Cooperative Binding of Estrogen Receptor to DNA Depends on Spacing of Binding Sites, Flanking Sequence, and Ligand†

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ABSTRACT: It has been suggested that cooperative binding of estrogen receptor (ER) may, in part, be responsible for the synergistic activation of transcription of estrogen-responsive genes that contain multiple estrogen-response elements (EREs). Experiments described here show that estradiol-liganded ER (E_2−ER) binds cooperatively to stereoaligned EREs that are surrounded by naturally occurring flanking sequences, such as an AT-rich region. In contrast, EREs lacking these sequences do not bind E_2−ER cooperatively, regardless of ERE spacing or stereoalignment. Moreover, binding is of lower affinity and capacity in the absence of these critical flanking sequences. By varying the sequence of nucleotides adjacent to the ERE, features important for the flanking sequence effect were characterized. Interestingly, when ER was liganded with 4-hydroxytamoxifen (4-OHT), the active metabolite of the widely used therapeutic antiestrogen tamoxifen, the antiestrogen-liganded ER complex (4-OHT−ER) did not bind cooperatively to multiple EREs, regardless of spacing or flanking sequence. We postulate that ERE flanking sequences bestow upon E_2−ER enhanced ERE binding capacity and cooperativity, but do not affect 4-OHT−ER−ERE binding.

Exposure to estrogens has been closely associated with a number of cancers, *e.g.*, endometrial, breast, and vaginal carcinomas. It is generally believed that the mitogenic effects of cumulative exposure to estrogens is the primary etiological factor in breast cancer (Henderson et al., 1991). Estrogens also play an important role in the regulation of reproduction and normal mammary gland, uterine, bone, and cardiovascular system homeostasis. The many diverse actions of estrogens are mediated by the estrogen receptor (ER),[1] a hormone-inducible transcription factor that stimulates the transcription of specific genes, including a number encoding proteins involved in the control of cell proliferation, *e.g.*, transforming growth factor α and the oncogene product c-fos (O'Malley, 1991; Evans, 1988; Gullick, 1990; Bates et al., 1988).

In the presence of ligand, *e.g.*, estradiol (E_2), ER dimerizes and binds with high affinity to specific DNA sequences, estrogen-response elements (EREs), containing the minimal core inverted repeat 5′-GGTCAnnnTGACC-3′. The binding of E_2-liganded ER (E_2−ER) to EREs, typically located 5′ to the promoter of estrogen-regulated genes, increases the efficiency of transcription initiation (Green & Chambon, 1991).

Antiestrogens inhibit estrogen action by competitive binding to ER (Lerner & Jordan, 1990; Klinge et al., 1992a). The current antihormonal treatment of choice for postmenopausal women with ER-positive breast cancer is tamoxifen, a non-steroidal agent which likely interferes with estrogen-mediated cell proliferation. An active metabolite of tamoxifen, 4-hydroxytamoxifen (4-OHT), binds to ER with high affinity and induces high-affinity ER−ERE binding, but fails to activate transcription (Lerner & Jordan, 1990). The exact mechanisms responsible for ER gene activation remain to be definitively elucidated, but may require functional interactions among receptor molecules as well as interactions between bound receptor and other transcription factors (Beato, 1989).

Many naturally occurring estrogen-responsive genes contain multiple copies of consensus or variant forms of the core inverted repeat, which function synergistically to regulate transcription (Klein-Hitpass et al., 1988; Martinez & Wahli, 1989; Ponglikitmongkol et al., 1990). For example, the estrogen response of the *Xenopus* vitellogenin B1 gene is mediated through two closely spaced EREs that have one- and two-nucleotide sequence variations, respectively, from the consensus (Martinez et al., 1987). In transient expression assays, the inductive capacity of the two EREs together was much greater than that of the sum of either alone, *i.e.*, the EREs act synergistically (Martinez & Wahli, 1989). Moreover, the two EREs, separated by two helical turns, were shown to bind ER cooperatively. These results prompted Martinez and Wahli to propose that the synergy of activation results from cooperative ER−ERE binding. Indeed, cooperative binding is well documented

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[1] Abbreviations: BSA, bovine serum albumin; c, 17-bp consensus; cxx, 13-bp consensus; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; E_2, estradiol; E_2−ER, E_2-liganded estrogen receptor; ER, estrogen receptor; ERE, estrogen-response element; $(ER-ERE)_n$, estrogen receptor−responsive element multimer, where *n* indicates the number of ER−ERE complexes; EDTA, ethylenediaminetetraacetic acid; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; m, monomer; NP-40, Nonidet P-40; PMSF, phenylmethanesulfonyl fluoride; PR, progesterone receptor; PRE, progesterone-responsive element; 4-OHT, 4-hydroxytamoxifen; 4-OHT−ER, 4-OHT-liganded estrogen receptor; (+), AT-rich; (−), AT-lacking.

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Table 1: Sequences of ERE Constructs

name[a]	DNA sequence
EREc(+)	5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′
ERE7.2(+)	5′-TCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAGCTAGCACTGACGCTAGCGAGCTAAAA-TAACACATTCAGCCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAA-3′
ERE7.2(−)	5′-CCTGGTCACTCTGACCGGGGTTGGAAATCGATAAGCTTGTTACAAGCTTGGATCCGGAGAGCTCC-CAACGCGTTGGATGCAGGTCACTCTGACCTGGTGCA-3′
GCEREc	5′-CCAGGTCAGAGTGACCTGAGCTGACAGGACTCGACCAG-3′
EREcxx(+)	5′-CCAGGTCAGAGTGACCGACGTTAAAATAACACATTCAG-3′
EREc(−)	5′-CCAGGTCAGAGTGACCTGAG-3′
EREm(−)	5′-CTGGTCACTCTGACC-3′
ERE1.4(−)	5′-CCGGTCACTCTGACCACGGTCACTCTGACC-3′
ERE2(−)	5′-CCGGTCACTCTGACCACTGACTGGTCACTCTGACC-3′
EREc2(+)	5′-CCAGGTCAGAGTGACCTGAGCCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′
EREc2.5(+)	5′-CCAGGTCAGAGTGACCTGAGCCAAAACAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′
EREc2(−)	5′-CCAGGTCAGAGTGACCTGAGCCAGGTCAGAGTGACCTGAG-3′
EREc2.5(−)	5′-CCAGGTCAGAGTGACCTGAGCCAAAACAGGTCAGAGTGACCTGAG-3′

[a] c = 17-bp consensus; cxx = 13-bp consensus; m = monomer; GCERE = GC-rich; (+) = AT-rich; (−) = AT-lacking; numbers indicate ERE center-to-center helical spacing.

for other transcription factors (Hochschild & Ptashne, 1986; Lebowitz et al., 1989). However, others (Klein-Hitpass et al., 1988; Ponglikitmongkol et al., 1990) have found no evidence for cooperative binding of ER to either consensus or variant ERE pairs.

In contrast, we previously demonstrated high-affinity cooperative binding of E_2−ER to three or four tandem EREs containing a 17-bp perfect consensus inverted repeat and a 3′ AT-rich region, positioned such that alternate EREs were ~7 helical turns apart (Klinge et al., 1992c). In contrast, binding was not cooperative to constructs with two such tandem EREs, where the EREs were 3.6 helical turns apart. This suggested that E_2−ER binds cooperatively only to EREs on the same face of the DNA helix (Klinge et al., 1992c). Moreover, we recently showed that E_2−ER binds non-cooperatively to a number of ERE dimers lacking the AT-rich flanking region, regardless of ERE spacing. Thus, cooperativity of ER−ERE binding may depend on the presence of specific flanking sequences that could facilitate ER-induced DNA bending or bind other transcription factors which stabilize ER binding (Anolik et al., 1993). On the basis of our results we postulated that the number, spacing, and sequence of EREs can produce fine tuning of the response of individual genes to increasing estrogen levels *in vivo*, through variations in the degree of cooperative binding.

High-affinity ER−ERE binding was also observed with antiestrogen-liganded receptor (4-OHT−ER), but the binding capacity was consistently approximately 50% lower than that for E_2−ER−ERE binding. Moreover, no cooperative 4-OHT−ER−ERE binding was detected, suggesting that antiestrogen (4-OHT) binding to ER induces a change in the ability of the receptor to interact with EREs (Klinge et al., 1992b).

Whereas there is a more general agreement that GR and PR can bind cooperatively to multiple GREs/PREs, contributing to observed functional synergism at the transcriptional level, the conflicting results summarized above indicate that the picture for ER−ERE binding remains unclear. In this report, we definitively demonstrate that ERE flanking sequences are essential for cooperative binding of ER to stereoaligned EREs. Moreover, the estrogen agonist estradiol is required for maximal saturation binding levels and cooperative binding of ER to these EREs. E_2−ER binding is cooperative to appropriately spaced EREs flanked by natural sequences, such as the consensus AT-rich region. In contrast, ER liganded with the estrogen antagonist 4-hydroxytamoxifen binds non-cooperatively, regardless of ERE spacing or flanking sequences. We suggest that ER is able to interact with the appropriate flanking sequences to achieve enhanced binding capacity and cooperative binding only when liganded with an estrogen agonist, not an estrogen antagonist.

EXPERIMENTAL PROCEDURES

Preparation of ERE-Containing Plasmids. Synthetic oligonucleotides were the generous gift of Dr. Robert Lechner (United States Biochemical, Cleveland, OH) or were purchased from Genosys Biotechnologies (Woodlands, TX). The sequences are given in Table 1 with nomenclature definitions. Note that (+) and (−) refer to with and without an AT-rich region, respectively. Double-stranded ERE oligomers were blunt-end ligated into the *Sma*I restriction site of the plasmid pGEM-7Zf(+) (Promega, Madison, WI) as described (Peale et al., 1988; Klinge et al., 1992c; Anolik et al., 1993). Construct ERE7.2(−) was obtained by subcloning a single ERE into the *Nsi*I site of construct EREm(−) and inserting linker DNA at the *Hin*dIII site. Construct EREc(−) was derived from the GCEREc by cleaving off the GC-rich region with *Alu*I, blunt-end ligating into the *Sma*I site of the plasmid, and transforming bacteria. EREc2(−) and EREc2.5(−) were similarly derived from EREc2(+) and EREc2.5(+), respectively. ERE7.2(+) was ligated at staggered ends into the *Eco*RI and *Hin*dIII sites of the plasmid.

Preparation of Estrogen Receptor. ER was partially purified from calf uterus according to the method of Weichman and Notides (1977) as previously modified (Klinge et al., 1987). The ammonium sulfate cytosol fraction (0−30%) was further purified by heparin agarose (Affi-Gel Heparin, Bio-Rad, Richmond CA) affinity chromatography and liganded with either 17β-[2,4,6,7,16,17-^{3}H]E_2 (145 Ci/mmol from Amersham) or *Z*-4[*N-methyl*-^{3}H]4-OHT (71 Ci/

mmol from Amersham). When 4-OHT was used, the [^{3}H]4-OHT-ER was protected from exposure to light during all steps of the procedure.

Microtiter Well Plate Assay of ER Binding to Plasmid DNA. Plasmid DNA was linearized with *Eco*RI for the microtiter well plate assay experiments presented here. Aliquots of *Eco*RI-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 *Escherichia coli* DNA polymerase I (New England Biolabs, Beverly, MA) and mixed with unlabeled DNA for the desired final concentration. The microtiter (well) plate assay for measuring [^{3}H]ER binding to DNA has been described (Ludwig et al., 1990; Klinge et al., 1992c). An advantage of this technique is that double label counting and calculation of the ratio of ^{3}H-liganded ER to ^{35}S-labeled DNA retained in the wells allows stoichiometric determination of ER binding to DNA.

For saturation binding analysis, various concentrations of heparin agarose affinity purified [^{3}H]E_2−ER or [^{3}H]4-OHT−ER were preincubated with one concentration (approx. 0.22 nM) of [^{35}S]DNA (plasmid DNA with or without ERE) for 2.5 h at 4 °C, with shaking in TDPK 100 buffer (40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 mM PMSF, 100 mM KCl) containing 0.1% NP-40. Fifty μL aliquots of the receptor-DNA equilibrium mixture were then incubated in histone/gelatin-coated microtiter wells (EIA/RIA 8 well strips, cat. no. 2580, Costar) for 2.5 h at 4 °C with shaking, until DNA binding (free DNA or DNA bound to ER) approached saturation. The histone/gelatin coating allows preferential retention of DNA or ER−DNA complexes compared to free ER (Ludwig et al., 1990). Unbound DNA or ER was removed with three washes of TDPEK 100 buffer (TDP buffer containing 1 mM EDTA and 100 mM KCl) containing 100 μg/mL carboxymethyl-BSA, and the radioactivity remaining in the wells was measured by scintillation counting.

The time course for ER−DNA binding in the microfuge tubes and binding to histone/gelatin-coated wells demonstrated that binding approached a maximum by 2−2.5 h, regardless of ligand, ER concentration, or DNA sequence and that binding was stable for at least 4 h (Ludwig et al., 1990; C. M. Klinge, personal communication; data not shown).

At each ER concentration, the receptor was incubated in parallel both with pGEM-7Zf(+) plasmid alone and with plasmid containing an ERE construct. The specific binding to EREs was calculated by taking the difference between the two binding values. The amount of [^{3}H]ER−[^{35}S]DNA binding was calculated by adjusting for that amount of [^{35}S]-DNA that did not bind to the well and for the background binding of the [^{3}H]ER preparation in wells without DNA added (Ludwig et al., 1990; Klinge et al., 1992c).

Gel Mobility Shift Assay. ERE-containing plasmid DNA (pGEM-7Zf(+)) was typically digested with *Eco*RI (13 bp 5′ to ERE insert) and *Bam*HI (22 bp 3′ to ERE insert) to free double stranded ERE-containing oligomers with recessed 3′-termini. ERE-containing oligomers were purified by polyacrylamide gel electrophoresis, electroeluted, and labeled by filling to blunt ends using [α-^{32}P]dATP (400 Ci/mmol from Amersham) and the Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by centrifugation through a G50 Sephadex spin column in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The sizes of the ERE oligomers used were 77, 115, 82, and 145 bp for the EREc(+) monomer, the EREc(+) and GCERE dimers, ERE1.4(−) and ERE7.2(+), respectively.

Typical binding reaction mixtures contained 10 fmol (10 000 dpm) of ^{32}P-labeled DNA, 91 fmol of liganded ER, and 2 μg of poly(dI-dC) in 40-μL reaction mixtures containing 56 mM KCl in TDP buffer and 14% glycerol. It was observed that the inclusion of a nonspecific protein such as BSA enhanced complex resolution, and hence BSA was included in some binding reactions, as indicated in the figure legends. Binding reactions were performed on ice for 1.5−2 h before samples were loaded on 4% nondenaturing polyacrylamide gels (12 cm in length) and subjected to electrophoresis at 150−200 V in 0.5× TBE (0.05 M Tris, 41 mM boric acid, and 0.5 mM EDTA, pH 8.3) at 4 °C. Gels were dried and exposed to X-ray film (XAR-5; Eastman Kodak Co., Rochester, NY) with intensifying screens (Lightning Plus from DuPont Co., Wilmington, DE) at room temperature or −80 °C.

Competitor DNA was prepared by *Eco*RI digestion of pGEM-7Zf(+)-derived plasmids or *Eco*RI/*Bam*HI digestion followed by electroelution of the ERE-containing fragment. In some instances, nonspecific salmon sperm DNA (Sigma) was used. In some experiments, ER monoclonal antibody (H222; Abbott Laboratories, North Chicago, IL) or control normal rat serum (Abbott) was added to the reaction mixtures.

RESULTS

Cooperative Binding of E_2−ER to an ERE Dimer Flanked by an AT-Rich Sequence. We have previously reported cooperative binding of E_2−ER to three (trimer) or four (tetramer) tandem EREs containing a 17-bp perfect consensus inverted repeat and a 3′ AT-rich region [EREc(+)], positioned such that alternate EREs were 7.2 helical turns apart (Klinge et al., 1992c). In contrast, E_2−ER binds noncooperatively to several dimeric EREs of varied spacing, containing a 13-bp perfect inverted repeat but lacking the AT-rich region (Anolik et al., 1993). The latter result may be due to characteristics of the center-to-center spacing, the number of tandem repeats, or the flanking sequence. The experiments described here, measuring binding of [^{3}H]E_2−ER to ERE dimers of varied spacing and flanking sequence, were designed to distinguish among these possibilities.

The specific binding of [^{3}H]E_2−ER to pGEM-7Zf(+) plasmid bearing EREc(+) dimer, ERE7.2(+), or ERE7.2-(−) inserts (sequences in Table 1) was measured at a fixed concentration of DNA and with increasing concentrations of [^{3}H]E_2−ER by a microtiter well plate assay (Ludwig et al., 1990). The results are shown in Figure 1. For EREc-(+) dimer and ERE7.2(+), which both contain AT-rich sequences flanking the inverted repeats, a 1:1 stoichiometry of [^{3}H]E_2−ER dimer bound per ERE was observed (Table 2). In contrast, E_2−ER binding at saturation was reduced by approximately 50% in the absence of an AT-rich region [ERE7.2(−)]. Moreover, the affinity of binding was significantly reduced as indicated by Scatchard analyses of the data, with K_d values of 0.24 and 0.50 nM for EREc(+) dimer and ERE7.2(−), respectively (Figure 1B and Table 3). Thus, the AT-rich region appears to be important for enhanced E_2−ER binding capacity and binding affinity, consistent with our previous results (Anolik et al., 1993).

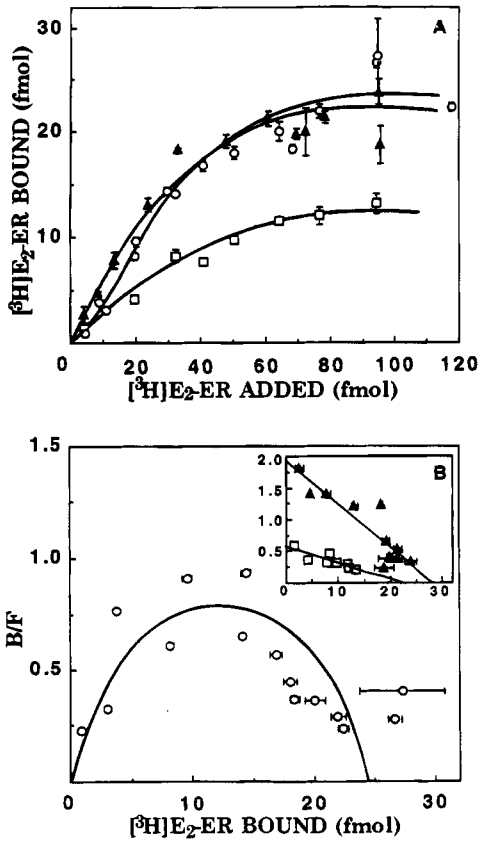

FIGURE 1: Saturation analysis of specific [^{3}H]E_2−ER binding to dimeric EREs. *Eco*RI-linearized [^{35}S]dATP-end-labeled plasmid DNA, either the parental plasmid alone or construct EREc(+) dimer (▲), ERE7.2(+) (○), or ERE7.2(−) (□) (A), was incubated with increasing concentrations of [^{3}H]E_2−ER. The data points shown are the average of quadruplicate determinations ± SEM from four to seven representative plate assay experiments and are calculated for binding to 11 fmol of DNA/well as described in Experimental Procedures. (B) Saturation analysis plotted according to the method of Scatchard. The lines were calculated by least squares regression analysis, and K_d values were derived from the slopes. K_d = 0.23 nM for EREc(+) dimer (inset, ▲) and 0.50 nM for ERE7.2(−) (inset, □).

The Scatchard plot of [^{3}H]E_2−ER binding to ERE7.2(+) was curvilinear convex in appearance, indicative of cooperative binding (Scatchard, 1949; McGhee & von Hippel, 1974; Schwarz & Watanabe, 1983). In contrast, the Scatchard plots for EREc(+) dimer and ERE7.2(−) are linear. The calculated Hill coefficients of ER binding to EREc(+) dimer, ERE7.2(+), and ERE7.2(−) are 1.16, 1.89, and 0.87, respectively, again indicative of cooperative binding to ERE7.2(+) (summarized in Table 3; Hill plots not shown). These results indicate that E_2−ER can bind cooperatively to an ERE dimer of the appropriate spacing and flanking sequence.

Analysis of E_2−ER Binding to EREs Flanked by a GC-Rich Sequence. E_2−ER binds cooperatively to ERE7.2(+), but non-cooperatively to ERE7.2(−). There are two critical differences between the flanking sequences of these two constructs. First, the 5 bp immediately 3′ of the 13-bp inverted repeat are different, such that ERE7.2(+) actually contains an extended 17-bp palindrome, whereas ERE7.2(−) has the shortened 13-bp palindrome. Additionally, there are significant differences in nucleotide sequence in the more distal flanking regions, such that the entire sequence separating the two inverted repeats is 60% AT in ERE7.2(+) but only 50% AT in ERE7.2(−).

Table 2: Stoichiometric Relationship of Specific E_2−ER or 4-OHT−ER ERE Interaction[a]

DNA	helical spacing[b]	ratio[c] E_2−ER-plasmid	ratio E_2−ER−ERE	ratio 4-OHT−ER-plasmid	ratio 4-OHT−ER−ERE
EREc(+) monomer		0.97	0.97	0.41	0.41
EREc(+) dimer	3.6	2.09	1.09	1.03	0.52
EREc(+) trimer	3.6, 7.2	3.15	1.05	1.78	0.59
EREc(+) tetramer	3.6, 7.2	4.10	1.03	2.14	0.54
GCEREc monomer		1.03	1.03	0.602	0.602
GCEREc dimer	3.6	1.98	0.99	1.06	0.53
GCEREc trimer	3.6, 7.2	2.40	0.80	1.49	0.50
GCEREc tetramer	3.6, 7.2	2.78	0.70	1.90	0.48
ERE7.2(+)	7.2	2.05	1.03	1.04	0.52
ERE7.2(−)	7.2	1.35	0.68	1.03	0.52
EREm(−)		0.52	0.52	0.40	0.40
EREcxx(+)		0.90	0.90	ND[d]	
EREc(−)		1.18	1.18	ND[d]	
EREc2(+)	2	1.32	0.66	ND[d]	
EREc2.5(+)	2.5	1.34	0.67	ND[d]	
EREc2(−)	2	1.06	0.53	ND[d]	
EREc2.5(−)	2.5	1.14	0.57	ND[d]	

[a] Saturation analyses were performed by microtiter well plate assay using a fixed concentration of plasmid DNA (11 fmol/well) and increasing concentrations of [^{3}H]E_2−ER or [^{3}H]4-OHT−ER, as described in the text. [^{3}H]E_2−ER or [^{3}H]4-OHT−ER binding to plasmid (pGEM-7Zf(+)), alone or containing each insert as indicated, was measured. The binding ratios presented were calculated from representative binding values taken at saturation (moles of receptor dimer bound per moles of plasmid or ERE), from which background and nonspecific binding to plasmid without inserts has been subtracted. [b] Distances are given as the number of helical turns, assuming center-to-center spacing and 10.4 bp/turn. [c] The ratio ER:plasmid represents the number of moles of ER dimer bound per mole of plasmid, with nonspecific binding to plasmid without ERE inserts subtracted. The ratio ER:ERE divides this number by the number of EREs present in the insert. [d] Not determined.

In order to determine whether the AT-richness of the EREc(+) 3′ flanking sequence was critical for cooperative binding of E_2−ER, a construct was designed (GCERE) that contains a GC-rich sequence flanking the ERE inverted repeat. However, the 17-bp extended palindrome was maintained. E_2−ER binding to pGEM-7Zf(+) plasmid bearing one (monomer), two (dimer), three (trimer), or four (tetramer) tandem copies of the GCERE was analyzed (Tables 2 and 3; saturation plots not shown). Surprisingly, stoichiometric levels of ER dimer are bound. For example, the ratios of E_2−ER dimer to plasmid DNA are 1.03 and 1.98 for the GCERE monomer and dimer, respectively (Table 2). Thus, it appears that the AT-richness *per se* of the flanking region is not critical for achieving maximal saturation binding capacity. However, it is interesting that we observe a somewhat reduced saturation binding capacity for the GCERE trimer and tetramer (Table 2).

Scatchard plots of the data reveal high-affinity binding, comparable to that of the analogous EREc(+) AT-rich constructs (Scatchard plots not shown; data summarized in Table 3). The Scatchard plots for E_2−ER binding to the GCERE trimer and tetramer are convex in shape, with Hill coefficients of 2.79 and 1.74, respectively, indicative of positive cooperative binding (summarized in Table 3; Hill plots not shown). In contrast, the Scatchard plots for the GCERE monomer and dimer are linear. Moreover, the calculated Hill coefficients for E_2−ER binding to the monomer and dimer are 1.27 and 1.03, respectively, suggestive of non-cooperative binding.

Table 3: Comparison of [^{3}H]E_2−ER or [^{3}H]4-OHT−ER Binding to ERE Constructs[a]

DNA	E_2−ER			4-OHT−ER		
	n[b]	K_d (nM)	Hill coefficient	n (nM)	K_d coefficient	Hill
EREc(+) monomer	17	0.24 ± 0.01	1.16 ± 0.03	24	0.16 ± 0.01[k]	<1[c]
EREc(+) dimer	12	0.23 ± 0.03	1.24 ± 0.04	22	0.69 ± 0.02[k]	0.74 ± 0.02
EREc(+) trimer	14	2.17 ± 0.46[g]		23	0.40 ± 0.01	0.71 ± 0.01
EREc(+) tetramer	32		1.86 ± 0.17[h]	22	3.55 ± 0.18	1.01 ± 0.04
GCEREc monomer	13	0.28 ± 0.01	1.27 ± 0.05	13	0.58 ± 0.11[k]	0.75 ± 0.36
GCEREc dimer	18	0.32 ± 0.03	1.03 ± 0.05	18	0.61 ± 0.08[k]	0.77 ± 0.19
GCEREc trimer	18		2.79 ± 0.15[i]	20		1.19 ± 0.05
GCEREc tetramer	22		1.74 ± 0.11[i]	24		1.05 ± 0.05
ERE7.2(+)	28		1.89 ± 0.20[h]	14	0.49 ± 0.04	0.91 ± 0.06
ERE7.2(−)	24	0.50 ± 0.02[e]	0.87 ± 0.01[j]	14	1.02 ± 0.20[f]	0.81 ± 0.03
EREm(−)	15	0.81 ± 0.04[e]	<1[c]		ND[d]	
EREcxx(+)	19	1.56 ± 0.15[e]	1.33 ± 0.03		ND[d]	
EREc(−)	22	2.02 ± 0.24[e]	1.27 ± 0.02		ND[d]	
EREc2(+)	21	0.40 ± 0.02[e]	0.80 ± 0.01		ND[d]	
EREc2.5(+)	26	0.87 ± 0.08[e]	0.93 ± 0.01		ND[d]	
EREc2(−)	27	0.53 ± 0.03[e]	0.77 ± 0.01		ND[d]	
EREc2.5(−)	25	0.74 ± 0.06[e]	0.86 ± 0.01		ND[d]	

[a] Saturation analyses were performed using the microtiter well plate assay, at a fixed concentration of plasmid DNA (11 fmol/well) and increasing concentrations of [^{3}H]E_2−ER or [^{3}H]4-OHT−ER as described in the text. Data was plotted according to the method of Scatchard and Hill to derive K_d values and Hill coefficients. The values shown were derived from the binding data in 3−7 separate experiments for a total of n different concentrations of receptor. [b] n = number of different concentrations of [^{3}H]E_2−ER or [^{3}H]4-OHT−ER assayed for DNA binding, each in quadruplicate. [c] Since binding is less than 1 ER dimer per plasmid, $\log(Y/1 - Y)$ is less than 0. The slope of the best fit line for the data was <1. [d] Not determined. [e−k] The value given is significantly different from one of the following: [e] K_d of E_2−ER binding to EREc(+) monomer and dimer ($p < 0.005$); [f] K_d of 4-OHT−ER binding to ERE7.2(+) ($p < 0.01$); [g] Hill coefficient of E_2−ER binding to EREc(+) monomer and dimer ($p < 0.05$); [h] Hill coefficient of E_2−ER binding to EREc(+) monomer and dimer ($p < 0.005$); [i] Hill coefficient of E_2−ER binding to GCERE monomer and dimer ($p < 0.005$); [j] Hill coefficient of E_2−ER binding to ERE7.2(+) ($p < 0.005$); [k] K_d of E_2−ER binding to the same ERE ($p < 0.005$).

In summary, E_2−ER binding to EREs flanked by an AT-rich region or a GC-rich region is comparable in most respects, including binding affinity and capacity and cooperativity of binding. These results suggest either that the extended palindrome is the critical feature for the previously observed flanking sequence effect or that the more distal AT-rich and GC-rich sequences share one or more common features that allow for enhanced E_2−ER binding capacity and affinity and cooperative binding.

Analysis of ER−ERE Complexes by Gel Shift Assay. Gel mobility shift assays were performed to visualize the complexes formed between ER and EREs of varying flanking sequence. Figure 2 compares ER binding to ERE dimers flanked by an AT-rich region (67% AT) [EREc(+) and ERE7.2(+)], a GC-rich region (65% GC) (GCERE), or plasmid DNA (50% AT) [ERE1.4(−)]. For comparison, lane 1 shows ER binding to the EREc(+) monomer, in which two prominent bands are observed. Although the exact nature of each of these ER−ERE complexes is unknown, all are specific since they are competed by unlabeled ERE-containing oligonucleotide (Figure 2, lanes 10−13). Moreover, all the complexes observed contained ER protein since they are supershifted with anti-ER antibody H222 (data not shown). Among the likely explanations for the appearance of multiple complexes are differential phosphorylation of the ER, association of other proteins with the ER (Curtis & Korach, 1990, 1991), or ER binding as a monomer and a homodimer.

Identical complexes are observed for ER binding to the ERE dimers, but an additional slower migrating complex is observed, representing two ER dimers bound [$(ER-ERE)_2$]. The intensities of the various complexes vary, with weaker binding to ERE1.4(−), which lacks both the extended palindrome and the AT-rich flanking region. Moreover, it is evident that the ER complexes formed with ERE1.4(−) are displaced by unlabeled EREc(+) tetramer much more readily compared to the other constructs, which contain the AT-rich region or a GC-rich region. At a 40-fold molar excess of competitor over labeled DNA, the ER−ERE1.4-(−) complexes are completely abolished, whereas there is some residual complex formation with the other constructs (data not shown). These results are consistent with plate

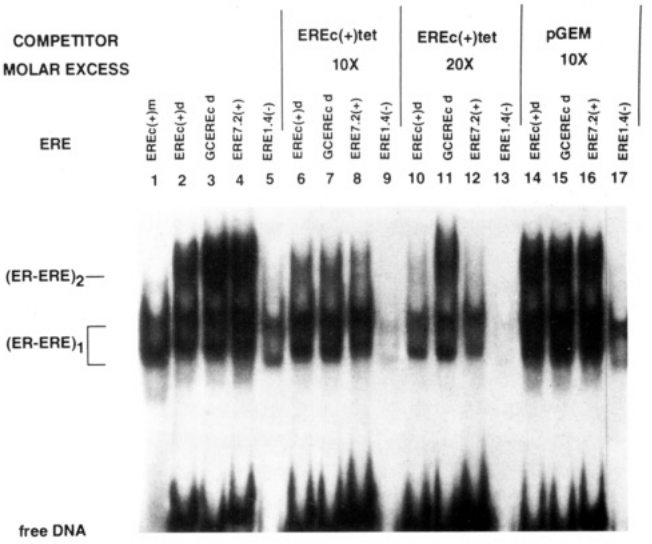

FIGURE 2: Gel mobility shift assay of E_2−ER binding to ERE dimers of various flanking sequences. ^{32}P-Labeled double-stranded oligomers (10 000 dpm, 10 fmol) containing the indicated EREs were incubated with partially purified E_2−ER (91 fmol), 2 μg of poly(dI-dC), 10 μg of BSA, and the indicated amounts of unlabeled competitor DNA (molar excess over [^{32}P]oligomer with respect to ERE sites), EREc(+) tetramer, or pGEM nonspecific competitor. The total reaction volume was 40 μL, containing 56 mM final KCl. Gel mobility shift analysis was performed as described in Experimental Procedures with 40 μL loaded per lane. $(ER-ERE)_n$ indicates that there are n number of E_2−ER−ERE complexes, *e.g.*, $(ER-ERE)_1$ indicates one dimeric E_2−ER bound to one ERE, as seen in lane 1 for EREc(+) monomer. Lanes 1−5 show complex formation in the absence of any added competitor.

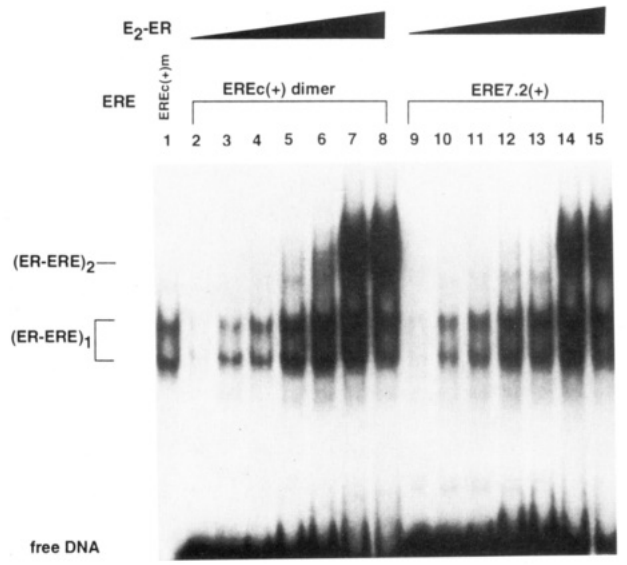

FIGURE 3: Gel mobility shift assay of ER−ERE binding: E_2−ER titration experiment with EREc(+) dimer *versus* ERE7.2(+). ^{32}P-Labeled double-stranded oligomers (10 000 dpm, 10 fmol) containing the indicated EREs were incubated with increasing concentrations of partially purified E_2−ER, 2 μg of poly(dI-dC), and 10 μg of BSA, in a total reaction volume of 30 μL and 37 mM KCl. Gel mobility shift analysis was performed as described in Experimental Procedures with 30 μL loaded per lane. $(ER-ERE)_n$, where n is the number of E_2−ER−ERE complexes, *e.g.*, $(ER-ERE)_1$ indicates one dimeric E_2−ER bound to one ERE, as seen in lane 1 for EREc-(+) monomer. The ER concentrations are as follows: (2) and (9), 3.4 fmol; (3) and (10) 7.7 fmol; (4) and (11), 13.5 fmol; (5) and (12), 24.9 fmol; (6) and (13), 45.5 fmol; (7) and (14), 68.2 fmol; and (8) and (15), 91 fmol.

assay experiments demonstrating lower affinity binding to the AT-lacking ERE1.4(−) (Anolik et al., 1993).

An ER titration experiment (Figure 3) was performed with EREc(+) dimer, where the spacing of EREs was such that binding was non-cooperative, and ERE7.2(+), which binds E_2−ER cooperatively as measured by the plate assay. At low concentrations of receptor, only $(ER-ERE)_1$ complexes are seen. As clearly seen in Figure 3, with increasing ER concentration the intensity of the $(ER-ERE)_1$ complexes is increased, and $(ER-ERE)_2$ complex begins to appear. The $(ER-ERE)_2$ complex does not become predominant until high concentrations of ER are attained. The results of these gel shift experiments contrast with those of Tsai et al. (1989) for PR interaction with a PRE dimer, in which binding of two PR dimers is strongly favored over binding of one PR dimer, even when only 30% of sites are occupied. Tsai et al. interpreted their results as indicative of cooperative binding of PR. It is curious that we see a similar pattern of complex appearance with increasing ER concentrations for both EREc(+) dimer and ERE7.2(+), the latter of which bound E_2−ER cooperatively in microtiter well plate assay experiments. As detailed in the Discussion, it remains possible that any ER−ER, ER−other protein, or ER−DNA interactions important for cooperative binding are weak relative to those of PR, such that they are disrupted under gel electrophoresis conditions.

Non-Cooperative Binding of 4-OHT−ER To EREs. In contrast to E_2−ER binding, 4-OHT−ER binding to EREs was similar in the presence [EREc(+) dimer and ERE7.2-(+)] and absence [ERE7.2(−)] of AT-rich flanking sequences (Figure 4). At saturation, 4-OHT−ER binding to the AT-rich EREs was about 50% of that for E_2−ER to the same

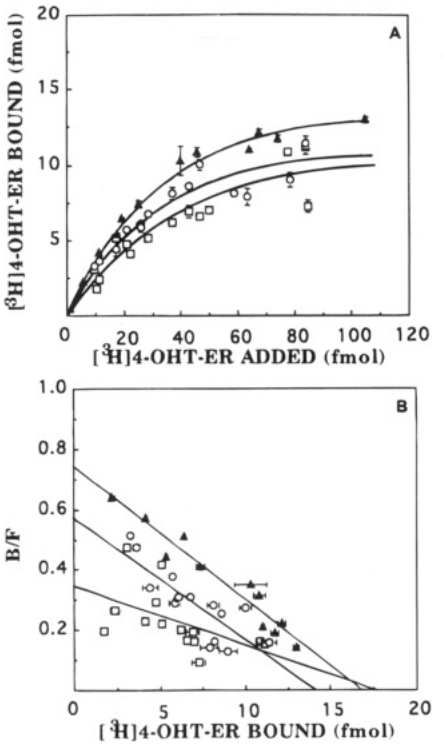

FIGURE 4: Saturation analysis of specific [^{3}H]4-OHT−ER binding to dimeric EREs. (A) *Eco*RI-linearized [^{35}S]dATP-end-labeled plasmid DNA, either the parental plasmid alone or construct EREc-(+) dimer (▲), ERE7.2(+) (○), or ERE7.2(−) (□), was incubated with increasing concentrations of [^{3}H]4-OHT−ER. The data points shown are the average of quadruplicate determinations ± SEM from five representative plate assay experiments and are calculated for binding to 11 fmol of DNA/well as described in Experimental Procedures. (B) Saturation analysis plotted according to the method of Scatchard. The lines were calculated by least squares regression analysis. K_d = 0.69 nM for EREc(+) dimer (▲), 0.49 nM for ERE7.2(+) (○), and 1.02 nM for ERE7.2(−) (□).

constructs, giving an apparent stoichiometry of 0.5 4-OHT−ER dimer, or 1 4-OHT−ER monomer, bound per ERE (Table 2; Figure 5). Saturation binding levels of 4-OHT−ER to both AT-rich and AT-lacking EREs were thus similar to those of E_2−ER binding to EREs lacking the AT-rich region. This suggests that E_2−ER is able to interact with the appropriate flanking region to achieve enhanced binding capacity, while 4-OHT−ER is not. Scatchard analyses (Figure 4B; K_d values are summarized in Table 3) demonstrated a lower affinity interaction for 4-OHT−ER compared to E_2−ER binding to the ERE dimers, irrespective of flanking sequence. Additionally, 4-OHT−ER binding is of lower affinity to AT-lacking EREs [K_d = 1.02 nM for ERE7.2-(−)] *versus* that to AT-rich ERE dimers [K_d = 0.49 nM for ERE7.2(+)].

In contrast to E_2−ER binding to ERE7.2(+), in which binding was strongly cooperative, as indicated by convex Scatchard plots and Hill coefficients > 1.5, binding of 4-OHT−ER is non-cooperative. Scatchard plots conformed to a linear distribution of points (Figure 4B), and the calculated Hill coefficient was 0.91. Thus, the nature of the ligand, as well as the ERE spacing and flanking sequence, is important for cooperative binding of ER.

We also quantitated the binding of 4-OHT−ER to the GCERE in one, two, three, or four tandem copies (saturation plots not shown; binding data summarized in Tables 2 and 3). In contrast to E_2−ER binding, where one dimeric E_2−

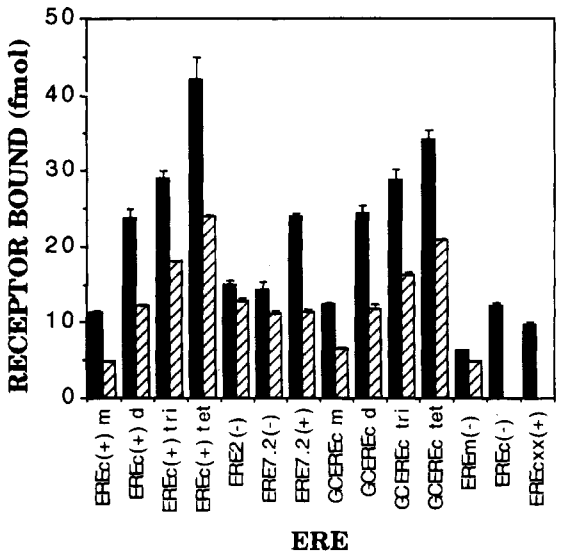

FIGURE 5: Comparison of binding of [^{3}H]E_2−ER *versus* [^{3}H]4-OHT−ER at saturation to a series of EREs. [^{3}H]E_2−ER (solid bars) or [^{3}H]4-OHT−ER (hatched bars) binding (fmol of ER dimer bound) was measured at saturation (60−110 fmol of receptor dimer added) to the indicated EREs. The sequences are shown in Table 1. The data shown are the average of quadruplicate determinations + SEM and are calculated for binding to 11 fmol of DNA/well.

ER was able to bind to each tandem GCERE, 4-OHT−ER binding at saturation was about 50% lower than for E_2−ER binding to each GCERE construct. Table 2 compares the stoichiometry of E_2−ER *versus* 4-OHT−ER dimer binding to the various ERE sequences. 4-OHT−ER:ERE binding ratios of 0.5 are consistently observed, regardless of the number of EREs, the spacing between EREs, or the flanking sequences. Thus, steric interference between ERE sites is not a likely explanation for the observed reduction in 4-OHT−ER:ERE binding compared to E_2−ER binding to EREs flanked by the AT-rich and GC-rich sequence. Other likely explanations are detailed in the Discussion.

The data were also analyzed by the method of Scatchard (plots not shown). Binding of 4-OHT−ER to the GCEREs was of significantly lower affinity compared to E_2−ER binding. For example, the K_d for 4-OHT−ER interaction with the GCERE dimer is 0.61 nM *versus* 0.32 nM for E_2−ER (Table 3). The Scatchard plots for 4-OHT-ER binding to the GCERE monomer and dimer were clearly linear, with Hill coefficients less than 1. Surprisingly, the Scatchard plots for 4-OHT−ER binding to three or four tandem GCEREs were convex in appearance (data not shown). However, the calculated Hill coefficients for binding to the GCERE trimer and tetramer were 1.19 and 1.05, respectively, values not indicative of cooperative binding (Table 3).

Characteristics of the Flanking Sequence Important for Enhanced E_2−ER Binding Capacity and Affinity. More discrete features of the ERE flanking region important for the flanking sequence effect were determined by design of two additional constructs. EREcxx(+) contains an AT-rich flanking region, but the 5 bp immediately 3′ to the inverted repeat have been altered, such that the 17-bp palindrome is shortened to a 13-bp palindrome. EREc(−) has the extended 17-bp palindrome but lacks the more distal AT-rich flanking region. E_2−ER binding to EREcxx(+), EREc(−), EREc-(+) monomer, and EREm(−), which contains a single 13-bp palindrome lacking the AT-rich flanking region, was compared.

E_2−ER binding at saturation was reduced by 50% for EREm(−) compared to EREc(+), which has the full palindrome *and* the AT-rich flanking region (Table 2; Figure 5). Surprisingly, saturation binding levels for EREc(−), which lacks the AT-rich flanking region, are similar to those for EREc(+), with a stoichiometry of approximately 1 E_2−ER dimer bound per ERE (Table 2). This indicates that the AT-rich sequence is not necessary for maximal saturation binding capacity, as was suggested by results of E_2−ER binding to the GCERE constructs. However, in the absence of the extended 17-bp palindrome, *i.e.*, for construct EREcxx-(+), the AT-rich region can substitute to achieve enhanced binding capacity (Table 3).

Scatchard plots of the data (plots not shown; data summarized in Table 3) reveal the highest affinity binding for EREc(+) (K_d = 0.24 nM), which contains both the extended palindrome and the AT-rich flanking regions. E_2−ER binding affinity is reduced either in the absence of the AT-rich region [K_d = 2.02 nM for EREc(−) and 0.81 nM for EREm(−)] or in the absence of the extended palindrome [K_d = 1.56 nM for EREcxx(−)]. Thus, the flanking sequence effect is complex, with both the immediately adjacent flanking region and elements in the more distal regions likely important for maximal E_2−ER binding affinity.

E_2−ER Binding to Closely Spaced ERE Dimers. We previously observed non-cooperative binding of E_2−ER to closely spaced ERE dimers lacking the AT-rich flanking region (Anolik et al., 1993). In light of the results described earlier, it was of interest to examine the effect of close ERE spacing on E_2−ER binding to tandem EREs in the presence of the appropriate AT-rich flanking regions. EREc2(+), EREc2(−), EREc2.5(+), and EREc2.5(−) all contain two 17-bp inverted repeats, separated by the indicated number of helical turns, and in the presence (+) or absence (−) of the AT-rich region. As indicated in Table 1, only the second ERE can contain the 3′ AT-rich flanking sequence due to spacing constraints in the region separating the two EREs.

Surprisingly, E_2−ER binding to these constructs at saturation was reduced from the expected stoichiometry of 1 E_2−ER dimer bound per ERE (Table 2; saturation plots not shown). This reduction in saturation binding capacity was evident regardless of whether the consensus AT-rich sequence was present or not, though it was somewhat more marked in the absence of the AT-rich flanking region (Table 2). Binding was of high affinity, however, with higher affinity binding to the more closely spaced EREs, consistent with our previous results (Anolik et al., 1993).

Interestingly, E_2−ER binding to these constructs appears to be non-cooperative, despite the presence of the extended palindrome and a single copy of the consensus 3′ AT-rich region for the (+) constructs. It remains possible that an AT-rich region must flank both EREs for E_2−ER to bind cooperatively. Alternatively, the reduced binding capacity and lack of cooperative binding may be due to steric interference between ER dimers bound to closely spaced EREs. This notion is supported by calculated Hill coefficients less than 1, which indicates negative cooperativity or interference between binding to neighboring sites. Moreover, the Hill coefficients increase as the ERE spacing increases from 2 to 2.5 helical turns.

DISCUSSION

Effect of ERE Flanking Sequences on ER Binding. In this report, we demonstrate that flanking sequences found adjacent to the ERE inverted repeat in naturally occurring estrogen-responsive genes have a significant impact on ER binding to EREs. Additionally, the nature of the ligand differentially affects ER−ERE binding. Results show that E_2−ER binds EREs much less effectively, *i.e.*, with lower affinity and capacity and non-cooperatively, in the absence of critical flanking sequences. In contrast, 4-OHT−ER binds non-cooperatively and with lowered binding capacity to EREs, regardless of ERE flanking sequence.

Experiments designed to determine characteristics of the flanking sequence that are important for enhanced E_2−ER binding capacity and affinity and for cooperative binding revealed that the flanking sequence effect is complex. Given that highly estrogen-responsive genes appear to contain EREs flanked by AT-rich sequences (Anolik et al., 1993), it was surprising to find that an ERE flanked by a GC-rich sequence bound E_2−ER in a similar manner to the AT-rich ERE, *i.e.*, with enhanced affinity and capacity compared to the EREs lacking either of these flanking sequences. Moreover, E_2−ER binding to the GCERE trimer and tetramer was cooperative. Thus, the AT-richness *per se* of the flanking region may not be critical for the flanking sequence effect. These results suggest that the AT-rich and GC-rich sequences share one or more common features that stabilize E_2−ER binding. Indeed, the 5 bp immediately flanking the ERE inverted repeat in these constructs are identical, creating an extended 17-bp inverted repeat. In addition, there are other common sequence features in the more distal flanking regions. These shared sequence features may allow the binding of a critical accessory protein which recognizes a specific DNA sequence or a particular sequence-directed DNA structure and subsequently stabilizes E_2−ER binding. Alternatively, DNA sequence features in the flanking region may facilitate the formation of a unique DNA conformation which stabilizes E_2−ER binding, independent of the binding of any other proteins. Experiments are in progress to further delineate which specific DNA sequence or structural features are critical for the flanking sequence effect.

Lannigan et al. (1993b) reported higher affinity binding of ER to the rat prolactin ERE in the presence of the natural ERE flanking sequences. They speculated that the flanking sequences assume a unique DNA conformation that stabilizes ER binding to the ERE. This is particularly intriguing in light of other results suggesting the presence of unusual DNA structure in the estrogen-responsive rat prolactin gene regulatory region (Lannigan et al., 1993a; Kladde et al., 1993). We have preliminary results that the AT-rich ERE flanking region used in experiments presented here may contain an intrinsic DNA bend (data not shown). Others have shown that ER bends DNA upon binding EREs (Nardulli et al., 1992, 1993; Sabbah et al., 1992). It is possible that ER may bind with higher affinity to DNA that is prebent (Kahn & Crothers, 1992). Additionally, interactions between multiple protein binding sites, leading to cooperative binding, may be facilitated by the DNA deformability in the regions between the ER binding sites.

Recently, Onate et al. (1994) showed that the DNA-bending protein HMG-1 enhances PR binding to PREs. They postulated that high-affinity PR−PRE binding is dependent on specific nucleotide sequence *and* a protein-induced conformational change, in this case DNA bending by the HMG-1 protein. Interestingly, this same group recently reported enhancement of binding of highly purified ER to EREs by HMG-1 protein (Prendergast et al., 1994). This raises the possibility that HMG-related proteins and/or other accessory proteins facilitate ER-induced DNA bending and that bending may be critical for a high-affinity ER−ERE interaction.

Accessory proteins may indeed bind to critical flanking sequences and stabilize the ER−ERE interaction. When we purify E_2−ER to homogeneity by DNA-affinity chromatography, the highly purified E_2−ER binds with 10-fold lower affinity, 50% saturation binding levels, and reduced cooperativity to EREs flanked by the AT-rich region, compared to partially purified E_2−ER binding (data not shown). Thus, saturation binding levels and binding affinities for the highly purified E_2−ER are very similar, regardless of flanking sequence. This suggests that one or more other proteins present in the partially purified ER preparation interact with the appropriate flanking sequences to achieve enhanced E_2−ER binding capacity, enhanced binding affinity, and cooperative binding.

Cato et al. (1988) demonstrated that sequences outside the chicken vitellogenin II ERE modulated steroid responsiveness by providing sites for other DNA binding proteins. Such DNA binding proteins may be important in regulating other estrogen-responsive genes by directly stabilizing ER−DNA binding or by intermediating ER interactions with other important transcription factors, *e.g.*, AP-1 (Pfahl, 1993), thus facilitating formation of a stable preinitiation complex. AP-1 bends DNA upon binding. This may impact ER−ERE binding and transactivation similarly to HMG-1−PR−PRE interactions described above. Additional accessory factors that enhance the DNA binding of a number of steroid hormone receptors have been described (Kupfer et al., 1993), including ER (Mukherjee & Chambon, 1990; Landel et al., 1994).

ER Cooperative Binding. There is controversy concerning whether ER can bind DNA cooperatively (Klein-Hitpass et al., 1988; Martinez & Wahli, 1989; Klinge et al., 1992c; Ponglikitmongkol et al., 1990). The fact that cooperativity is a complex phenomenon, dependent on several variables, may account for the conflicting results of other investigators. We observed cooperative binding of E_2−ER to three or four tandem copies of a 38-bp consensus ERE with a 3′ AT-rich region (Klinge et al., 1992c) or a GC-rich region, positioned such that every other ERE was on the same side of the DNA helix (~7 helical turns apart). Experiments described here further show that ER also binds cooperatively to an ERE dimer in which the EREs are approximately 7 helical turns apart. In contrast, binding is non-cooperative to an ERE dimer in which the EREs are 3.6 helical turns apart. Thus, cooperative binding of E_2−ER depends on spacing and possibly stereoalignment of EREs. Moreover, E_2−ER binds non-cooperatively to EREs lacking the appropriate flanking sequences, regardless of ERE spacing. When ER is liganded with the antiestrogen 4-OHT, binding is non-cooperative, regardless of ERE spacing *or* flanking sequences. Thus, cooperative binding is also dependent on the nature of the ER ligand as well as the ERE flanking sequences.

Curiously, using gel shift assays, we do not detect cooperative binding of ER to EREs that bind ER coopera-

tively as measured in two distinct equilibrium binding assays: a microtiter well plate assay (Ludwig et al., 1990) and a gel filtration method (Peale et al., 1988; Klinge et al., 1992c). Thus, we suggest that the detection of cooperative binding *in vitro* may be highly dependent on the experimental technique and conditions used. Others have consistently observed cooperative binding of PR to PREs (Tsai et al., 1989; Ponglikitmongkol et al., 1990) and variably that of E_2−ER to EREs (Martinez & Wahli, 1989) using gel shift assays. It is possible that the ER−ER, ER−DNA, or ER−accessory protein interactions important for cooperative binding are weaker than those for PR, such that they may be disrupted in the gel shift assay conditions. Moreover, the amount of critical accessory protein(s), which may contribute to the observed cooperative binding of ER, could vary with receptor preparation, thus differently impacting *in vitro* binding assays.

Our detection of cooperative E_2−ER binding is based on S-shaped saturation binding plots, convex Scatchard plots, and Hill coefficients greater than 1.5. However, the molecular mechanisms responsible for this observation are unknown. The apparent dependence of cooperativity on ERE spacing and stereoalignment suggests that protein−protein interactions between DNA-bound ER dimers may be important. Additionally, as discussed above, interactions between ER and other proteins may enhance and stabilize ER−ERE binding, resulting in apparent cooperativity. Alternatively, interactions between ER monomers in the dimeric ER complex may account for cooperativity (Notides et al., 1981). It remains possible that the cooperativity we observe is different from the classical cooperativity observed with the λ-repressor, for example (Hochschild & Ptashne, 1986), where binding of one protein molecule increases the affinity of binding of a second protein molecule to another DNA site. Preliminary DNase I footprinting experiments suggest that the cooperative phenomenon is not simply a stabilizing interaction between ER dimers bound on the same face of the DNA helix (M. D. Driscoll, personal communication). Moreover, the saturation curves obtained when ER binds with apparent cooperativity to multiple EREs are right-shifted at low E_2−ER to ERE ratios (compare binding to GCERE trimer *versus* dimer, for example). One explanation for this shift is that E_2−ER binding to one ERE inhibits binding to another ERE. As the amount of E_2−ER increases past a critical ER−ERE ratio, the inhibition may be overcome, such that the amount of ER−ERE bound rapidly rises. This would result in an S-shaped saturation binding profile and a curvilinear Scatchard plot. Thus, binding would appear cooperative in the absence of classical cooperative interactions between ER dimers. Importantly, the end result would be the same: that is, small changes in ER or ligand concentration would cause large changes in the occupancy of binding sites. This could allow sensitive fine-tuning of the response of individual genes to increasing estrogen levels *in vivo*.

Role of Ligand in ER Action. Although a number of groups have shown that ligand is required for ER binding to EREs (Kumar & Chambon, 1988; Metzger et al., 1988; Skafar & Notides, 1985), others have observed no effect of ligand on ER−ERE binding (Furlow et al., 1993; Murdoch et al., 1990; Lees et al., 1989; Curtis & Korach, 1990). However, estrogens and antiestrogens clearly have different effects *in vivo*. It is likely that ligand binding induces changes in ER conformation, thus affecting ERE binding and interaction with other proteins important for transcription (Giambiagi & Pasqualini, 1988; Ing et al., 1992).

Results presented here, and previous results from our laboratory (Klinge et al., 1989, 1992b; Anolik et al., 1993) and others (Giambiagi & Pasqualini, 1991), indicate that 4-OHT binding to ER changes the ability of the receptor to interact with EREs, although binding is still of high affinity. For example, 4-OHT−ER has a lowered binding capacity for certain EREs compared to E_2−ER. We observed that the E_2−ER−ERE saturation binding ratios for the AT-rich EREs are 1 E_2−ER dimer bound per ERE. This agrees with the stoichiometry of 1 ER monomer binding to each half of the palindromic ERE predicted by NMR and crystallographic studies of steroid receptor structure and interaction with DNA (Schwabe et al., 1990, 1993). Although we initially expected that, at saturating ER protein concentrations, 1 ER dimer would bind per ERE, regardless of the DNA flanking sequence, the ligand, or the receptor purity, the stoichiometry of E_2−ER binding to the AT-lacking EREs and 4-OHT−ER binding to all EREs is approximately 0.5 ER dimer bound per ERE, equivalent to 1 ER monomer bound per ERE.

There are at least three explanations for the observed reduction in binding to one-half of the expected stoichiometric saturation binding values: (1) ER binds as a monomer under certain ligand and ERE flanking sequence conditions; (2) ER binds as a dimer, but the dimeric ER dissociates once bound to an ERE, such that only monomeric ER remains bound; or (3) ER binds as a dimer, but one molecule of ligand dissociates from the ER dimer due to allosteric changes in protein conformation upon ER binding to DNA (Klinge et al., 1992a,c). Since the plate assay detects ER by the labeled ligand, explanation 3 would result in lower apparent ER−ERE binding. The ER conformational changes induced by DNA binding may differ in magnitude for E_2−ER *versus* 4-OHT−ER. Moreover, ER conformation and ligand stability may depend on ERE flanking sequence because of DNA structural effects and/or the binding of accessory proteins which could stabilize the interaction of ligand with ER.

The first two explanations appear to conflict with the reported high stability of ER dimers (Kumar & Chambon, 1988; Sasson & Notides, 1988) and the low-affinity binding of monomeric ER to EREs (Klinge et al., 1992c; Fawell et al., 1990). Additionally, gel mobility shift assay experiments presented here show similar sized complexes regardless of ligand or ERE flanking sequences. Sucrose density analysis of E_2−ER or 4-OHT−ER revealed that the receptor was a 5.2−5.3-S dimer in solution (C. M. Klinge and A. M. Traish, personal communication). Finally, DNase I footprinting experiments show protection of the 13-bp ERE inverted repeat, and 5 bp flanking on either side, by both E_2−ER and 4-OHT−ER (M. D. Driscoll, unpublished observation). Thus, our results are consistent with ER dimer binding.

In support of hypothesis 3, we previously showed that the binding of [^{3}H]4-OHT−ER to EREc(+) results in a greater loss of free ^{3}H-labeled ligand than [^{3}H]E_2−ER−ERE binding in plate assay experiments (Klinge et al., 1992b; C. M. Klinge, personal communication). Moreover, when ER is liganded with the antiestrogen tamoxifen aziridine (TAz), which covalently attaches to the ligand-binding domain, saturation binding levels are identical to those of E_2−ER−ERE binding. Additionally, no dissociation of [^{3}H]TAz ligand was detected. Remarkably, ligand dissociation ap-

pears to have a critical impact on ER binding kinetics, as both E_2–ER and TAz–ER, but not 4-OHT–ER, bind certain multiple EREs cooperatively (C. M. Klinge, personal communication).

We have previously shown that, in addition to ligand choice, the ERE core sequence can affect the degree of cooperative E_2–ER binding (Klinge et al., 1992c). Results presented here further suggest that sequences *outside* the core inverted repeat influence receptor binding kinetics and cooperativity and suggest a basis for the differences in action between estrogen agonists and antagonists, partly at the level of ER–DNA binding. The estrogen agonist estradiol is required for maximal saturation binding levels and cooperative binding of ER to certain EREs. Cooperativity depends additionally on the spacing between ERE binding sites and the presence of appropriate flanking sequences. We suggest that the conformation of ER when bound by the antagonist 4-OHT may be altered, such that stabilizing interactions with accessory proteins bound at critical ERE flanking sequences are precluded. Such differences in interactions with accessory proteins may clearly have important consequences on ER binding and promoter induction.

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