or E$_2$ added. E$_2$ (1.2 × 10^{-8} M), lane 1, no ligand (ethanol) was added to lane 13. Estratriene: 1.2 × 10^{-5} M, lane 3; 1.2 × 10^{-6} M, lane 4; 1.2 × 10^{-7} M, lane 5; 1.2 × 10^{-8} M, lane 6. Estratrien-17β-ol: 1.2 × 10^{-6} M, lane 8; 1.2 × 10^{-7} M, lane 9; 1.2 × 10^{-8} M, lane 10; 1.2 × 10^{-9} M, lane 11. ^{32}P-radiolabeled φX174 HaeIII fragments were run in lanes 2, 7 and 12. (B) Effect of 1-hydroxyestratrien-17β-ol and 3-hydroxyestratriene on the mobility of the ER–ERE$_c$ complex in the gel shift assay. The following levels of 1-hydroxyestratrien-17β-ol were added to the incubations: 1.2 × 10^{-5} M, lane 3; 1.2 × 10^{-6} M, lane 4; 1.2 × 10^{-7} M, lane 5; 1.2 × 10^{-8} M, lane 6. 3-Hydroxyestratriene was added to the incubations at the following concentrations: 1.2 × 10^{-6} M, lane 8; 1.2 × 10^{-7} M, lane 9; 1.2 × 10^{-8} M, lane 10; 1.2 × 10^{-9} M, lane 11. E$_2$ (1.2 × 10^{-8} M) was added to incubation in lane 1 and ethanol, lane 13. φX174 HaeIII fragments were run in lanes 2, 7 and 12. Only the three highest molecular weight markers and complex-a are shown in this figure.

either the ligand binding domain (H222) or the hinge region of ER (D547) bound to proteins in complex-a with high enough affinity to cause a marked decrease in its electrophoretic mobility [Fig. 2(A)]. The mAbs had no effect on migration of complexes-b and -c.

Additional evidence that complex-a was a specific complex between ER and ERE_c was obtained by adding unlabeled ERE_c to the reaction mixture. In this experiment the amount of radiolabel in all three complexes was diminished [Fig. 2(B)]. However, a 12.5-fold molar excess of unlabeled ERE_c was sufficient to completely block the binding of detectable amounts of radiolabeled ERE_c while a 500-fold (900 ng) excess of unlabeled ERE_c was required to displace radiolabeled ERE_c bound to proteins in complexes-b and -c. This result is consistent with the identification of complex-a as a complex formed by specific binding between ER and the ERE_c. It also indicates some specificity of binding of ERE_c to the proteins in complexes-b and -c, since addition of as much as 10 μg of poly dI-dC reduced but did not completely block ERE_c binding. Thus, it appears that these proteins must either be present in much higher concentration than ER or have a much higher capacity for DNA binding.

Taken together, these results reconfirmed previous reports that estrogenic compounds (E_2 and DES) had a different effect on the conformation of ER–ERE_c complexes than antiestrogens [4–6]. However, they also suggested that compounds with greatly differing chemical structure (E_2 and DES) could induce the same configurational change in the ER–ERE_c complex. Since it is obvious that ligand binding is not necessary for ER–ERE_c complex formation [17], one explanation for this result could be that the presence of ligand in the reaction mixture is sufficient to stably alter the conformation of the complex and that continued binding of ligand is not necessary to maintain the altered conformation. Since our aim was to determine the effect of subtle structural alterations in ligand on the ER–ERE complex, it was important to determine whether these ligands catalyzed a conformational change without stable binding or whether they remained associated with the complex, thus retaining the possibility of affecting its ultimate conformation. To obtain ligand of sufficient specific activity and radiation energy to allow detection of ligand in complex-a, 16α-[^{125}I]iodo-3,17β-estradiol (2200 Ci/mmol) was added to the reaction mixture at 2.8 nM. The presence of labeled 16α-iodoestradiol was detected in a complex with the same mobility as ER-[^{32}P]ERE_c complex formed in the presence of 12 nM E_2 [compare Fig. 3(A) and (B)].

Effect of binding of structurally altered estrogens on the conformation of the ER–ERE complex

Removal of, or relocation of, the hydroxyl groups on E_2 had variable effects on the ability of ligand to alter the mobility of the ER–ERE_c complex. Estratriene, a hydroxyl free estrogen, which possesses an affinity for ER too low to measure [8], did not cause an alteration in the mobility of complex-a until its concentration was 10^{-5} M [Fig. 4(A)]. Even at this high concentration, the effect of estratriene was minor, causing complex-a to migrate only slightly faster than complex-a formed in the absence of ligand. Restoration of the D-ring alcoholic hydroxyl group on the estrogen nucleus (estratrien-17β-ol) greatly increased its affinity for ER-binding (RBA = 0.11, relative binding affinity compared to $E_2 = 1$, $K_a = 3.7 \times 10^9\ M^{-1}$; ref. [8]). However, even at micromolar concentrations, the effect of this monohydroxyestrogen on the mobility of

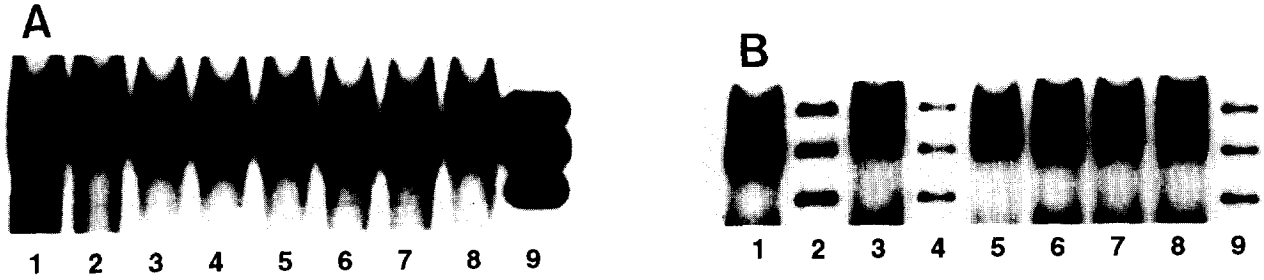

Fig. 5. (A) Effect of 4-hydroxyestratrien-17β-ol on the mobility of the ER–ERE_v complex in the gel shift assay. Extracts of MCF-7 cells were incubated with ^{32}P-radiolabeled ERE_v and estrogen as described in "Materials and Methods" with the exception that γ^{32}P-ATP (6000 Ci/mmol) and 8.2 ng ERE_v were used in the procedures. The following levels of 4-hydroxyestratrien-17β-ol were added to the incubations: 1.1×10^{-5} M, lane 3; 1.1×10^{-6} M, lane 4; 1.1×10^{-7} M, lane 5. E_2 was added to the incubations in following concentrations: 1.1×10^{-9} M, lane 1; 1.1×10^{-8} M, lane 6; 1.1×10^{-9} M, lane 7; 1.1×10^{-10} M, lane 8. Ethanol was added to the incubation in lane 2 and ^{32}P-radiolabeled ϕX174 HaeIII fragments were run in lane 9. (B) Effect of 2-hydroxyestratrien-17β-ol on the mobility of the ER–ERE_v complex in the gel shift assay. Extracts of MCF-7 cells were incubated with ^{32}P-radiolabeled ERE_v and estrogen as described in "Materials and Methods" with the exception that γ^{32}P-ATP (6000 Ci/mmol) and 1.1 ng ERE_v were used in the procedures. The following levels of 2-hydroxyestratrien-17β-ol were added to the incubations: 1.1×10^{-7} M, lane 5; 1.1×10^{-8} M, lane 6; 1.1×10^{-9} M, lane 7; 1.1×10^{-10} M, lane 8. E_2 (1.1×10^{-9} M) was added to the incubation in lane 1. ^{32}P-radiolabeled ϕX174 HaeIII fragments were run in lanes 2, 4 and 9 and ethanol in lane 3. Only the three highest molecular weight markers and complex-a are shown in this figure.

complex-a was minimal [Fig. 4(A)]. Similarly, micromolar concentrations of a ligand with an RBA approaching that of E_2, 3-hydroxyestriene (RBA = 0.80), had no more than a minor effect on the migration of ER–ERE_c [complex-a, Fig. 4(B)] in the gel shift assay.

A-ring isomers of E_2 also demonstrated variations in their effect on mobility of the ER–ERE_c complex. 1-Hydroxyestratrien-17β-ol (RBA = 0.005) caused an increase in mobility of complex-a comparable to that induced by E_2 (10^{-9} M) when present at a micromolar concentration during equilibrium with the ER–ERE_c complex prior to electrophoresis [Fig. 4(B)]. Relocation of the phenolic hydroxyl group of E_2 to one or the other ortho positions on the A-ring had quite different effects on the conformation of the ER–ERE_c complex. Even at the elevated concentration of 10^{-5} M, 4-hydroxyestratrien-17β-ol (RBA = 0.07) had a minor effect on the mobility of the complex between ER and ERE_c (data not shown) or the variant pS2 ERE_v [Fig. 5(A)]. In contrast, 2-hydroxyestratrien-17β-ol (RBA = 0.71), at concentrations of 10^{-7} M or greater, increased the mobility of ER complexes with either ERE_c (data not shown) or ERE_v [Fig. 5(B)] to approx. 50% of that observed for the E_2–ER–ERE_c complex at 10^{-9} M or greater.

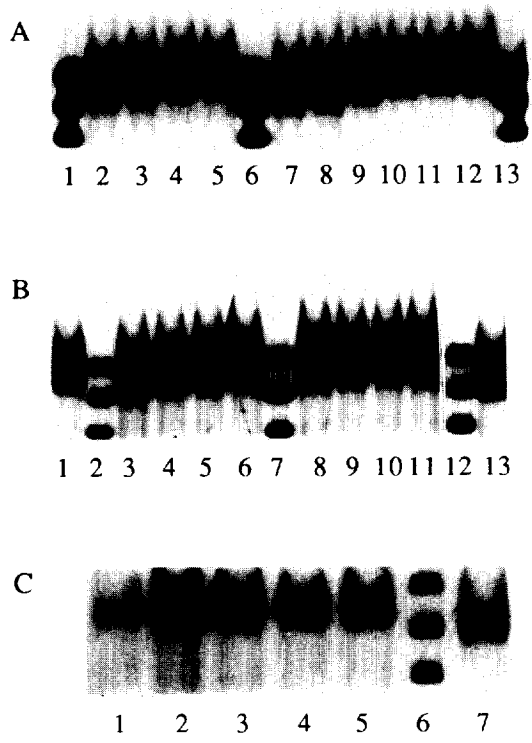

Fig. 6. (A) Effect of estrone and estriol on the mobility of the ER–ERE_c complex in the gel shift assay. The following levels of estrone were added to incubations: 1.2×10^{-8} M, lane 2; 1.2×10^{-9} M, lane 3; 1.2×10^{-10} M, lane 4; 1.2×10^{-11} M, lane 5. Various concentrations of estriol were added to the incubations in the following lanes: 1.2×10^{-8} M, lane 8; 1.2×10^{-9} M, lane 9; 1.2×10^{-10} M, lane 10; 1.2×10^{-11} M, lane 11. E_2 (1.2×10^{-8} M) was added to the incubation run in lane 7 and ethanol in lane 12. ^{32}P-radiolabeled ϕX174 HaeIII fragments were run in lanes 1, 6 and 13. (B) Effect of estradiol-16α and estradiol-17α on the mobility of the ER–ERE_c complex in the gel shift assay. The following levels of estradiol-16α were added to the incubations: 1.2×10^{-8} M, lane 3; 1.2×10^{-9} M, lane 4; 1.2×10^{-10} M, lane 5; 1.2×10^{-11} M, lane 6. Estradiol 17α was added to incubations at the following concentrations: 1.2×10^{-8} M, lane 8; 1.2×10^{-9} M, lane 9; 1.2×10^{-10} M, lane 10; 1.2×10^{-11} M, lane 11. E_2 (1.2×10^{-8} M) was added to the incubation run in lane 13 and ethanol in lane 1. ^{32}P-radiolabeled ϕX174 HaeIII fragments were run in lanes 2, 7 and 12. (C) Effect of 5-androstene-3,17β-diol on the mobility of ER–ERE_c complex in the gel shift assay. The following concentrations of 5-androstene-3,17β-diol were added to the incubations: 1.2×10^{-5} M, lane 2; 1.2×10^{-6} M, lane 3; 1.2×10^{-7} M, lane 4; 1.2×10^{-8} M, lane 5. E_2 (1.2×10^{-8} M) was added to the incubation run in lane 7 and ethanol in lane 1. ^{32}P-radiolabeled ϕX174 HaeIII fragments were run in lane 6. Only the three highest molecular weight markers and complex-a are shown in this figure.

Changes in the D-ring hydroxyl group also caused differential effects on the ability of ligand to mediate conformational changes in the ER–ERE_c complex. Estrone at 5×10^{-10} M (RBA = 0.22) and estriol (RBA = 0.17) at 5×10^{-9} M both induced increases in mobility of complex-a comparable to that induced by E_2 [10^{-9} M; Fig. 6(A)]. However, placement of the D-ring hydroxyl at position 17α (estradiol-17α, RBA = 0.22), brought about a complete loss of the ligand's ability to alter the mobility of complex-a [Fig. 6(B)], even though the binding affinity of this analog for ER was comparable to that of estrone and estriol. In contrast, placement of the D-ring hydroxyl at position 16α (estradiol-16α, RBA = 0.80), still allowed this E_2 analog to bring about a measurable increase in the mobility of complex-a at concentrations as low as 5×10^{-9} M [Fig. 6(B)].

The saturated A-ring of the active estrogen, 5-androsten-3β,17β-diol (RBA = 0.007), prohibited this ligand from altering the mobility of the ERE–ERE_c complex in native polyacrylamide gels [Fig. 6(C)].

DISCUSSION

Identification of the ER–ERE complex

The experiments described herein confirm a previous report that whole cell extracts of MCF-7 cells contain several proteins (i.e. those in complex a, b and c) which bind to and decrease the migration of ds-ODNs containing ERE_c and ERE_v [18]. They further demonstrate that even though ER in these extracts had not been exposed to salt concentrations >15 mM or temperatures >4°C during extraction and incubation with the ERE, complex formation occurred in the absence of added ligand. The ER–ERE complex was identified by: (1) its ability to undergo different conformational changes when formed in the presence of E_2 or ICI 164,384; (2) the presence of ligand (16α[^{125}I]iodo-3,17β-estradiol) in the complex with altered mobility; (3) the ability of protein in the complex to bind

monoclonal Abs specific for ERE; and (4) the specific and high affinity binding of ERE_c in the complex.

Since ER has been shown to bind as a dimer to the palindromic ERE_c in solution [18], complex-a may consist of an ER homodimer bound to ERE_c. This supposition was supported by the observation that detectable levels of complex-a did not form until the concentration of ER in the binding reaction was greater than 1 nM (1.8–5.1 nM). Notides *et al.* [19, 20] have reported that the positive cooperativity and Hill coefficient obtained at ER concentrations between 1 and 10 nM are characteristic of homodimerization of the activated ER. At concentrations below 0.3 nM, dimer formation occurs less readily. On the other hand, Furlow *et al.* [21] have demonstrated that 1 mol of ERE_c is complexed with 1 mol ER in these *in vitro* binding reactions. These authors conclude that the protein–DNA complex is composed of an ER monomer or a heterodimer of ER and another protein. The data presented here demonstrate that ER was present in complex-a, but give no information as to whether it was present as a monomer, a dimer or in complex with another protein.

None of the characteristics expected of a protein–DNA complex containing ER were displayed by complexes-b and -c. These complexes did not bind ligand or undergo alterations in mobility in their presence, nor did they bind either of the mAbs to ER (H222 or D527). Thus, although protein–DNA complexes-b and -c were formed more readily with ERE than with non-specific DNA and were present at higher concentrations than ER–ERE (complex-a), it is unlikely that ER is one of the proteins involved in their formation. DNA–protein cross-linking experiments (data not shown), indicated that the most abundant MCF-7 protein bound to ERE_c had an apparent molecular weight of approx. 70,000 and was not immunoprecipitable by either of the anti-ER mAbs used in our studies. This suggests the possibility that either, or both complex-b and -c contain Ku protein. The ubiquitous protein NHP1 [22, 23], the PSE1 protein from K562 cells [24, 25] and the Ku protein from primate cells [26–28] all appear to bind to ERE with low affinity and may be similar or identical proteins. Ku is a hererodimer of 70 and 80 kDa proteins that make up a regulatory component controlling a kinase that phosphorylates RNA polymerase II [28]. Ku protein, which neither enhances nor impairs interaction of ER and ERE, is present in whole cell extracts of MCF-7 cells (F. E. Murdoch, pers. comm.).

Relation of gel mobility to transcription activation

It is assumed that the conformational alteration elicited upon binding of E_2 to the ER–ERE_c complex is associated with this ligand's activation of transcription [2, 4]. This alteration brought about the maximal increase in the mobility of complex-a when E_2 was present in the *in vitro* binding reaction at concentrations of at least 10^{-9} M (Fig. 1). When E_2 is added to cultures of MCF-7 cells, it causes maximal stimulation of growth rate at 10^{-11} M [15] and maximal expression of the estrogen responsive pS2 and cathepsin D genes at 10^{-10} M [9]. Peak stimulation of an ERE regulated transfected CAT reporter gene also occurs at an E_2 concentration of 10^{-10} M [10]. Thus, it appears that E_2 activation of transcription *in vivo* occurs at concentrations (in the medium) 10–100-fold lower than those required for E_2 to have maximal effect on the conformation of the ER–ERE_c complex *in vitro*. However, it should be noted that the native polyacrylamide gel electrophoresis system used to detect ER–ERE_c complexes does not "freeze" the complexes in such a way as to allow separation of ER–ERE_c complexes with or without bound ligand. Rather, since the mobility of the complex gradually increased with increasing concentration of ligand (Fig. 1), it appears that the mobility of the complex depended on the percent of ER–ERE bound to ligand at equilibrium, i.e. a full shift in electrophoretic mobility will only be observed when, at equilibrium, most complexes contain ligand. If, as has been found in a number of systems, only a small fraction of ER need to bind E_2 in order to activate transcription, but full occupancy of ER–ERE_c is needed to detect conformational changes by gel electrophoresis, the basis for this discrepancy is evident. Nevertheless, it has been reported that nanomolar concentrations of E_2 are required to increase the rate of transcription from the vitellogenin promoter *in vitro* [29] compared to 10–100 pM concentrations which are effective with endogenous transfected genes [8–10]. Thus, the possibility remains that cellular concentration of estrogen or certain factors acting within the cell, but not *in vitro*, allow activation of genes by lower levels of E_2.

The concept that ligand-induced alterations in the mobility of ER–ERE complexes detectable in gel shift assays do not always correlate with transcription activity has already been established through studies of interaction of E_2 and antiestrogens with ERs mutated in the ligand binding domain [30, 31]. The results reported here confirm this concept and demonstrate that agonist-induced increases in the mobility of ER–ERE complexes formed with a wtER depend on specific structural features of the ligand and that these structural features are not the same as those previously reported to be essential for stimulation of the transcriptional activation function of ER.

By comparing the results of the studies of the effect of estrogen analogs on the electrophoretic mobility of ER–ERE complexes reported herein with previous studies [8–10] of the effects of these analogs on gene expression in MCF-7 cells (Table 1), several general conclusions can be reached.

Table 1. Relationship between estrogen structure, gel mobility and gene stimulation

Estrogen	RBA*	Gel shift† complex-a	Gene stimulation‡ pS2	Gene stimulation‡ Cath D
Estratriene	<0.001	–	–	–
3-Hydroxyestratriene	0.800	–	+ +	+ +
Estratrien-17β-ol	0.110	–	+ +	+ +
E_2	1.000	+ +	+ +	+ +
1-Hydroxyestratrien-17β-ol	0.005	+ +	+ +	+ +
2-Hydroxyestratrien-17β-ol	0.710	+	+	–
4-Hydroxyestratrien-17β-ol	0.070	–	–	–
Estrone	0.220	+ +	+ +	+ +
Estriol	0.170	+ +	+ +	+ +
Estradiol-16α	0.800	+ +	+ +	+ +
Estradiol-17α	0.220	–	+ +	+ +
5-Androstene-3β,17β-diol	0.007	–	+ +	+ +

*RBA, relative binding affinity; $E_2 = 1$ with $K_a = 3.7 \times 10^9\,M^{-1}$ [ref. 8].

†Gel shift of complex-a in the presence of ligand concentrations great enough to compensate for receptor affinity differences. No shift, –; 50% shift of E_2, +; shift equal to that of E_2, + +.

‡Gene stimulation by each ligand at a concentration great enough to compensate for receptor affinity differences [ref. 9]. Gene transcription not stimulated, –; moderate level of gene transcription, +; EC_{50} of gene transcription stimulated by ligand is equal to that of E_2, + +. Cath D = cathepsin D.

1. There was no direct relationship between the relative binding affinity of the analogs and their ability to alter the conformation of the ER–ERE complex as measured by migration in the gel shift assay. Similarly, although relative binding affinity of the analogs correlates with their ability to activate transcription of a transfected reporter gene regulated by an ERE upstream of a minimal TK promoter [10], it is not directly related to the capacity of an analog to activate transcription of some endogenous estrogen regulated genes. For example, estriol with an RBA of 0.17, maximally altered ER–ERE_c conformation at 5×10^{-9} M and pS2 transcription at 10^{-10} M [9], while 3-hydroxyestratriene with an RBA of 0.80 activates pS2 transcription [9] at the same concentration as estriol but caused only minor conformational changes at micromolar concentrations.
2. Several analogs with excellent ability to activate transcription of endogenous genes (3-hydroxyestratriene, estradiol 17-α and estratrien-17β-ol; refs [8–10]) had little or no effect on ER–ERE_c conformation as detected by gel electrophoresis.
3. None of the analogs with ability to alter ER–ERE_c conformation at concentrations $<10^{-6}$ M fail to activate transcription of the pS2 gene at nanomolar concentrations [9].
4. The presence, location and enantiomeric form of the hydroxyl groups on the estratriene nucleus were of importance in determining whether conformational changes in ER–ERE complexes occur, as well as the gene regulation activity of the ligand. Estratriene, with no hydroxyl groups, has extremely low binding affinity for ER and lacked the ability to cause either conformational change in ER–ERE_c complexes (above) or activation of E_2 regulated genes [8–10]. Replacing one or the other of the hydroxyl groups of E_2 (3 or 17β) on estratriene created ligands with minimal effect on ER–ERE_c complex conformation even at micromolar concentrations [Fig. 4(A and B)]. Nevertheless, nanomolar levels of 3-hydroxyestratriene or estratrien-17β-ol stimulate the accumulation of pS2 and cathepsin D mRNAs in MCF-7 cells to a level similar to that induced by 10^{-10} M E_2 [9]. At micromolar concentrations, the binding affinity of these analogs is high enough to allow occupancy of all receptors in the binding reaction. Thus, if a conformational change was induced by these analogs, the conformation was different than that induced by E_2. Clearly, efficient stimulation of estrogen responsive genes does not require the same ligand induced conformational change in the ER–ERE_c complex as that mediated by E_2 binding. These data also suggest that the conformational change induced by E_2 required both the 3- and 17β-hydroxyl groups.

In fact, a number of dihydroxyestrogens were capable of influencing the conformation of the ER–ERE_c complex in the same way as E_2. Alterations in the position or oxidative state of D-ring oxygens on E_2 yielded ligands which brought about the maximum gel-shift of complex-a at concentrations near 10^{-8} M [Fig. 6(A and B)]. These estrogens (estrone, estriol and estradiol-16α) have been shown to actively stimulate estrogen responsive genes [8–10]. Only the estrogenic estradiol-17α was ineffective in altering the conformation of ER–ERE_c gel shift complexes. This suggests that the 17α-hydroxyl group did not interact with

ER, since this dihydroxyestrogen was as inefficient as 3-hydroxyestratriene in altering ER–ERE_c complex conformation [Figs 4(B) and 6(B)] and equally effective in activating estrogen responsive genes [8–10]. The influence of a hydroxylated aromatic A-ring on the conformation of the ER–ERE_c complex was apparent from the result that the dihydroxyandrostene, 5-androsten-3β,17β-diol, did not display the capacity for increasing the mobility of complex-a [Fig. 6(C)]. Nevertheless, this steroid has been shown to stimulate the accumulation of mRNAs for pS2 and cathepsin D in MCF-7 cells [9].

Location of the A-ring phenolic hydroxyl group had a dramatic influence on the conformation induced by binding of estrogen analogs to ER–ERE_c complexes. Similarly, these isomers vary greatly in their ability to activate different E_2 responsive genes [8–10]. A 1-hydroxyl group (1-hydroxyestratrien-17β-ol, RBA = 0.005), diminished the affinity of estratrien-17β-ol (RBA = 0.11). However, at micromolar concentrations, this analog affected the conformation of ER–ERE_c in a manner that increased its mobility to the maximum obtained with E_2 at 10^{-9} M [Fig. 4(B)]. This result suggests that 1-hydroxyestratrien-17β-ol induced a conformational change in the ER–ERE_c similar to that induced by E_2 at a concentration consistent with its reduced affinity for receptor. This A-ring isomer was also active in stimulating estrogen responsive genes at elevated concentrations [8–10].

2-Hydroxyestratrien-17β-ol, which binds ER with an RBA of 0.71, did not alter the migration of ER–ERE_c or ER–ERE_v [Fig. 5(B)] complexes until a concentration of $>10^{-8}$ M was achieved, and even then, failed to increase their mobility to more than 50% that obtained with E_2 at 10^{-9} M. The effect on complexes with ERE_c and ERE_v (the response element of the pS2 gene) was identical. 2-Hydroxyestratrien-17β-ol maximally stimulates accumulation of pS2 [9] in MCF-7 cells at 10^{-8} M. Even lower levels of this E_2 isomer (10^{-11} M) stimulate expression of an ERE-regulated CAT gene in a plasmid with a minimal promoter [10]. In contrast, 2-hydroxyestratrien-17β-ol is ineffective in activating cathepsin D expression in MCF-7 cells exposed to concentrations as high as 10^{-7} M [9].

The finding that 2-hydroxyestratrien-17β-ol was unable to alter the mobility of complex-a to the same extent as E_2, even when present at a concentration which should be sufficient to ensure binding to all ER–ERE complexes in the binding reaction mixture, suggests that the conformational change elicited by the 2-hydroxyl group in the ER–ERE complex was different to that brought about by the 3-hydroxyl group of E_2. The difference in response of the pS2 and CAT reporter genes and the absence of a response of the cathepsin D gene to 2-hydroxyestratrien-17β-ol may thus be the result of an inability of the ligand–ER–ERE complex to interact with transcriptional regulatory factors necessary for expression of the cathepsin D gene.

4-Hydroxyestratrien-17β-ol did not affect the mobility of complex-a (containing either ER–ERE_c or ER–ERE_v) at concentrations as high as 10^{-5} M [Fig. 5(A)]. This isomer is also ineffective in regulating estrogen responsive genes in MCF-7 cells (pS2, cathespin-D and progesterone receptor; refs [8 and 9]). Nevertheless, 4-hydroxyestratrien-17β-ol is capable of stimulating growth (maximal effect is a 3-fold stimulation at 10^{-7} M, unpublished results from this laboratory), transcription of an E_2 responsive CAT gene [10] and synthesis of several as yet unidentified proteins in MCF-7 cells (10^{-8} M; ref. [32]). This indicates that a ligand bound ER–ERE with a conformation that differs from that of ER–ERE with bound E_2 is still capable of activating some endogenous ER genes. Taken together, the results from experiments with the three A-ring isomers demonstrate that there was no correlation between the ability of these isomers to increase the mobility of ER–ERE_c or ER–ERE_v complexes and their ability to activate at least some endogenous genes in MCF-7 cells.

The A-ring isomers of E_2 possess a modulated positioning of the electronegative isopotential above the A-ring [8]. We have postulated that this electronegative cloud (present above the unsubstituted aromatic A-ring and positioned differently above the A-ring isomers) may influence the position or conformation of the AF-2 in the estrogen binding domain of ER [8–10]. AF-2 is reported to form an amphoteric α-helix which has been proposed to lie near the A-ring of the bound estrogen ligand [33]. The negative side of the α-helix could conceivably be repulsed or distorted by the proximity of the electronegative isopotential above the A-ring of these analogs, resulting in a conformational change in the ER–ERE. The natural estrogen (E_2) would produce a conformation favorable for the interaction with other transcriptional regulatory factors, whereas the A-ring isomers could either fail to alter ER–ERE conformation or yield conformational changes which do not interact effectively with certain of these factors.

In conclusion, our results clearly demonstrate that a dihydroxyestrogen ligand is required to induce a conformational change in the ER–ERE that confers an equivalent increase in electrophoretic mobility to that obtained with E_2. On the other hand, the finding that estrogen analogs active in stimulating expression of certain endogenous estrogen responsive genes did not cause conformational changes detectable in gel-shift assays, leaves open the question as to whether these analogs induced changes that are too subtle to be detected or whether some AF-2 interactions can occur without ligand-induced conformational changes. In either case, on the basis of the results presented here and in our previous reports [8–10, 32], it is reasonable to conclude that the conformational requirements for productive interactions between the ligand

bound ER–ERE complex and other transcription factors depend on the nature of the transcription factors involved.

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