ABSTRACT

Specific binding of estradiol-ligated, partially purified calf uterine estrogen receptor (ER) to a 38-base pair estrogen responsive element (ERE) consensus sequence, containing the inverted repeat 5'-GTCAnnnTGACC-3', was measured in vitro. The ERE sites were inserted as single or multiple tandem copies in a plasmid vector [pGEM-7Zf(+)]. Results showed that one dimeric ER can interact with one ERE, and steric constraints do not inhibit binding of ER to adjacent EREs. Molybdate-stabilized monomeric (4S) ER did not bind to EREs. ER bound to single and tandem double EREs with Kₐ values of 0.24 and 0.23 nM, respectively. When the plasmid contained three or more tandem copies of the ERE, ER bound in a cooperative manner, as indicated by convex Scatchard plots and Hill coefficients greater than 1.5. To determine those characteristics of the consensus sequence that are important for maximal high-affinity ER binding, ten variant ERE oligomer sequences were synthesized and cloned into pGEM consensus ERE and was reduced for variants containing one or two nucleotide changes in the inverted repeat. The number of nucleotides separating the inverted repeat in the ERE was critical for high-affinity ER binding. Certain sequence-variant EREs when cloned as single copies bound less ER compared to the consensus ERE, yet when cloned as four tandem copies, ER binding displayed cooperativity by Scatchard and Hill analyses. Results demonstrate that cooperative interactions noted in vitro by others are present when measured in vitro. Results strongly imply that the number, spacing, and nucleotide sequence of EREs could precisely control the amount of ER binding to estrogen-responsive genes.

INTRODUCTION

Estrogens are thought to be essential for the initial development of most mammary tumors (1–4). The exact mechanism by which estrogens modulate such growth is unknown. In addition to direct effects on cell growth, recent evidence points to the potential involvement of estrogens in regulating the transcription of paracrine or autocrine growth factors (1–5).

Transcription of estrogen-regulated genes is mediated via a specific and high-affinity interaction of estrogen with ER, forming a homodimeric ER complex that, in turn, interacts with specific regions of DNA called estrogen-responsive elements located either 5', 3', or within estrogen-regulated genes (reviewed in Refs. 6–9). By a still unknown mechanism, these interactions at EREs signal RNA polymerase II to initiate transcription more efficiently at a promoter located within several kilobases of the ERE. Studies in vivo have demonstrated that transcription may require functional interactions among receptor molecules as well as interactions between the bound receptor and other essential transcription factors (8–11).

Recently, the DNA sequences to which steroid hormone receptors bind have been intensively investigated. For ER, analysis of the 5' regulatory regions of several well-characterized estrogen-responsive genes, e.g., those of Xenopus laevis vitellogenin (12–14), very low density chicken apo lipoprotein (15), and chicken vitellogenin (16, 17), has suggested a 13-base pair core consensus sequence to which ER binds: 5'-GGTCAnnnTGACC-3'. However, many naturally occurring copies of the 13-base pair consensus ERE are imperfect (13, 18–27). Others have investigated the effects of point mutations in a natural ERE on ER-DNA interaction, using gel mobility shift assays (28, 29) and transient expression assays with recombinant plasmids and an ER expression vector transfected into HeLa cells (22, 29). Single nucleotide substitutions in a single copy of the 13-base pair perfect consensus inverted repeat ER destroyed its ability to induce transcription (14). However, many naturally occurring estrogen-responsive genes have two or more imperfect core consensus sequences, located 5' to the promoter, which function synergistically to regulate transcription of a reporter gene construct in transfected cells (14, 18, 23, 24).

As one approach to study the mechanism of estrogen-responsive signal transduction at the DNA level, we developed assays in vitro to measure binding of partially purified ER to a plasmid containing a 38-base pair consensus sequence representing full-length EREs from estrogen-responsive genes (30, 31). The consensus sequence contains the core ER inverted repeat (5'-CAGGTCAAGTGACCCTG-3') and a 3' AT-rich region. Plasmids containing a single copy of this sequence bound ER in vitro in a salt-dependent, saturable, specific manner, exhibiting a Kₐ of 0.5 nM (30).

In the experiments reported here, we measured binding of ER to tandem series of this consensus ERE and to a series of ERE variants containing specific nucleotide changes that reduced the ER binding capacity. Results demonstrate that there is cooperative binding to EREs displaying either high or low affinity for ER. Consequently, precise hormonal control of specific genes in vivo may be achieved through the use of EREs with sequence alterations that exhibit different degrees of affinity and cooperativity of ER binding.

MATERIALS AND METHODS

Preparation of Oligomers. Synthetic single-stranded oligonucleotides, which were designed variants of the original ERE consensus sequence (30), were generous gifts from Drs. Michael Kotick and David Leland (Biotechnology Products Division, Miles, Inc., Elkhart, IN). The sequence of these oligomers is given in Table 1. The synthetic primer (5'-CTGTAAGT) was annealed to the 3' end of the oligomers and extended with Klenow fragment of Escherichia coli DNA polymerase I (Promega, Madison, WI).
were determined by absorbance. Plasmid DNA was linearized with Xhol and BstNl-BamHl followed by electrophoresis on a 10% polyacrylamide gel. The sizes of the restriction band fragments generated were definitive of either a forward or backward orientation.

The size of the intact plasmid DNA was visualized after 0.8% agarose gel electrophoresis on 6% acrylamide gels were used in a head-to-tail orientation using HaellI (5'-CC-3') and PvuII (5'-CAG-3') half-recognition sequences at the end of the oligomer, in the presence of these restriction endonucleases and T4 ligase (New England Biolabs, Beverly, MA).

Large-Scale Preparation of Plasmid DNA. Plasmid DNA was prepared from 2-liter cultures of bacteria by alkaline-sodium dodecyl sulfate lysis (33) and purified by cesium chloride gradient centrifugation. The DNA was dialyzed extensively against 10 mM Tris-HCl (pH 8.0) 1 mM EDTA buffer prior to use. DNA concentration and purity were determined by absorbance. Plasmid DNA was linearized with EcoRI-mediated uptake of procedure (32). Plasmid DNA was prepared from individual bacterial colonies by a standard "mini-prep" protocol (32). The size of the intact plasmid DNA was visualized after 0.8% agarose gel electrophoresis. Plasmids that demonstrated EcoRI/HindIII digestion patterns consistent with the incorporation of intact single or multimeric EREs by gel electrophoresis on 6% acrylamide gels were further examined as follows. Because all sequence variants have asymmetrically located BsrNI or Ddel sites, orientation of insertion of the oligomers into pGEM-7Zf(-) was determined by digestion with BsrNI-Xhol and BsrNI-BamHl followed by electrophoresis on an 10% polyacrylamide gel. The sizes of the restriction band fragments generated were definitive of either a forward or backward orientation.

The sequences of the ERE variants were confirmed by direct DNA sequencing using dideoxynucleotide incorporation (K/R Sequencing System; Promega).

Microtiter Plate Assay of [3H]ER Binding to Plasmid DNA. The microtiter (well) plate assay for measuring [3H]ER binding to DNA has been described (31). Briefly, for saturation binding analysis, various concentrations of heparin-agarose affinity-purified [3H]ER were preincubated with one concentration (see figures and tables) of 35S-DNA (plasmid DNA with or without ERE) for 2.5 h at 4°C, with shaking, in TDPK 100 buffer containing 0.1% NP40 and then incubated in histone/gelatin-coated microtiter wells for 2 h at 4°C, with shaking. Wells were washed with TDP buffer containing 1 mM EDTA and 100 mM KCl plus 100 μg/ml carboxymethyl bovine serum albumin (38). The column was developed with a peristaltic pump at 16 ml/h.

The time course for ER-DNA binding to histone/gelatin-coated wells, as used in each of the plate assays reported here, demonstrated that binding approached a maximum by 2 h and that ER-ERE binding was stable for at least 4 h (31). In an additional experiment, ER binding to pGEM-7Zf(+) (plasmid alone) or to plasmid containing monomer or tetramer inserts of the consensus ERE showed ER binding was 98%, 97%, and 97% of control after incubation with TDP buffer containing 1 mM EDTA and 100 mM KCl plus 100 μg/ml carboxymethyl bovine serum albumin for 4 h following the usual 2-h incubation of ER plus DNA in the wells (data not shown). Thus, rinsing of the wells does not decrease the detected amount of ER or DNA bound to the wells. These controls demonstrate that results from the experiments described herein represented measurements made under equilibrium conditions.

Calculation of Specific [3H]ER Binding to ERE versus Plasmid. For each determination of ER-DNA binding, [3H]ER was incubated in parallel with pGEM-7Zf(+) plasmid alone and with plasmid containing one or more EREs. The specific binding of [3H]ER to the EREs was calculated by taking the difference between the two binding values. In column assays, each specific [3H]ER binding profile was normalized to correct for artificial underestimation [mean = 55 ± 1% (SD), n = 131 individual column runs] of ER-DNA complexes that occurs secondary to overlap between DNA and ER elution profiles. For plate assays, the amount of [3H]ER binding was calculated by adjusting for that amount of 35S-DNA that did not bind to the well and for the background binding of the [3H]ER preparation in wells without DNA added (32). Nonspecific binding of [3H]ER to plasmid DNA, pGEM-7Zf(+) sites, represented 1–10% of the total counts bound for [3H]ER from 0.18–0.72 pM and 11–36% from 0.8–1.8 pM. The calculated amount of [3H]ER added (total) was adjusted to correct for the fact that only 82% of added heparin-agarose-purified [3H]ER was retained by hydroxyapatite (data not shown). For Scatchard analysis (40) of [3H]ER binding data from column assays, the amount of total functional [3H]ER in a reaction was assumed to be 50% of the amount of added [3H]ER, as determined by hydroxyapatite assay described above, since only 50% of ammonium sulfate-fractionated [3H]ER was capable of binding DNA (data not shown).
RESULTS

Saturation Analysis of [3H]ER Binding to Multiple Inserts of the ERE Consensus Sequence in Vitro. The specific binding of [3H]ER to pGEM-7Zf(+) plasmid bearing one (monomer), two (dimer), three (trimer), or four (tetramer) tandem copies of the original consensus ERE sequence (Z16 in Table 1) was measured at a fixed concentration of DNA and with increasing concentrations of [3H]ER by the two assay methods described above. Results from the plate assay are shown in Fig. 1A; results from the column assay are shown in Fig. 2A. Although ammonium sulfate-fractionated ER was used in the column assay and heparin-agarose affinity-purified ER was used in the plate assay, quantitatively similar and qualitatively identical results were obtained. In each assay, the 1:1 stoichiometry of [3H]ER dimer bound per ERE is conserved for monomer, dimer, trimer, and tetramer (Table 2). The binding of [3H]ER to one ERE did not appear to interfere with [3H]ER binding to an adjacent site.

Scatchard analyses of heparin-agarose affinity-purified [3H]ER binding to the consensus ERE sequence as measured by both assays were apparently linear for monomer and dimer inserts (Figs. 1B and 2B). Data from plate assays provided estimated $K_a$ values of 0.24 and 0.23 nM for the monomer and dimer, respectively (Table 1). The Scatchard plots of [3H]ER binding to trimer and tetramer were convex in appearance, indicative of the cooperative binding of ER when more than two sites are cloned in tandem. The calculated Hill coefficients of ER binding to monomer, dimer, trimer, and tetramer are 1.16, 1.24, 2.17, and 1.86, respectively (Table 2, plots not shown). The latter two values suggest cooperative binding to the trimer and tetramer.

Similarly, Scatchard analyses of the saturation binding data from column assays yielded apparently straight lines for the monomer and the dimer and convex curves for the trimer and tetramer (Fig. 2B). The calculated Hill coefficients for ER binding to the monomer, dimer, trimer, and tetramer were 1.1, 0.9, 2.5, and 1.4, respectively (plots not shown).

Binding of ER to Monomer ERE Inserted in Either Orientation. As discussed earlier, the consensus ERE we have used is derived from natural sequences larger than the core inverted repeat and is partially asymmetrical. Since an enhancer element increases transcriptional efficiency in either orientation relative to the promoter (14), we measured the binding capacity and affinity of [3H]ER to ERE cloned in either a forward and backward orientation relative to the rest of the plasmid. By plate assay, binding of [3H]ER was orientation independent; [3H]ER binding capacity was virtually identical, and the affinity of [3H]ER binding was 0.20 versus 0.24 nM for the forward and backward orientations, respectively (data not shown). Similar results were obtained with the column assay (30). Thus, orientation of the ERE relative to the plasmid did not significantly affect [3H]ER binding.

Binding of Molybdate-stabilized ER to ERE. The binding of [3H]ER was compared with the binding of molybdate-stabilized...
The binding affinity of [3H]ER binding to these sequence variants could not be determined (data not shown). Together these results demonstrate that discrete alterations in nucleotide sequence can markedly affect [3H]ER binding in vitro. [3H]ER Binding to Tetrameric Inserts of Variant ERE Sequences. The plate assay was used to measure binding of heparin-agarose affinity-purified [3H]ER to tetramers of those ERE sequence variants that had measurable binding when cloned as monomers (Figs. 4 and 5). There was no difference in the

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Table 2  Summary of ER-ERE interaction

<table>
<thead>
<tr>
<th>DNA</th>
<th>Ratio of ER-plasmid</th>
<th>Ratio of ER-ERE</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z16</td>
<td>Monomer</td>
<td>0.97</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>2.09</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Trimer</td>
<td>3.15</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>4.10</td>
<td>1.10</td>
</tr>
<tr>
<td>Z22</td>
<td>Monomer</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>2.49</td>
<td>0.62</td>
</tr>
<tr>
<td>Z23</td>
<td>Monomer</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>3.64</td>
<td>0.91</td>
</tr>
<tr>
<td>Z25</td>
<td>Monomer</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>1.05</td>
<td>0.26</td>
</tr>
<tr>
<td>Z26</td>
<td>Monomer</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>1.14</td>
<td>0.35</td>
</tr>
<tr>
<td>Z27</td>
<td>Monomer</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>1.65</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Trimer</td>
<td>2.63</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>2.95</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* Sequences of the ERE variants are given in Table 1.
* Number of tandem copies of ERE: monomer, 1; dimer, 2; trimer, 3; tetramer, 4.
* Significantly different (P < 0.10) from the Hill coefficient of [3H]ER binding to monomer or dimer.
* Significantly different (P < 0.05) from the Hill coefficient of [3H]ER binding to monomer or dimer.
* Hill coefficients could not be calculated from the binding data.
* Significantly different (P < 0.10) from the Hill coefficient of [3H]ER binding to Z16 tetramer.
* Significantly different (P < 0.10) from the Hill coefficient of [3H]ER binding to Z22 tetramer.

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Fig. 3. Saturation analysis of ER binding to single copies of ERE sequence variants in vitro. EcoRI-linearized, 32P-dATP-end-labeled plasmid DNA, either the plasmid alone or containing one copy of the 38-base pair consensus ERE Z16 (●) or ERE sequence variants Z27 (▲), Z20 (O), Z23 (●), Z21 (□), Z22 (▲), Z25 (●), Z26 (△), Z25 (▲) (sequences provided in Table 1), were incubated with increasing concentrations of heparin-agarose affinity-purified [3H]ER and assayed as described in Fig. 1. Points, average of duplicate samples and represent specific binding of [3H]ER to ERE (as in Fig. 1); bars, SEM. B, saturation analysis plotted according to the method at Scatchard (40). The lines were calculated by least squares regression analysis.
saturating amount of $[^{3}H]ER$ bound to a monomer of the 15-base pair inverted repeat consensus ERE, Z16, compared to the 13-base pair inverted repeat, Z27 (Fig. 3A). Scatchard analysis of specific $[^{3}H]ER$ binding to a tetramer of Z27 revealed a convex plot (Fig. 4B), indicative of cooperative binding with a calculated Hill coefficient of 3.0 (data not shown).

The saturation binding curves revealed that there was a greater than proportional increase in $[^{3}H]ER$ binding to tetramers of the sequence variants containing one or two nucleotide changes compared to $[^{3}H]ER$ binding to monomers of these sequences. A representative saturation plot for the Z23 tetramer is shown in Fig. 5A. Scatchard analysis of these data revealed convex plots similar to that detected for $[^{3}H]ER$ binding to trimeric and tetrameric copies of the consensus ERE (Z16), as represented in Fig. 5B. The calculated Hill coefficients for ER binding are presented in Table 2. Likewise, $[^{3}H]ER$ binding to tetramers of the ERE sequence variants containing variant spacing between the halves of the inverted repeat, Z25 and Z26, also appeared to be more than additive when compared to ER binding to monomers of these sequence variants (Fig. 4A). Although Scatchard plots of $[^{3}H]ER$ binding to Z25 and Z26 revealed apparently convex curves indicative of some cooperative binding (Fig. 4B), Hill coefficients could not be calculated because the line plotted from the data never crossed the “zero value” on the y-axis (data not shown). The slopes of the computer-drawn best fit lines for the Hill plots were 1.6 for Z25 and 2.0 for Z26.

Together, these results suggest that cooperative binding occurred when four copies of ERE were cloned in tandem, even though variant ERE sequences had one or two base pair alterations compared to the consensus ERE. However, the amount of $[^{3}H]ER$ binding to tetramers of sequence variants was invariably reduced compared to that of consensus ERE.

DISCUSSION

Cooperative Binding of ER to EREs. It appears that hormone receptors work synergistically to regulate transcription in vivo (8, 29, 41-47) and that synergy between hormone receptors and other transcription factors may also be important in modulating gene transcription (11, 46, 48-55). Functional synergism of steroid hormone receptor interaction with multiple responsive elements has been demonstrated by measuring levels of reporter gene transcription in transient transfection assays with GR (57), PR (43), ER (13, 18, 24, 29), and thyroid hormone receptor (56). In addition, gel retardation assays indicated cooperative binding of PR with PRE (42), GR with GRE (57, 58) and ER with ERE (23). However, not all investigators have demonstrated cooperative ER binding to DNA (13, 14, 29). Here, we report a quantitative analysis of the affinity of ER binding and the cooperative nature of that binding to tandem series of consensus EREs and selected sequence variants of the consensus ERE.

Three imperfect EREs are located upstream of the Xenopus vitellogenin B1 gene (23), and one perfect plus one imperfect EREs are located upstream of the chicken vitellogenin II gene (16, 17). The human oxytocin gene contains one imperfect ERE and two half-palindromic sequences, 5'--TGACC-3' located 5' to the promoter (26). Estrogen-responsive transcription is dependent on the presence of two closely spaced 13-base pair imperfect EREs in Xenopus vitellogenin B1 and B2 (13, 18, 58, 59). The dependence of transcriptional synergy on close spacing

Cooperative Estrogen Receptor Binding to DNA.
(less than 131 base pairs) prompted the suggestion that two ER dimers could recognize the "estrogen-responsive unit" formed by these two EREs (13, 18). Whether this synergy results from altered DNA conformation induced by ER binding or by protein-protein interactions between DNA-bound ER dimers is not known (18, 24).

Our results suggest that ER binds cooperatively to consensus EREs containing inverted repeats located on the same side of the DNA helix. Cooperative binding was detected with three or four but not two tandem EREs. Counting from the center of the perfect inverted repeat of the consensus ERE, there are 37 base pairs between the first and second ERE and 74 base pairs between the first and third ERE. Since there are 10.4 base pairs/helical turn of DNA (60), there are 3.5 turns between the first and second ERE and 7.05 turns between the first and third ERE. Therefore, the first and third or second and fourth ERE sites are on the same side of the helix. Such an arrangement may allow protein-protein interaction between ER dimers to facilitate ER binding. Alternatively, or perhaps simultaneously, binding of one ER dimer to one ERE may induce a localized change in DNA conformation that increases the accessibility to an adjacent ERE on the same side of the helix.

The importance of the location of HREs on the same side of the DNA helix was suggested by the finding that the stability of the GR-GRE complex was maximal when GRES were close together (center distance, 21 base pairs) and positioned on the same side of the DNA helix (56).

Ponglikitmongkol et al. (29) investigated the effect that varying the center-to-center distance between two adjacent perfect 17-base pair consensus Xenopus vitellogenin A2 EREs had on transcription and on gel mobility. In an extensive series of experiments, no synergistic effect of ER on transcription was detected when the EREs were located adjacent to the TATA box (29). However, if the EREs were located 175 base pairs upstream of the TATA box, synergy was dependent on helical spacing of the EREs, with maximal synergy resulting when the EREs were located on the same side of the DNA helix (29). In contrast to these results, helical-spacing-dependent synergy was detected when paired imperfect EREs were located adjacent to the TATA box (29). Despite such findings, there is no evidence for cooperative ER binding as analyzed by gel retardation assay using any of the dimeric EREs studied (29). We believe that the discrepancy between our conclusion and that of Ponglikitmongkol et al. lies either in the spacing between adjacent EREs in the plasmid constructs, the binding assay methodology, or the ER preparation.

Untransformed ER Does Not Bind ERE with High Affinity. After estradiol binding to ER in vivo, "activation" and dimerization occur, i.e., the monomeric 4S ER is converted to dimeric 5S ER, rendering the ER capable of binding to chromatin in a saturable and highly specific manner (reviewed in Ref. 61). Ammonium sulfate fractionation of calf uterine cytosol also activates the ER (34), but inclusion of 10 mM molybdate anion during this step prevents the 4S to 5S conversion (62). The presence of 10 mM molybdate ion stabilizes the 4S form, which was not found to interact with isolated nuclei, but did not impair the nuclear binding of 5S ER, prepared first in the absence of molybdate (35). Here, we found that significantly less molybdate-stabilized ER bound to a trimer ERE in vitro than did 5S ER. Moreover, the apparent $K_d$ value calculated from these data is 450 nM for the 4S monomer versus 0.21 nM for the 5S ER. Thus, untransformed, monomeric ER binds very weakly to EREs. Our result contrasts with the reported equal affinity of 4S and 5S ER binding to non-sequence-specific DNA cellulose (63). Likewise, untransformed glucocorticoid receptor GR from HeLa cell extracts did not bind to a 326-base pair fragment of the mouse mammary tumor virus long terminal repeat DNA, containing two clusters of binding sites for GR and PR (48, 64), whereas heat-activated GR bound stably to the mouse mammary tumor virus long terminal repeat DNA (64). Recent evidence suggested that dimerization of ER is necessary for high-affinity binding to DNA (65).

Alterations in ERE Sequence Affect ER Binding and Cooperativity. To determine characteristics of the ERE that are important for ER binding, we examined specific binding of ER to 11 ERE sequence variants, inserted in single or multiple tandem copies. Results demonstrated that the number of bases separating the two halves of the inverted repeat is critical for efficient ER binding. Separation of the sides of the inverted repeat by 22 base pairs along with the removal of one C in the 3' half of the repeat (Z31) resulted in a large decrease in ER binding that is almost certainly the result of the separation, since it is much larger than expected for a single nucleotide change, as discussed below. The latter result suggests that allowing the sides of the inverted repeat more possibilities for orientation in 3-dimensional space, e.g., by looping out of the intervening sequence, does not improve ER binding. That spacing between the inverted repeat sequences is critical for ER recognition of ERE is supported by the evidence that the thyroid hormone-responsive element (5'-GGTCAgagTGACC-3') is identical to the ERE without separation of the two sides of the inverted repeat (66, 67). ER does not bind to the thyroid hormone-responsive element; however, unlike ER-ERE binding, inclusion of 1-6 base pairs between the sides of the inverted repeat of the thyroid hormone-responsive element did not affect thyroid hormone receptor binding (66). Recent studies documented that orientation and spacing of the core binding motif, 5'-TCAGGTCAG-3', differentiate DNA binding and transcriptional activation between ER, thyroid hormone receptor, and retinoic acid receptor (68, 69). These results have been attributed to very subtle differences in the amino acid sequence between the first and second zinc fingers of the DNA-binding domains of the ER and thyroid hormone receptor (70).

We found that a single base pair change in the most highly conserved nucleotide A (5'-GGTCAgagTGACC-3') to C (Z20) decreased ER binding by 24-30% and reduced the affinity of binding slightly. Klein-Hitpass et al. (13) suggested that a purine/purine or a pyrimidine/pyrimidine exchange in the conserved 13-base pair inverted repeat of the ERE would be permissible for synergy, but a purine/pyrimidine exchange was found to decrease the inducibility of a reporter gene. Our results suggest that ER recognizes this sequence variant despite this purine/pyrimidine exchange. Interestingly, the 5' flanking region of the estrogen-regulated rabbit uteroglobin gene has a C in this position of an ERE containing an imperfect 13-base pair inverted repeat (21), and ER binds it in vitro (71). A cell extract from Chinese hamster ovary cells transfected with the ER expression vector HEO retarded the mobility of a 42-base pair oligomer containing a hybrid (ERE/GRE) HRE, 5'-GGGTCAgagTGACCCT-3' (71), but purified PR did not bind to this sequence.

The 5' flanking region of the human pS2 gene contains an ERE with the inverted repeat 5'-GGTCAgagTGACC-3'. This ERE displayed 5-fold lower affinity for ER than the consensus ERE (28) but nevertheless conferred estrogen inducibility on a heterologous promoter (22, 25). In fact, the pS2 ERE was the
first example of an ERE containing an imperfect inverted repeat capable of inducing transcription, albeit with an efficiency that is 3–4 times lower than that of the consensus ERE (25). Klein-Hitpass et al. (72) also observed that this A to G mutation reduced ER binding 5- to 10-fold. In this case, the purine/purine substitution affected both ER binding and gene induction.

A single nucleotide change in the 3’ half of the inverted repeat from an A (5′-GGTCagTGACC-3’) to a T (Z23), which makes the ERE more like a GRE, reduced ER binding by 80%. Slater et al. (73) suggested that since no natural ERE has a T at this position, recognition of the DNA double helix by ER may be hindered by the presence of a 5′ methyl group of thymidine. Our results suggest that ER recognizes this sequence variant, even though this is a purine/pyrimidine exchange, but that binding is not as efficient as that to the consensus inverted repeat (Z16) or the single base C variant (Z20). It is interesting to note that Truss et al. (71) observed that either PR or ER bound to a 42-base pair oligomer containing the hybrid HRE 5′-GGGTCAcagTGTCCT-3′ and transfected CAT expression was activated in the presence of either diethylstilbestrol or dexamethasone (71). Interestingly, whereas ER binding to one copy of variant Z23 was only 20% of that to Z16, ER binding to a tetramer of this sequence variant reached the same saturating level as to a tetramer of the consensus ERE, Z16. Both the Scatchard analysis and the derived Hill coefficient imply a highly cooperative binding of ER to the Z23 tetramer.

Changes of two nucleotides in the 3’ half of the consensus inverted repeat (TGACC, i.e., TGCC (Z21) and TAGCC (Z22), considerably reduced total ER binding and the affinity of ER binding. The vitellogenin B1 imperfect ERE-1 (deviating from the consensus ERE by 2 base pairs) has a lower affinity for ER than ERE-2 (containing a 1-base pair deviation from the consensus ERE) (23). Only 2.5 of the 4 ERE sites of the tetramer of Z22 were occupied at saturation. Since the Z22 variant tetramer has exactly the same spacing between core palindromic sequences as the Z16 consensus tetramer, the difference in ER binding is not a consequence of a change in the relative location of ER binding sites on the DNA helix.

ER binding to the single copy of the sequence variant containing the 13-base pair consensus inverted repeat, but not the immediately adjacent nucleotides (Z27), was nearly identical to that of ER binding to a monomer of Z16. However, ER binding to a tetramer of Z27 showed 75% of the ER binding versus a tetramer of Z16. The reason for the decreased ER binding to Z27 is unknown, although this sequence is 2 nucleotides shorter than the consensus ERE (Z16), and steric constraints for binding of ER to adjacent EREs may play a role. However, this seems unlikely, since the dimer and trimer of Z27 showed ER-ERE binding ratios of 0.83 and 0.88, respectively (Table 2). The Scatchard plot had a convex shape indicative of cooperative binding. In contrast to a tetramer of Z16, in which the first and third (or second and fourth) ERE inverted repeats are on the same side of the DNA helix, only the first and fourth ERE, separated by 10 helical turns, are located on the same side of the helix in a tetramer of Z27.

The idea that spacing between EREs is important in determining ER binding and subsequent transcriptional activation was suggested earlier (29). The number of bases separating two perfect consensus 13-base pair EREs was critical for the synergistic induction of a reporter gene (13). Functional synergism of ER induction of CAT expression was detected with dimers of certain mutant EREs, those containing either purine/purine or pyrimidine/pyrimidine exchanges, separated by 20 base pairs between the centers of the inverted repeat. The authors suggested that synergism of induction did not require the cooperative binding of ER, even though they reported a higher relative binding affinity to dimers of either the wild type (2-fold) or mutant EREs (4-fold) than to a single ERE (13).

The only previous observation of cooperative ER binding was made by means of gel mobility shift competition experiments using the vitellogenin B1 ERE. Results showed that the binding of one ER dimer to ERE-2 increased the affinity of ER binding to neighboring ERE-1 by 4 to 8-fold (23).

High-Affinity ER-ERE Binding. The close agreement among the $K_a$ values that we obtained by Scatchard analyses of specific ER binding to one ERE by column assay and by the plate assay implies a high degree of reliability of each assay. The values obtained are somewhat lower than the $K_a$ value of 0.5 nM calculated previously from gel filtration assays (30) or the $K_a$ of 0.7 nM for mouse uterine ER-vitellogenin A2 binding (74) and 0.58 nM for rat uterine ER (27) estimated from gel retardation assays, but are higher than the 10 pm value reported for ER binding to the 5′ flanking sequence of the coding strand of the rat prolactin gene (20). The values that we obtained are most similar to the $K_a$ value of 0.39 nM for ER binding to vitellogenin A2 ERE as detected by avidin-biotin complex with DNA (ABCD) assay (75). Improvements in the assays used here, e.g., inclusion of poly[d(B)-poly[d(C)]] to reduce nonspecific DNA binding and 35S end-labeling of DNA, provided a way to measure high-affinity ER-ERE binding more accurately.

In summary, the data presented demonstrate that (a) ER binds cooperatively to perfect consensus EREs or imperfect EREs located on the same side of the DNA helix; (b) the degree of cooperativity appears to vary with ERE sequence and may be higher for EREs with certain imperfect inverted repeats than for the consensus sequence; (c) single nucleotide changes in the perfect inverted repeat of the consensus ERE sequence have modest effects on ER binding to a single ERE, but two nucleotide changes markedly reduce ER binding; (d) spacing between the two half-sites of the inverted repeat is critical for ER binding, with reduction by one or an increase by two nucleotides resulting in little or no ER binding either to a single ERE or to four tandem copies; (e) molybdate-stabilized monomeric (4S) ER does not bind detectably to EREs.

Based on our results, we suggest that choice of imperfect nucleotides in the consensus sequence can influence both the degree of cooperativity and the concentration of liganded-ER at which higher-affinity binding resulting from cooperativity is manifest. The number, sequence, and spacing of EREs can provide fine tuning of the response of individual genes to increasing estrogen levels in vivo. This type of control could offer a central mechanism by which the change in concentration of a hormone could differentially affect expression of a variety of genes.

Although we have described DNA binding studies here, it is clearly of interest to compare these results with those of transcriptional activation studies using these ERE sequences. Others demonstrated that while mutations in individual nucleotides of the HRE of mouse mammary tumor virus caused a 3-fold decrease in GR binding, there was a 10-fold reduction in transcriptional activity (48). Studies to clarify the relationship between ER-ERE binding and subsequent initiation of transcription are under way. In addition, the role of nucleosomal struc-
tecture, as well as other levels of chromatin organization, on these events in vivo is unknown.

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COOPERATIVE ESTROGEN RECEPTOR BINDING TO DNA


Cooperative Estrogen Receptor Interaction with Consensus or Variant Estrogen Responsive Elements in Vitro

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