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ANTIESTROGEN-LIGANDED ESTROGEN RECEPTOR INTERACTION WITH ESTROGEN RESPONSIVE ELEMENT DNA *IN VITRO*

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Summary—The mechanism whereby antiestrogens alter the ability of the estrogen receptor (ER) to enhance transcription of estrogen-regulated genes is largely unknown. The effect that selected estrogenic and antiestrogenic ligands have on binding of ER to specific DNA sequences, estrogen responsive elements (EREs) has been quantitated. No differences in purification properties of calf uterine ER liganded with 4-hydroxytamoxifen (4-OHT-ER), ICI 164,384 (ICI 164,384-ER) or estradiol (E_2-ER) were detected. A microtiter well plate assay was employed in which liganded ER bound to plasmid DNA is preferentially retained compared to free liganded ER. Binding of E_2-ER, 4-OHT-ER, or ICI 164,384-ER was measured to plasmids containing or lacking a 38bp consensus ERE *in vitro*. The EREs tested contain an inverted repeat (5′-CAGGTCAGAGTGACCTG-3′). Both E_2-ER and 4-OHT-ER showed similar high affinity specific binding (K_d = 0.24 and 0.16 nM, respectively) to one copy of the ERE. ICI 164,384-ER did not bind to plasmids containing one ERE. At saturation, however, 4-OHT-ER binding was about 50% of that observed for E_2-ER. When the plasmid contained 3 or 4 tandem copies of the ERE, binding of E_2-ER, 4-OHT-ER, and ICI 164,384-ER binding was measurable. E_2-ER bound in a cooperative manner as suggested by convex Scatchard plots and Hill coefficients > 1.5. In contrast, 4-OHT-ER binding displayed much reduced cooperativity, and ICI 164,384-ER did not display cooperative binding. From these results, we propose that the conformation of ER induced by 4-OHT reduces its binding capacity to this consensus ERE without altering its affinity of binding. Furthermore, higher order protein–protein interactions between antiestrogen-liganded ER bound to DNA differ from those of E_2-ER bound to ERE.

INTRODUCTION

The growth of mammary tumors is thought to be initially dependent on estrogens [1]. Although much effort has focused on the role of estrogens in this process, the exact mechanism by which estrogens modulate cell replication is unknown. Recently, evidence was obtained to support the suggestion that estrogens regulate the transcription and/or secretion of certain peptide growth factors that, in turn, act in an autocrine or paracrine manner to promote cell replication [1–4].

At the molecular level, the transcription of estrogen-regulated genes is mediated by binding of estrogen to estrogen receptors (ERs), forming a homodimeric ER complex that, in turn, binds to specific DNA sequences, called estrogen responsive elements (EREs) [reviewed in 5–7]. EREs are usually located upstream of the promoter of estrogen-regulated genes, and often contain a core inverted repeat sequence, 5′-AGGTCAnnnTGACCT-3′, that acts as an enhancer [reviewed in 5–7]. Using partially purified calf uterine ER and a 38bp consensus ERE, derived from a number of genes that are highly responsive to estrogen, and cloned into a plasmid vector, we quantitated high affinity (K_d = 0.24 nM) estradiol-liganded ER (E_2-ER)–ERE binding *in vitro* [8, 9].

The non-steroidal antiestrogen tamoxifen (TAM) is widely used in the treatment of women with advanced breast cancer or in an

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Abbreviations: AER, antiestrogen-liganded estrogen receptor; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; DES, diethylstilbesterol; DEX, dexamethasone; DTT, dithiothreitol; E_2, estradiol; E_2-ER, E_2-liganded estrogen receptor; ER, estrogen receptor; ERE, estrogen responsive element; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HAP, hydroxylapatite; HRE, hormone responsive element; ICI 164,384-ER, ICI 164,384-liganded estrogen receptor; PMSF, phenylmethylsulfonylfluoride; PR, progesterone receptor; 4-OHT-ER, 4-hydroxytam-oxifen-liganded estrogen receptor; TAF, transcriptional activation function; TAM, tamoxifen; 4-OH-TAM, *trans*-4-hydroxytamoxifen.

adjuvant setting [10]. However, depending on the tissues and species, TAM displays partial agonist as well as antagonist activity. One active metabolite of TAM, *trans*-4-hydroxytamoxifen (4-OH-TAM), was shown to compete with E_2 for binding to ER with a relative binding affinity that is 310% that of E_2 [11–13]. Both TAM aziridine (an estrogen antagonist) and ketonon-estrol aziridine (an estrogen agonist), used as affinity labeling agents, were covalently attached to the same cysteine residue in ER from MCF-7 cells, thus suggesting that differences in agonist- vs antagonist-liganded receptor are not attributable to differential attachment in the ligand binding domain [14]. Several investigators reported that the conformation of antiestrogen-liganded ER (AER) differs slightly from that of E_2-ER [15–17]. As visualized by electron microscopy, ER in TAM-treated oocyte nuclei, i.e. presumably liganded with TAM, did not bind to a 2.7 kb DNA fragment of the 5′ end of vitellogenin B2 gene that contained an ERE [18]. It was suggested that the TAM-induced change in receptor protein conformation does not activate transcription [19, 20].

Although both TAM- and 4-OH-TAM-liganded ER appear, at least in some systems, to retain ERE binding ability [19–27], only E_2-ER fully induced transcription of a reporter gene [24, 27]. Two regions of the ER were shown to have transcriptional activation function (TAF) [28]. The N-terminal A/B region of the ER contains a constitutive, hormone-independent TAF-1, while the carboxy-terminal E region, the hormone binding domain, contains the hormone-inducible TAF-2 [27, 28]. ER-mediated agonist activity of 4-OH-TAM is cell-type and promoter-context-dependent [27]. Berry *et al.* [27] proposed that 4-OH-TAM acts as an estrogen agonist when it promotes ER binding to a promoter from which transcription can be activated by TAF-1 alone. Studies with chimeric receptors, having the DNA binding region of yeast transcriptional activator *GAL4* and the E region of the ER protein, showed that E_2- but not 4-OH-TAM-liganded chimeric receptor, induced transcription from a reporter plasmid [24, 27]. Thus, it is not clear how AER differs from E_2-ER in ERE binding characteristics, and why, in some cases, transcription is not activated.

We previously demonstrated that [^{3}H]4-OH-TAM-liganded partially purified calf uterine ER ([^{3}H]4-OHT-ER) binds to calf uterine nuclei [29] or rat mammary tumor nuclei [30] with high affinity, but to approx. 40% fewer sites than [^{3}H]E_2-liganded ER ([^{3}H]E_2-ER). One possible explanation suggested for those results was that 4-OHT-ER binding precluded E_2-ER binding because 4-OHT-ER bound to a larger region of DNA. Others reported a similar decrease in the ability of AER vs ER to bind to DNA cellulose [31].

Aside from TAM and other non-steroidal, triphenolic derivatives, other antiestrogenic compounds are also being investigated for anti-tumor efficacy. Wakeling and Bowler [32, 33 reviewed in 34] examined 7-alpha alkylamide analogs of E_2 including ICI 164,384, an anti-estrogen seemingly devoid of estrogen agonist activity. In cell-free extracts, ICI 164,384 had an affinity for ER comparable to that of E_2 [32, 35], but displayed no agonist activity in rats or mice [32, 33]. Moreover, the binding of ICI 164,384 to either human or porcine ER did not increase the affinity of the receptor complex for binding to DNA cellulose [35, 36]. Lees *et al.* [19] suggested that the receptor binding of ICI 164,384 produces a distinct conformational change in the ER that precludes use of TAF-1 and 2, whereas 4-OH-TAM allows TAF-1 function. Similarly, neither ER nor chimeric receptor *GAL4*/ER when liganded with ICI 164,384 induced transcription from various reporter gene constructs in two different cell systems [27].

We have examined the role of the ligand on the ability of ER to interact with consensus EREs or sequence variants of ERE *in vitro*. A microtiter well plate assay in which histone/gelatin-coated wells selectively retain E_2-ER–DNA complexes [9] was used to accurately quantitate E_2-ER–ERE interaction. Recently we showed that E_2-ER bound cooperatively to consensus and certain sequence variant EREs located on the same face of the DNA helix [37].

In this report, we present a quantitative comparison of the binding of 4-OHT-ER, ICI 164,384-ER vs E_2-ER to a 38bp ERE consensus sequence and to several variants of this consensus ERE. We observed high affinity binding of 4-OHT-ER to ERE, but the binding capacity of the ERE for 4-OHT-ER vs E_2-ER was consistently lower. No binding of ICI 164,384-ER was detected to one ERE. Further, and in contrast to our earlier findings of binding cooperativity of E_2-ER to multiple tandem EREs [37], little or no cooperative binding was detected for 4-OHT-ER or ICI 164,384-ER–ERE binding.

EXPERIMENTAL

Preparation of plasmids containing EREs

Synthetic single stranded oligonucleotides that are variants of the original ERE consensus sequence [8] were cloned into the plasmid pGEM-7Zf(+) (Promega, Madison, WI) and sequenced [37].

The sequences of the oligonucleotides containing variations in the consensus sequence [8,37] are given below. Half-sequences of the inverted repeat are underlined, changes in nucleotide sequence are printed in bold face type, ⌒ denotes nucleotides inserted, and x denotes nucleotides deleted:

No. bp	Name	DNA sequence
38	Z16	5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′
38	Z20	5′-CCAGGTCAGAGTGCCCTGAGCTAAAATAACACATTCAG-3′
38	Z21	5′-CCAGGTCAGAGTGCACTGAGCTAAAATAACACATTCAG-3′
38	Z22	5′-CCAGGTCAGAGTGGCCTGAGCTAAAATAACACATTCAG-3′
38	Z23	5′-CCAGGTCAGAGTGTCCTGAGCTAAAATAACACATTCAG-3′
40	Z25	5′-CCAGGTCAGAGA⌒ATGACCTGAGCTAAAATAACACATTCAG-3′
37	Z26	5′-CCAGGTCAGxGTGACCTGAGCTAAAATAACACATTCAG-3′
36	Z27	5′-CCxGGTCAGAGTGACCxGAGCTAAAATAACACATTCAG-3′
38	Z30	5′-CCCTAAAGGAGTGACCTGAGCTAAAATAACACATTCAG-3′
38	Z31	5′-CCAGGTCAGAGCATTTCAAAATAACACATTTGACTCAG-3′
38	Z33	5′-CCAGGTCAGAGCATTTCGAGCTAAAATAACACATTCAG-3′

Plasmid DNA was linearized with *Eco*R I for all experiments presented here. Aliquots of *Eco*R I-digested DNA were labeled by incorporation of [^{35}S]dATP (>600 Ci/mmol, Amersham, Arlington Heights, IL) at the recessed 3′ termini using the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA). Aliquots of [^{35}S]DNA were mixed with unlabeled DNA for the desired final concentration.

Preparation of ER

ER was partially purified from calf uterus according to the method of Weichman and Notides [38] as previously modified [39]. In brief, the ammonium sulfate cytosol fraction (0–30%) was desalted [40] and the concentration of ER was determined by hydroxylapatite (HAP) assay (see below) [41, 42]. 17β-[2,4,6,7,16,17-^{3}H]E_2 (151 Ci/mmol from Amersham), Z-4[*N*-methyl-^{3}H]4-OHT (81.1 Ci/mmol from NEN or 77 Ci/mmol from Amersham), or [^{3}H]ICI 164,384 (91.1 Ci/mmol, a generous gift from Dr A. E. Wakeling of ICI, Macclesfield, Cheshire) was added in at least 5 nM excess prior to incubation with Heparin Agarose (Affi-Gel Heparin, BioRad, Richmond, CA). When using 4-OHT, the [^{3}H]4-OHT-ER was protected from exposure to light during all steps of the procedure. Liganded receptor was eluted from the heparin–agarose column with a linear gradient from 175 to 500 mM KCl in TDP (40 mM Tris–HCl, pH 7.5, 1 mM DTT, 0.5 mM PMSF). Active fractions were pooled, the salt concentration measured by conductivity and diluted with TDP buffer containing 40% glycerol to 111 mM KCl [37].

HAP assay of ER

The concentration of E_2-ER, 4-OHT-ER, or ICI 164,384-ER was determined by adsorption to HAP [41]. For quantitating the amount of E_2-ER, 4-OHT-ER, or ICI 164,384-ER used in saturation analysis, NP-40 was omitted from a set of parallel tubes containing the liganded receptor. Inclusion of NP-40 inhibited E_2-ER binding to HAP (data not shown).

Microtiter plate assay of ER or AER binding to plasmid DNA

The microtiter (well) plate assay for measuring [^{3}H]E_2-ER binding to DNA has been described previously [9]. Briefly, for saturation binding analysis, various concentrations (range 0.1–2.8 nM dimeric ER) of heparin agarose affinity purified [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER were preincubated with one concentration (approx. 0.22 nM, see figure or table legends for specific amount) of [^{35}S]DNA (plasmid DNA with or without ERE) for 2.5 h at 4°C, with shaking in TDPK 100 buffer containing 0.1% NP-40 and then incubated in histone/gelatin-coated microtiter wells for 2.5 h at 4°C with shaking. Wells were rinsed with TDPEK100 buffer (TDP buffer containing 1 mM EDTA and 100 mM KCl) containing 100 μg/ml carboxymethyl BSA [42] and the radioactivity remaining in the wells was counted.

Calculation of specific [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER binding to ERE

For each determination of E_2-ER, 4-OHT-ER, or ICI 164,384-ER binding to DNA, [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER was incubated with *Eco* R I digested [^{35}S]end-labeled pGEM-7Zf(+) plasmid alone and plasmid containing one or more EREs. The amount of [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER [^{35}S]DNA binding was calculated as described previously [37] and was corrected for the background binding of the [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER preparation in wells without DNA added [9]. The specific binding of [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER to the EREs was then calculated by subtracting the binding to pGEM-7Zf(+) from binding to plasmid containing EREs. All calculations were accomplished using QUATTRO: The Professional Spreadsheet (Borland International, Scotts Valley, CA).

RESULTS

Effect of ligand on heparin–agarose affinity purification of ER

When the binding of E_2-ER vs 4-OHT-ER to isolated rat tissue nuclei was quantitated previously, receptor preparations were used after ammonium sulfate precipitation of calf uterine cytosol [30]. Now we are using a more extensive purification of ER including heparin–agarose chromatography [42]. Because others reported subtle differences in the physical properties of E_2-ER vs AER [reviewed in 13, 43, 44], we were concerned that the type of ligand employed would alter ER purification parameters. Consequently, for each ligand employed, we determined the KCl concentration that eluted the peak of [^{3}H] hormone binding activity from the heparin–agarose affinity column used in partial purification of ER. These KCl concentrations were 305 ± 4 mM (15 purifications) for [^{3}H]E_2-ER, 305 ± 4 mM (10 purifications) for [^{3}H]4-OHT-ER, and 316 ± 5 mM (2 purifications) for [^{3}H]ICI 164,384-ER. Thus, the heparin–agarose binding affinities of the various liganded ER preparations used in the experiments reported here were essentially equivalent.

Effect of KCl concentration on 4-OHT-ER, E_2-ER, or ICI 164,384-ER binding to EREs

The influence of salt concentration on the specific binding of E_2-ER, 4-OHT-ER, or ICI 164,384-ER to the ERE was examined *in vitro*. In this experiment, *Eco* R I-linearized, [^{35}S]dATP-end labeled plasmid DNA [either the parental pGEM-7Zf(+) plasmid alone or containing four copies of the 38bp consensus ERE] was incubated with increasing concentrations of KCl and a single concentration of the appropriate receptor preparation. The highest specific binding to EREs was observed at 100 mM KCl for E_2-ER and 75–100 mM KCl for 4-OHT-ER, with a sharp decrease in receptor binding at greater than 200 mM KCl (Fig. 1). This is similar to the findings of Brown and Sharp [20] who showed that addition of 200–400 mM KCl resulted in decreased detection of E_2-ER–ERE complex by gel retardation assay. It is also consistent with the results of Murdoch *et al.* [45] who detected maximum differences in ER binding affinity for perfect vs imperfect EREs between 100–150 mM KCl. The peak of ICI 164,384-ER binding was at 150 mM KCl. It is also apparent that, compared to specific E_2-ER–ERE binding, the amount of specific 4-OHT-ER binding is significantly lower, and ICI 164,384-ER binding is lower yet. This difference is evident over nearly the entire range of tested ionic strength.

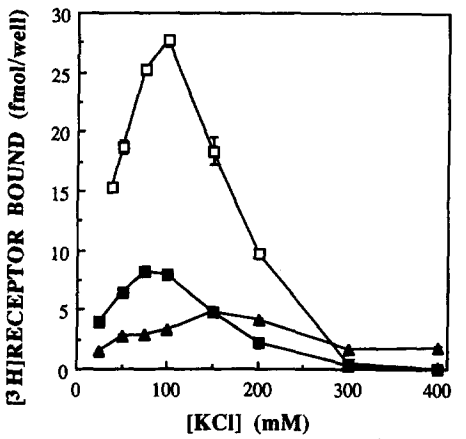

Fig. 1. Effect of salt concentration on specific [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER binding to ERE *in vitro*. *Eco* R I-linearized, [^{35}S]dATP-end labeled plasmid DNA, either the parental plasmid alone or containing four copies of the 38bp consensus ERE (Z16 in Experimental), was incubated with increasing concentrations of KCl and heparin–agarose purified [^{3}H]E_2-ER (□, 27.3 fmol receptor dimer/well), [^{3}H]4-OHT-ER (■, 21.8 fmol receptor dimer/well), or [^{3}H]ICI 164,384-ER (▲, 14.3 fmol receptor dimer/well). 50 μl aliquots of the receptor–DNA mixtures, containing 11 fmol DNA, were then pipetted into histone/gelatin-treated wells and incubated as described in Experimental. Radioactivity remaining in the wells after the washes was counted. The data points shown are the average of quadruplicate determinations ± SEM.

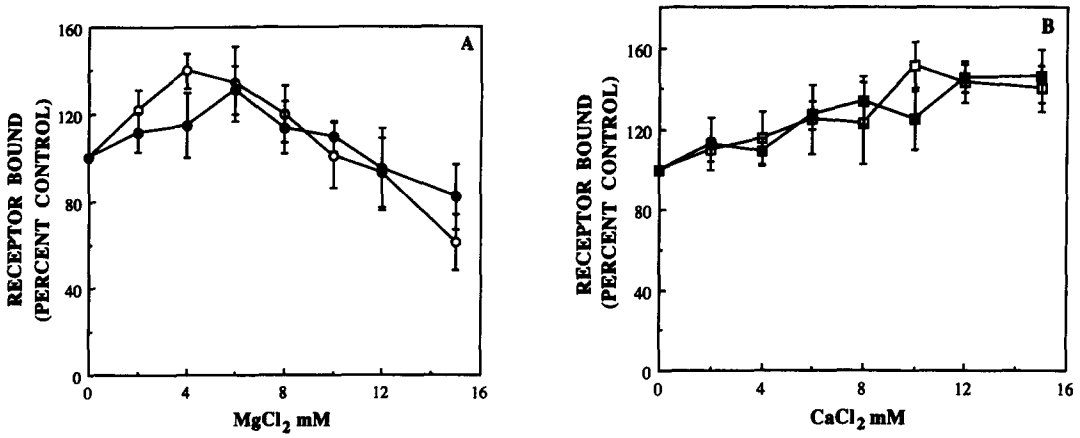

Fig. 2. Effect of divalent cation concentration on specific [^{3}H]E_2-ER or [^{3}H]4-OHT-ER binding to ERE *in vitro*. *Eco*R I-linearized, [^{35}S]dATP-end labeled plasmid DNA, either the parental plasmid alone or containing two copies of the 38bp consensus ERE (Z16 in Experimental) was incubated with increasing concentrations $MgCl_2$(A) or $CaCl_2$(B) in TDPK111 buffer and [^{3}H]ER (61–113 fmol receptor dimer/well, open symbols) or [^{3}H]4-OHT-ER (74–114 fmol receptor dimer/well, closed symbols). The data points shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol of DNA/well.

Effect of divalent cations on E_2-ER vs 4-OHT-ER binding to the ERE dimer

We examined whether addition of increasing, but physiological, concentrations of the divalent cations Mg^{2+} or Ca^{2+} would reveal differences in ER vs AER binding to EREs. Similar to the above experimental design, but using plasmids containing two tandem copies of the 38bp consensus ERE, we examined the effects of increasing concentrations of $MgCl_2$ or $CaCl_2$ (Fig. 2). A fixed concentration of the appropriate receptor preparation was chosen in order to saturate the ERE sites, based on our previous work [37].

The specific binding of either E_2-ER or 4-OHT-ER to ERE was identically affected by addition of the divalent cations Mg^{2+} or Ca^{2+}. For both receptor preparations, an increase in specific ERE binding capacity was detected with the addition of 2–10 mM Mg^{2+}, while reduced binding occurred with addition of 12 or 15 mM Mg^{2+} [Fig. 2(A)]. The addition of Ca^{2+} increased specific E_2-ER–ERE or 4-OHT-ER–ERE binding throughout the range of concentrations tested [Fig. 2(B)]. Thus, unlike reports for PR [46], we observed no differential effect of these divalent cations on agonist vs antagonist-liganded ER binding to the ERE dimer.

As one control, the effect of each divalent cation on the binding of [^{35}S]DNA to the histone/gelatin-coated wells in the absence of added receptor was examined. Mg^{2+}, over a 2–10 mM range, had no effect on DNA binding capacity, although 12 and 15 mM Mg^{2+} caused a modest (approx. 4%) decrease in DNA binding (data not shown). Addition of 2–15 mM Ca^{2+} had no effect on [^{35}S]DNA binding (data not shown). Moreover, since [^{35}S]DNA retention in wells is determined, and any difference is normalized by calculation, any effects of Mg^{2+} and Ca^{2+} on E_2-ER or 4-OHT-ER–ERE binding would not be attributable to altered DNA binding to the wells.

Specific binding of 4-OHT-ER, E_2-ER, or ICI 164,384-ER to the consensus ERE

To determine whether 4-OHT-ER–ERE binding is of lower affinity than E_2-ER–ERE binding, we quantitated the binding of 4-OHT-ER, E_2-ER, or ICI 164,384-ER to one copy of the 38bp consensus ERE (Table 1, Z16) by saturation analysis (Fig. 3). 4-OHT-ER binding was about 50% of that observed for E_2-ER–ERE binding, whereas ICI 164,384-ER did not appear to bind to one ERE. Scatchard analysis revealed that both E_2-ER and 4-OHT-ER showed similarly high specific binding affinity ($K_d = 0.24$ and 0.16 nM, respectively, Table 1). It should be noted that all preparations of E_2-ER, 4-OHT-ER, and ICI 164,384-ER used here displayed similar binding profiles, indicating that differences in ERE-binding of E_2-ER vs 4-OHT-ER or ICI 164,384-ER were independent of the particular receptor preparation.

Saturation binding analyses were also performed for E_2-ER, 4-OHT-ER, and ICI 164,384-ER binding to multiple tandem (i.e. directly repeated, head-to-tail) copies of the 38bp consensus ERE (Z16). A representative saturation

Table 1. Comparison of [^{3}H]E_2-ER or [^{3}H]4-OHT-ER binding to consensus ERE

		E_2-ER			4-OHT-ER	
DNA	n[a]	K_d (nM)	Hill coefficient	n	K_d (nM)	Hill coefficient
Monomer	17	0.24 ± 0.01	1.16 ± 0.03	24	0.16 ± 0.01[e]	<1[b]
Dimer	12	0.23 ± 0.03	1.24 ± 0.04	22	0.69 ± 0.02[e]	0.74 ± 0.02
Trimer	14		2.17 ± 0.46[c]	23	0.40 ± 0.01	0.71 ± 0.01
Tetramer	32		1.86 ± 0.17[d]	22	3.55 ± 0.18	1.01 ± 0.04

Saturation analyses were performed using a fixed concentration of plasmid DNA (Z16 in Experimental) and increasing concentrations of heparin–agarose purified [^{3}H]E_2-ER or [^{3}H]4-OHT-ER as described in Experimental.

[a] n = number of different concentrations of [^{3}H]E_2-R or [^{3}H]4-OHT-ER assayed for DNA binding, each in duplicate.

[b] Since 4-OHT-ER binding to monomer ERE is less than one 4-OHT-ER dimer per ERE, LOG (Y/1-Y) is less than zero. The slope of the best fit line for the data was < 1.

[c] Significantly different ($P < 0.01$) from the Hill coefficient of E_2-ER binding to monomer or dimer.

[d] Significantly different ($P < 0.005$) from the Hill coefficient of E_2-ER binding to monomer or dimer.

[e] Significantly different ($P < 0.005$) from the K_d value for E_2-ER binding to the same ERE.

plot of 4-OHT-ER consensus ERE binding is shown in Fig. 4 (A and B). Although at saturation one dimeric E_2-ER was capable of binding to each tandem ERE (i.e. one E_2-ER dimer bound one ERE, two E_2-ER dimers bound two tandem EREs, etc.) [37], 4-OHT-ER binding was consistently about 50% lower than that for E_2-ER binding (Figs 4 and 5). A comparison of the stoichiometric relationship of E_2-ER vs 4-OHT-ER dimer binding to ERE sequences is given in Table 2. The ratio of 4-OHT-ER bound per ERE is about the same irrespective of the number of Z16 EREs per plasmid. Although specific ICI 164,384-ER binding to EREs increased with the number of tandem copies of ERE, the binding remained significantly lower than that of 4-OHT-ER or E_2-ER to the same ERE construct (Fig. 5).

In contrast to E_2-ER binding to 3 or 4 tandem consensus EREs, in which cooperative binding was indicated by convex Scatchard plots [37], Scatchard analysis of 4-OHT-ER interaction with 3 tandem copies of the consensus ERE conformed to a linear distribution of points [Fig. 4(B)]. Likewise, ICI 164,384-ER binding to multiple copies of the consensus ERE was not found to be cooperative (data not shown). The calculated Hill coefficient of 1.01 for 4-OHT-ER binding to the ERE tetramer indicates a minor amount of cooperative binding, if any (Hill plots not shown, Hill coefficients summarized in Table 1). For comparison, Scatchard analysis of ICI 164,384-ER–ERE binding gave estimated K_d values of 0.45, 1.56, or 3.58 nM for binding to 2, 3, or 4 copies of ERE, respectively. Thus, the binding of ICI 164,384-ER to ERE is

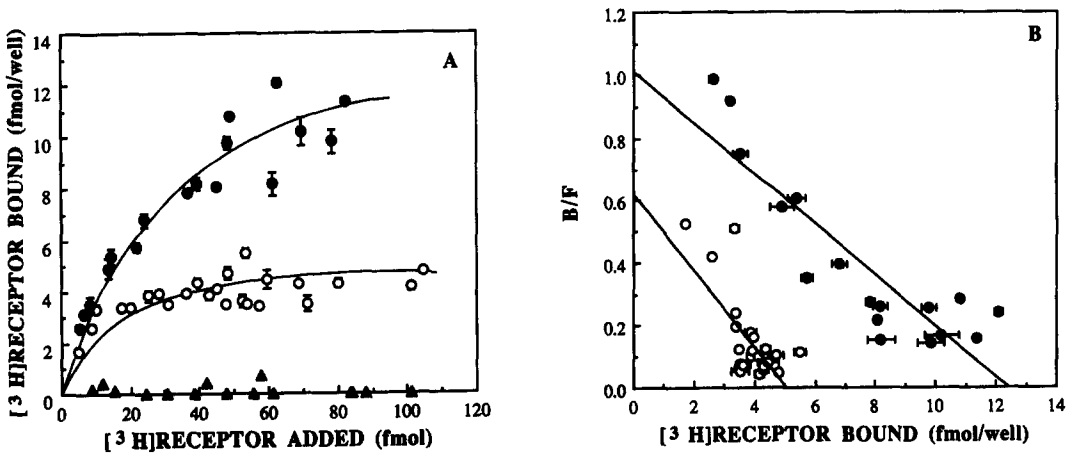

Fig. 3. Saturation analysis of specific [^{3}H]E_2-ER, [^{3}H]4-OHT-ER, or [^{3}H]ICI 164,384-ER binding to monomer ERE consensus sequence *in vitro*. *Eco*R I-linearized, [^{35}S]dATP-end labeled plasmid DNA either the parental plasmid alone or containing one copy of the 38bp consensus ERE (Z16 in Experimental) was incubated with increasing concentrations of heparin–agarose purified [^{3}H]E_2-ER (●), [^{3}H]4-OHT-ER (○), or [^{3}H]ICI 164,384-ER (▲). (A) The data points shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol of DNA/well. (B) Saturation analysis plotted according to the method of Scatchard. The lines were calculated by least square regression analysis. $K_d = 0.24$ for [^{3}H]E_2-ER–ERE (○) and 0.16 nM for [^{3}H]4-OHT-ER–ERE (●) binding.

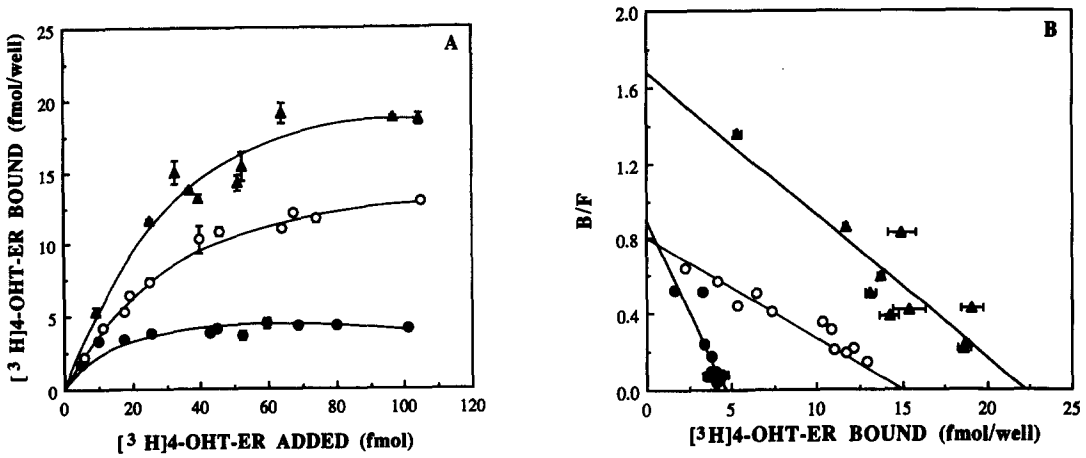

Fig. 4. Saturation analysis of specific [^{3}H]4-OHT-ER binding to monomer, dimer, or trimer ERE consensus sequences *in vitro*. *Eco*R I-linearized, [^{35}S]dATP-end labeled plasmid DNA either the parental plasmid alone or containing one (●), two (○), or three (▲) (A) tandem copies of the 38bp consensus ERE (Z16 in Experimental) was incubated with increasing concentrations of heparin–agarose purified [^{3}H]ER. The data points shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol of DNA/well. This graph is representative of data from 19 separate plate assay experiments. (B) Saturation analysis plotted according to the method of Scatchard. The lines were calculated by least square regression analysis. $K_d = 0.10$ nM for monomer (●), 0.45 nM for the dimer (○) and 0.26 nM for the trimer (▲).

generally of lower affinity than that of either E_2-ER or 4-OHT-ER.

Specific binding of 4-OHT-ER, E_2-ER, or ICI-ER to sequence variant EREs

Since most naturally occurring HREs have an imperfect palindrome structure [6], we examined 4-OHT-ER binding to 10 different sequence variants of the original 38bp consensus ERE (sequences in Experimental). At saturation, 4-OHT-ER binding was significantly lower than E_2-ER binding to each of the tested variants (Fig. 6). However, the ratio of E_2-ER to 4-OHT-ER binding varied depending on the sequence. The stoichiometric relationship of E_2-ER vs 4-OHT-ER binding to ERE sequence variants at saturation is presented as a ratio of E_2-ER or 4-OHT-ER dimers bound to total plasmid DNA molecules, as well as the ratio of receptor dimers bound per ERE, i.e. the ratio of receptor/plasmid divided by the number of copies of ERE in the plasmid (Table 2). We found that 4-OHT-ER binding to Z16, Z27, and Z22 was always lower than E_2-ER binding, whereas the binding of 4-OHT-ER to Z23 and Z25 was similar to that of E_2-ER. Neither 4-OHT-ER nor E_2-ER bound to a single copy of the Z25 sequence variant that contains 5bp, instead of 3bp, separating the half inverted repeats, whereas 4-OHT-ER binding to Z26, which contains 2bp separating the half inverted repeat, was about 50% lower than E_2-ER binding (Fig. 6). Interestingly, among the sequence variants, 4-OHT-ER binding to Z23 was most comparable to E_2-ER binding (71% of the E_2-ER binding value, Fig. 6). Z23 is the ERE sequence most similar to a GRE [37]. There was virtually no detectable specific binding of 4-OHT-ER to Z30 and Z33, which contain perfect 3′ and 5′ half ERE sites, respectively, or to Z31, which contains one imperfect 3′ half site located 22bp downstream of a perfect 5′ half site.

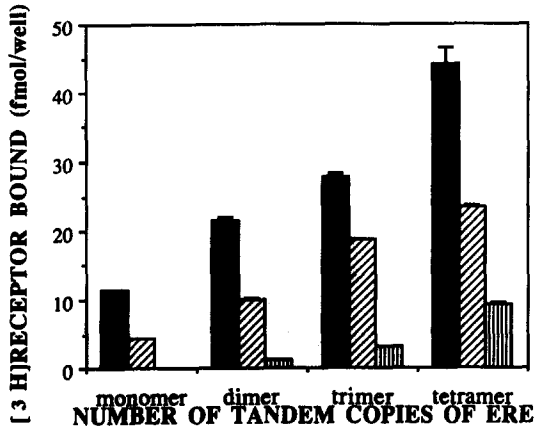

Fig. 5. Comparison of specific binding of [^{3}H]E_2-ER *vs* [^{3}H]4-OHT-ER vs [^{3}H]ICI 164,384-ER to multiple tandem copies of the ERE consensus sequence. [^{3}H]E_2-ER (■), [^{3}H]4-OHT-ER (▨), or [^{3}H]ICI 164,384-ER (▥) binding was measured at saturation (80–110 fmol receptor dimer added) to the indicated number of tandem copies of the consensus ERE (Z16 in Experimental) as described in Experimental and Fig. 1. The data shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol DNA/well.

Table 2. Stoichiometric relationship of E_2-ER or 4-OHT-ER–ERE interaction

DNA	No. tandem copies of ERE	Ratio ER–plasmid	Ratio ER–ERE	Ratio 4-OHT-ER–plasmid	Ratio 4-OHT-ER–ERE
Z16					
Monomer	1	0.97	0.97	0.41	0.41
Dimer	2	2.09	1.09	1.03	0.52
Trimer	3	3.15	1.05	1.78	0.59
Tetramer	4	4.10	1.10	2.14	0.54
Z27					
Monomer	1	1.00	1.00	0.31	0.31
Tetramer	4	2.95	0.74	0.87	0.22
Z22					
Monomer	1	0.89	0.89	0.42	0.42
Tetramer	4	2.49	0.62	1.90	0.48
Z23					
Monomer	1	0.26	0.26	0.18	0.18
Tetramer	4	2.30	0.58	2.72	0.68
Z25					
Monomer	1	0	0	0	0
Tetramer	4	1.05	0.26	0.40	0.10

Saturation analyses were performed using a fixed concentration of plasmid DNA and increasing concentrations of heparin–agarose purified [^{3}H]E_2-ER or [^{3}H]4-OHT-ER as described in Experimental. [^{3}H]E_2-ER or [^{3}H]4-OHT-ER binding to plasmid, [pGEM-7Zf(+)], alone or containing each insert as indicated (see Experimental for sequences) was measured as detailed in Experimental and Fig. 1. The binding ratios presented were calculated from binding values taken at saturation (77–110 fmol/well [^{3}H]E_2-ER or [^{3}H]4-OHT-ER dimer added) from which background and non-specific binding to plasmid without inserts has been subtracted.

4-OHT-ER binding was also measured to four tandem copies of selected sequence variants. The stoichiometric ratio of 4-OHT-ER binding per ERE is consistently less than one (Table 2), suggesting that binding is always ERE specific. Interestingly, sequence variant Z23 appeared to bind more 4-OHT-ER at saturation than did the consensus ERE, Z16 (Fig. 7). Furthermore, the ratio of 4-OHT-ER bound per ERE for sequence variant Z23 increased more than 3-fold between the monomer and tetramer constructs. This result strongly suggests cooperative binding of 4-OHT-ER to four tandem copies of Z23. In fact, a Scatchard plot of the data from experiments using this construct appears convex (data not shown) and the calculated Hill coefficient of 1.88 is suggestive of cooperative binding (Table 3). However, there was little evidence of cooperative binding of 4-OHT-ER to the other multiple tandem inserts examined, particularly compared to that of E_2-ER binding (Table 3).

Effect of ER–ERE interaction on ligand binding to ER

The rate of specific binding of [^{3}H]E_2-ER and [^{3}H]4-OHT-ER to EREs is identical and binding is stable for at least 12 h at 4°C ([9], data

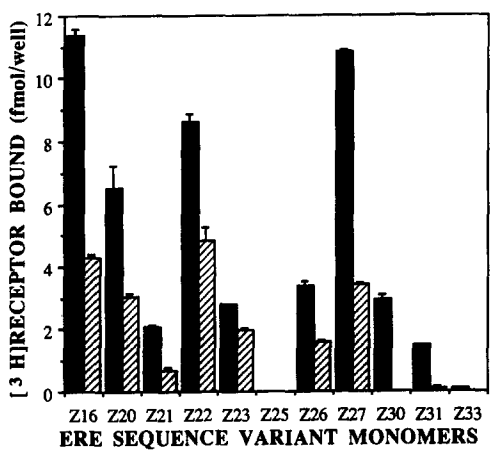

Fig. 6. Comparison of specific binding of [^{3}H]E_2-ER vs [^{3}H]4-OHT-ER binding to monomer ERE sequence variants. [^{3}H]E_2-ER (■) or [^{3}H]4-OHT-ER (▨) binding was measured at saturation (80–110 fmol receptor dimer added/well) to single copies of the indicated sequence variants (sequences are given in Experimental) as described in Experimental and Fig. 1. The data shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol DNA/well.

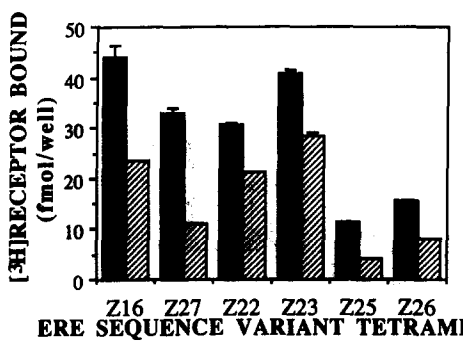

Fig. 7. Comparison of specific binding of [^{3}H]E_2-ER vs [^{3}H]4-OHT-ER binding to four tandem copies of ERE sequence variants. [^{3}H]E_2-ER (■) or [^{3}H]4-OHT-ER (▨) binding was measured at saturation (60–110 fmol receptor dimer added/well) to four tandem copies of the indicated sequence variants (sequences are given in Experimental) as described in Experimental and Fig. 1. The data shown are the average of quadruplicate determinations ± SEM and are calculated for binding to 11 fmol DNA/well.

Table 3. Relationship of [^{3}H]E_2-ER or [^{3}H]4-OHT-ER binding to tetramers of ERE sequence variants and the distance between centers of inverted repeats

ERE sequence variant[a]	Adjacent	First and third (second and fourth)	First and fourth	E_2-ER Hill coeff.	4-OHT-ER Hill coeff.
Z16	3.6	7.1	10.7	1.86 ± 0.17	1.01 ± 0.9[e]
Z22	3.6	7.1	10.7	1.84 ± 0.36	1.44 ± 0.04
Z23	3.6	7.1	10.7	2.59 ± 0.34[c]	1.88 ± 0.19[e]
Z25	3.8	7.5	11.3	ND[b]	
Z26	3.4	6.7	10.1	ND[b]	
Z27	3.4	6.7	10.1	3.00 ± 0.42[c,d]	0.84 ± 0.09

Distances are given as the number of helical turns, assuming 10.4 bp/turn.
[a]Sequences of the ERE variants are given in Experimental.
[b]ND, Hill coefficients could not be determined from the binding data.
[c]Significantly different ($P < 0.01$) from the Hill coefficient of [^{3}H]E_2-ER binding to Z16.
[d]Significantly different ($P < 0.01$) from the Hill coefficient of [^{3}H]E_2-ER binding to Z22.
[e]Significantly different ($P < 0.05$) from the Hill coefficient of [^{3}H]E_2-ER binding to the same sequence variant.

not shown). In order to determine whether a differential loss of ligand occurs upon binding of 4-OHT-ER vs E_2-ER to EREs, [^{3}H]E_2-ER or [^{3}H]4-OHT-ER were incubated with trimer or tetramer ERE constructs. Following the usual 2.5 h incubation in the wells, aliquots of unbound material were removed, incubated with 10% HAP, and the receptor concentration was determined by the standard HAP assay protocol [41, 42]. Analyses indicated that $6 \pm 1.3\%$ of the input [^{3}H]E_2-ER counts and $32 \pm 2.6\%$ of the input [^{3}H]4-OHT-ER counts were not retained by either the wells or HAP, thus representing free [^{3}H]4-OHT ligand. As one control, 100% of [^{35}S]DNA counts were recovered. These results indicate that [^{3}H]4-OHT-ER binding to ERE results in a greater loss of free ^{3}H ligand than does [^{3}H]E_2-ER–ERE binding.

DISCUSSION

Antiestrogens inhibit the proliferative effect of estrogens [47–49], an effect most probably mediated by competitive binding of antiestrogens to ER. Our results show that the specific ERE binding ability of calf uterine ER liganded with either the non-steroidal antiestrogen 4-OH-TAM or with the steroid analog ICI 164,384 is significantly lower than that of E_2-ER. We believe this is the first report to quantitate antiestrogen-liganded receptor binding to EREs *in vitro*.

Earlier reports documented differences in binding of E_2 vs 4-OH-TAM-liganded receptor to isolated nuclei [29, 30], or E_2-ER vs ICI 164,384-liganded receptor to DNA-cellulose [35, 36]. Our results corroborate the findings of others that 4-OHT-ER can bind to the ERE [21–26], but we observed a significant reduction in the total specific binding of 4-OHT-ER, a difference that was not detected by gel retardation assays. When comparing specific binding at saturation, using one, two, three, or four tandem copies of the consensus ERE, 4-OHT-ER binding was about 50% that of E_2-ER binding to that ERE. Our results are consistent with those of Giambiagi and Pasqualini [50] who reported a 38% decrease in DNA-cellulose binding of fetal guinea pig uterine cytosol incubated with OH-TAM vs E_2. Of further interest is our observation that, in contrast to E_2-ER binding to three or four tandem copies of the consensus ERE [37], 4-OHT-ER binding does not appear to be cooperative. This difference in cooperativity may account for the lower amount of total 4-OHT-ER–ERE binding. We suggested that when the inverted repeat portion of alternate consensus core EREs were positioned on the same side of the DNA helix, i.e. trimer and tetramer ERE constructs (distances given in Table 3), protein–protein interaction may occur between bound E_2-ER dimers [37]. Binding of antiestrogens to ER may induce conformational changes that preclude receptor dimers from interacting.

There are at least two explanations for the approximate 50% reduction in total specific binding of 4-OHT-ER to ERE compared to E_2-ER binding to ERE. First, it is possible that 4-OHT-ER binds each ERE as a monomer. Binding of 4-OHT may impose conformation changes that prevent dimerization or cause the dimeric complex to dissociate so that only monomeric 4-OHT-ER remains bound to ERE. This explanation seems unlikely. First, in contrast to the detection of low ($K_d = 450$ nM) affinity binding for molybdate-stabilized monomeric ER [37], the binding of 4-OHT-ER to ERE is of high affinity (0.16–2.56 nM, Table 1). Secondly, the binding mechanism of E_2 and 4-OHT to ER was shown to be identical and cooperative, indicating a preference for dimer

formation [51]. Others reported a direct correlation between the ability of ER mutants to dimerize and their ability to bind DNA [52]. Third, the mobilities of E_2-ER–ERE and AER–ERE complexes, seen in gel retardation assays are nearly identical, indicating that the ERE-bound receptor form is of virtually identical molecular size [21–26]. However, Murdoch and Gorski [53] have called for a re-examination of the oligomeric state of DNA-bound receptor, since binding of E_2 to ER in intact cells is not cooperative.

A second explanation for our results is that, following 4-OHT-ER binding, the resulting protein conformational changes allow ER to dimerize but during that process, one molecule of 4-OHT ligand dissociates from the receptor while the other remains bound. The ERE-bound receptor would remain as a dimer, but with only one ER liganded by 4-OHT. We tested this hypothesis by adding [^{3}H]E_2 to pre-equilibrated [^{3}H]4-OHT-ER–ERE. After incubation overnight to allow any additional ligand association to occur, ^{3}H receptor binding was equal to that expected for E_2-ER rather than for 4-OHT-ER. Although this experiment cannot measure an exchange of ^{3}H ligands, i.e. whether all receptors are occupied by [^{3}H]E_2, or whether dimeric receptors are liganded with one molecule of 4-OHT and one molecule of [^{3}H]E_2, incubation at 4°C does not favor exchange.

An alternate method to answer this question was also used. In two separate experiments, the concentration of [^{3}H]E_2-ER or [^{3}H]4-OHT-ER that was not bound to DNA in the histone/gelatin-coated wells was determined by HAP assay and compared to the amount of [^{3}H]E_2-ER or [^{3}H]4-OHT-ER bound to [^{35}S]DNA in the wells. If all the ^{3}H ligand remained bound to ER, all the ^{3}H counts would be recovered either bound to the wells or bound to HAP. However, this was not the case. After incubation of [^{3}H]4-OHT-ER with plasmid DNA, only 68% of total input ^{3}H ligand was recovered complexed to protein. For [^{3}H]E_2-ER, 94% of input ligand was recovered. Thus, the binding of [^{3}H]4-OHT-ER to ERE resulted in a greater loss of free ^{3}H ligand than did [^{3}H]E_2-ER–ERE binding. One interpretation of these results is that the binding of [^{3}H]4-OHT-ER to ERE results in a conformational change in the receptor dimer that lowers the affinity of one molecule of the ligand and it dissociates from the ER–ERE complex. Future experiments will examine these possibilities.

In support of this notion, the binding of heat-activated rat uterine cytosolic ER to vitellogenin A2 ERE was recently reported to cause a 2-fold increase in the rate of E_2 dissociation [54]. Changes in the conformation of the steroid binding domain induced by DNA binding were suggested to be involved in regulation of ER interactions with other proteins involved in transcriptional activation of target genes.

The K_d values determined by Scatchard analyses revealed that the binding affinity of 4-OHT-ER to one copy of the 38bp consensus ERE, Z16, appeared to be significantly higher than that for E_2-ER binding. Previously, we reported higher affinity binding of 4-OHT-ER vs E_2-ER to nuclei isolated from rat tissues and R3230AC mammary tumor [30]. In contrast, E_2-ER vs 4-OHT-ER binding to two tandem copies of the consensus ERE showed the opposite relationship. The biological significance of these statistical differences is unknown. Gel retardation assays revealed no difference in the affinity of ER binding to an oligomer containing the vitellogenin A2 ERE, when ER was liganded with E_2, OH-TAM, or ICI 164,384 [55].

Gel retardation assays visualize the effect of ligand on the ability of ER to bind to ERE *in vitro*. TAM-liganded ER [19–26], antiestrogen LY117018-liganded ER [56, 57] and 4-OHT-ER [21, 23, 25] form specific complexes with EREs that migrate slower than the corresponding E_2-ER–ERE. The slower migration of 4-OHT-ER was interpreted as indicating that 4-OHT-ER has a different shape than E_2-ER [21, 23, 25]. This difference could be the basis for our observation of lower and non-cooperative 4-OHT-ER–ERE binding. Likewise, mouse ER translated *in vitro* and liganded with ICI 164,384 formed a complex with the ERE that migrated slower than E_2 or DES-liganded ER, but similarly to 4-OHT-ER [19, 22]. On the other hand, a whole cell extract from ICI 164,384-treated insect cells, into which a baculovirus construct containing mouse ER cDNA was transfected, failed to bind ERE [26], and ICI 164,384 added to calf uterine cytosol did not produce a slower migrating complex [58]. We did not detect binding of ICI 164,384-ER to one copy of consensus ERE, Z16, and although we observed ICI 164,384-ER binding to two, three, or four tandem copies of ERE, that binding was of lower capacity and affinity than that for E_2-ER or 4-OHT-ER to the same construct.

ICI 164,384 is thought to prevent or destabilize the dimerization of ER [26]. Our results are

compatible with this interpretation if the binding of monomeric ICI-ER to two or more tandem EREs results from receptor–receptor interaction or receptor interaction with other proteins present in the ER preparation. In contrast to E_2-ER–ERE binding [37], binding of ICI 164,384-ER to multiple EREs gave no evidence of cooperativity.

We found that Mg^{2+} and Ca^{2+} had little effect on the interaction of E_2-ER or 4-OHT-ER with ERE *in vitro*, similar to results of gel retardation assays by Brown and Sharp [20]. In contrast, 2–10 mM $MgCl_2$ decreased the ability of Z-DNA to elute PR liganded with either the progesterone agonist R5020 or antagonist RU486 from DNA cellulose [46]. Addition of 5 mM $MgCl_2$ decreased in RU486-liganded PR binding to Z-DNA by 50% but had no significant effect on R5020-liganded PR binding to the same plasmid [46]. We conclude that Ca^{2+} and Mg^{2+} have different effects on ER–ERE interactions than those reported for PR-Z DNA interactions. The oligomeric state of PR vs ER was recently postulated to account for differences in their DNA binding parameters *in vitro* [59]. Thus, different classes of steroid hormone receptors may exhibit subtle differences in specific HRE-binding that remain to be elucidated.

The effect of ligand on ER binding to variants of the consensus ERE revealed quantitative differences in the specific ERE binding capacity for E_2-ER vs 4-OHT-ER. Similar to results of binding to one copy of the consensus ERE, Z16, 4-OHT-ER binding to sequence variants containing one or two nucleotide changes in the 3′ half of the inverted repeat, i.e. Z20, Z21, and Z22, was about 50% of that seen for E_2-ER binding. Sequence variant Z20 is a functional ERE in the promoter of the rabbit uteroglobin gene [60].

However, 4-OHT-ER binding to one copy of sequence variant Z23, which has one nucleotide change in the 3′ half site: 5′-TGTCC-3′, making it most similar to a GRE [37], showed binding most comparable to that of E_2-ER. Truss *et al.* [61] observed that this sequence, 5′-TGTCC-3′, bound both ER and PR in gel retardation assays and elicited enhanced CAT activity in transient transfection assays upon addition of either DES, dexamethasone, or R5020. They suggested that this sequence may be related to the nGRE (negative GRE, which, when bound by GR, inhibits transcription) and may bind GR and PR in a non-productive form [61]. Perhaps 4-OHT-ER-liganded ER competes for GR and PR binding to such a sequence, depending on the ratio of the individual receptors and their ligands in a given cell.

The role of ligand on ER interaction with the ERE is curiously unresolved. Earlier studies *in vitro* showed that E_2 binding induced a change in ER conformation that led to increased affinity for DNA cellulose [62]. More recently, similar levels of ER were crosslinked to chromatin of MCF-7 cells incubated with E_2 or ICI 164,384 or in the absence of hormone [63]. On the other hand, either E_2 or the antiestrogen nafoxidine were required to promote ER binding to DNA in yeast cells *in vivo* [64]. In contrast, a recent report showed no effect of added E_2 on the ERE binding capacity of heat-activated rat uterine ER [65]. A model was proposed in which ER is always bound to EREs; ligand binding promotes a conformational change in the receptor that affects the dimerization domain, enhancing ER interaction with nuclear proteins and hence, altering transcription [53].

The results reported here, and those on 4-OHT-ER or TAM-ER binding to EREs published by others, indicate that 4-OH-TAM binding to ER induces a change in the ability of the receptor to interact with ERE. There is an increasing consensus that steroid receptors mediate the activation of gene transcription in cooperation with other transcription factors [7, 52, 66–72]. The observation that synergism between ER bound to EREs requires their stereoalignment, i.e. the same face of the DNA helix, suggests that protein–protein interactions between ER domains or between factors bound to the receptor are critical to transcriptional activation [73]. However, one recent report showed no apparent synergism between GR and the basal transcription factors SP-1, Oct-1, or CTF-1 [74]. Since the transcription of reporter gene constructs containing ERE is not activated by extracts from cells treated with 4-OH-TAM [21, 27] or ICI 164,384 [27], one explanation is that the protein conformation of AER, while capable of binding DNA with affinity comparable to that of E_2-ER, at least for 4-OHT-ER, does not permit stable interaction of the DNA-bound ER with nascent transcription factors or adaptor molecules that are critical to the initiation of gene transcription. Studies examining these suggestions should provide important information on estrogen agonist vs antagonist action and could form the basis for development of new compounds to treat women with breast cancer.

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