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Properties of a high-affinity DNA binding site for estrogen receptor

(gene regulation/steroid receptor)

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ABSTRACT It has been shown previously that a short sequence from the 5′ regulatory region of the *Xenopus laevis* vitellogenin gene *A2*, when appropriately placed, can confer estrogen responsiveness to another gene. Using the *Xenopus* sequence and similar sequences from the 5′ regulatory regions of other estrogen-responsive genes, we derived a consensus sequence 38 nucleotides long. The sequence contains an inverted repeat (5′ C-A-G-G-T-C-A-G-A-G-T-G-A-C-C-T-G 3′) and an A/T-rich region. Plasmids carrying a single copy of the sequence bound 3-fold-more partially purified estrogen receptor (ER) than did control plasmids when assayed by gel filtration. Maximum specificity for ER binding occurs at 100–150 mM ionic strength and pH 7.5–8.0. Plasmids carrying multiple copies of the sequence bound correspondingly more ER. The dissociation constant for ER bound to the sequence is 0.5 nM. This value is lower by a factor of about 400 than the dissociation constant for ER bound to an equivalent length of plasmid DNA. Portions of the consensus sequence were evaluated for binding efficiency. Plasmids containing the inverted repeat alone bound ER, though less efficiently than did plasmids containing the entire sequence. The A/T-rich region alone was ineffective in binding ER. Linearization of the plasmid DNA did not enhance specific binding efficiency for ER. This model system represents an effective tool for characterization of ER binding to DNA sequences involved in the regulation of gene expression.

The estrogen receptor (ER) protein modulates the structure of chromatin in a manner that results in detectable changes in the nuclease hypersensitivity, DNA methylation, histone acetylation, and transcription rate of hormonally responsive genes (1). Although the completely functional interaction between ER and chromatin must involve accessory proteins, there is compelling evidence that specific DNA sequences are essential components of estrogen-dependent regulatory sites in chromatin. Repeated occurrences of the sequence 5′ G-G-T-C-A-N-N-N-T-G-A-C-C 3′ are found in the 5′ regions of estrogen-induced proteins in frogs and chickens (2–4). Early competitive DNA-binding experiments demonstrated that a 300-base-pair (bp) fragment of the chicken vitellogenin gene containing this sequence preferentially binds ER (5). The ER binding ability of this fragment was abolished by digestion with *Msp* I, which cuts between the last two 3′ nucleotides of the sequence 5′ G-G-T-C-A-G-C-G-T-G-A-C-C 3′. A fragment of the *Xenopus laevis* vitellogenin *A2* gene containing this inverted repeat functions as an estrogen-dependent cis-acting enhancer when transfected into human breast tumor cells (6). The same sequence functions in mouse, rat, or human cells lacking endogenous ER if co-transfected with a cDNA coding for the human ER (7). Clearly, the mechanism by which ER recognizes estrogen-responsive elements in chromatin is strongly conserved among species and depends in part on discrete DNA sequences. We have derived a short consensus sequence from the regulatory regions of several vertebrate estrogen-responsive genes and have demonstrated high-affinity binding of the calf uterine ER to this sequence. This paper describes the model system, which will allow the study of the functional consequences of ER binding to DNA.

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MATERIALS AND METHODS

Reagents. [2,4,6,7,16,17-^{3}H]Estradiol-17β ([^{3}H]E_2) (140 Ci/mmol; 1 Ci = 37 GBq) was supplied by Amersham. Dithiothreitol and Tris (ultrapure) were purchased from Boehringer Mannheim. DNA-grade hydroxylapatite was from Bio-Rad. Sephacryl S-1000 was from Pharmacia Fine Chemicals. The 2a70 preblended scintillant (2,5-diphenyloxazole/*p*-bis(*o*-methylstyryl)benzene, 49:1 wt/wt) was from Research Products International (Mt. Prospect, IL). Restriction enzymes, phage T4 DNA ligase, and *Escherichia coli* DNA polymerase I (Klenow fragment) were from New England Biolabs. The pGEM-1 plasmid was from Promega Biotech. All other chemicals were reagent grade.

Preparation of Calf ER. ER was partially purified from calf uteri by ammonium sulfate precipitation as described by Weichman and Notides (8) and modified by Klinge *et al.* (9). Samples of receptor protein were resuspended on ice for 1 hr in 2.0 ml of TDP buffer (40 mM Tris·HCl, pH 7.5 at 20°C/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride) containing 111 mM KCl. The pH of TDP buffer at 4°C is ≈8.1; the pH at 25°C is ≈7.4. The protein solution was clarified in a microcentrifuge for 15 min and desalted on Sephadex G-50 spin columns (10) equilibrated in TDP containing 111 mM KCl. Receptor solutions were labeled with 4–10 nM [^{3}H]E_2 either alone to detect total binding or in the presence of 200-fold excess of unlabeled E_2 to detect nonspecific binding. Incubations were at 0–4°C for at least 12 hr prior to conducting DNA binding assays.

Preparation of the Estrogen-Responsive Consensus Sequence Oligomer. The 5′ flanking sequence of the chicken vitellogenin II gene (3) and the chicken very low density apolipoprotein II gene (4) contain multiple copies of the interrupted inverted repeat 5′ G-G-T-C-A-N-N-N-T-G-A-C-C 3′ (2) shown by Klein-Hitpass *et al.* to be an essential component of their "estrogen-responsive element" (6). The chicken vitellogenin gene has one complete inverted repeat centered at 621 nucleotides upstream from the start of transcription. In addition, each gene has one imperfect inverted repeat, differing by a single transition from the original sequence. Half-inverted repeats (5′ G-G-T-C-A 3′ or 5′ T-G-A-C-C 3′) are found in four copies in the vitellogenin gene and in three copies in the apolipoprotein gene. A consensus sequence was derived by aligning the full and half

Abbreviations: ER, estrogen receptor; E_2, estradiol-17β.

inverted repeats from these 10 examples and the inverted repeat from the *Xenopus* vitellogenin gene *A2* (324 nucleotides upstream from the start of transcription); the most frequently occurring nucleotides were noted at each position (Fig. 1).

This sequence contains an extended inverted repeat at the "upstream" (i.e., 5') end (5' C-A-G-G-T-C-A-G-A-G-T-G-A-C-C-T-G 3') and an A/T-rich region further downstream. An *Alu* I restriction site two nucleotides downstream of the end of the inverted repeat separates the two regions. The sequence is homologous at 23 of 35 nucleotides with the estrogen-responsive element from *Xenopus* and at 20 of 24 nucleotides with the sequence that was noted by Jost *et al.* to bind ER with high affinity (5). The *Hae* III (5' C-C 3') and *Pvu* II (5' C-A-G 3') half-recognition sequences at the ends of the oligomer were added to facilitate head-to-tail polymerization of the monomeric sequence (see below). Separate strands of the sequence were chemically synthesized and purified by gel electrophoresis. These were hybridized and then ligated at their blunt ends into the *Hin*cII site in the pGEM-1 vector. Head-to-tail concatamers of the monomeric site were generated by ligation in the presence of *Hae* III and *Pvu* II restriction enzymes. These were then ligated into the vector as described above. To generate partial recognition sites, the purified double-stranded 38-bp oligomer was first digested with *Alu* I, which cleaves the sequence as indicated in Fig. 1 into 20-bp and 18-bp fragments. These were then cloned separately as above. Identification and orientation of inserts were determined by diagnostic restriction patterns. All plasmids used were purified from mass cultures on CsCl gradients. DNA was quantitated by spectrophotometry, assuming 50 μg of DNA per A_{260} unit.

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                 Xenopus vitellogenin A2 (2)

 1  (-324)  TCAGGTCACAGTGACCTGATCAAAGTTAATGTAACCTC

                 Chicken vitellogenin II (3)

 2  (-770)  AGGTTCTAGGCTGACCTGCACTTCTATCCCTCTTGCCT
 3  (-621)  CCTGGTCAGCGTGACCGGAGCTGAAAGAACACATTGAT
 4  (-361)  TTGGGTCAGGTGCCAGGTCAACATAACCTGGGCAAAAC
 5  (-349)  CCAGGTCAACATAACCTGGGCAAAACCAGTCTCATCTG
 6  (-293)  GCAGCCAGCCGTGACCCAATCTAGGAAAGCAAGTAGCA
 7  (-191)  TCTGGTCAATCAGAAAAAGGTTTTTATCAGAGATGCC

    Chicken very low density apolipoprotein II (4)

 8  (-278)  ACTGGTCAATACCACGTAGACTTTATTTAACAGTGAGA
 9  (-205)  AGGGGGCTCAGTGACCCAGGAGCTGCCTTCCACGTCTC
10  (-161)  TCAGGTCAGACTGACTTCCATTACCAAATCCGAACAAC
11  ( -46)  AGCAGGACCTTTGACCCCTCACTATATTAGTTCTGCAT

               Most frequently used nucleotides

            A       C         AAA  A         AA
12          TCAGGTCAG*GTGACCTGGGCTATAATAACCACTTCAC
13          474997884*48810975444665446454544454545

                    Consensus sequence

14        5'-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3'
                                 |
                               AluI
                         recognition site
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FIG. 1. Derivation of a consensus sequence for the ER binding site. The functional ER binding site determined by Klein-Hitpass *et al.* (6) was used as a reference in which to align the other genes by using the inverted repeats (2) that occur in each sequence (underlined). Line 1 shows the *Xenopus* vitellogenin *A2* sequence (2); lines 2–7 show sequences from the chicken vitellogenin II gene (3); lines 8–11 show chicken very low density apolipoprotein II gene sequences (4). The numbers in parentheses indicate the position of the middle nucleotide of the inverted repeat relative to the start of transcription for each gene. Lines 12 and 13 show the most commonly occurring nucleotide at each position. Asterisks in these lines indicate positions at which no nucleotide occurred more than 3 times in 11 cases. Line 14 shows the consensus sequence based on these frequencies. The A-C pair seven and eight nucleotides from the 3' end was made to conform to the binding sequence described by Jost *et al.* (5). The *Alu* I recognition site was used to divide the sequence prior to cloning individual halves.

Hydroxylapatite Assay. The concentration of ER was quantitated by adsorption to hydroxylapatite (11). After incubation of ER with $[^3H]E_2$ alone (total samples) or with $[^3H]E_2$ and excess unlabeled E_2 (nonspecific samples), 90-μl aliquots were mixed with 250 μl of hydroxylapatite [10% (wt/vol) in TDP buffer] on ice for 15 min. The hydroxylapatite with bound receptor was drained and resuspended three times in 1 ml of buffer and then drained and assayed in 1.3 ml of scintillation fluid (330 ml of Triton X-100, 670 ml of toluene, and 6.3 g of 2a70 preblended scintillant per liter). Counting efficiency was 24%. Specific binding was calculated by subtracting cpm for the nonspecific samples from cpm for the total samples.

DNA Binding Assay. The standard DNA binding reaction mixtures contained 180 μl of ER solution in TDP buffer/111 mM KCl and 20 μl of DNA in 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The final concentration of ER and plasmid DNA in the reactions was 3 nM and 1.5 nM (in whole molecules), respectively. The final KCl concentration in the binding reaction was 100 mM (except as indicated in Fig. 3). Samples were mixed briefly, incubated for 30 min at 25°C, and then loaded (180 μl) onto the Sephacryl S-1000 column at 4°C. The Sephacryl column was equilibrated and developed with TDP buffer containing 100 mM KCl. Under these conditions, the effective pH is 7.4 during the binding reaction and 8.1 during gel filtration.

Gel filtration columns were constructed from 5-ml serological pipettes (Falcon 7543) capped with 200-μl micropipette tips containing 3-mm disks of plastic frit (70-μm pore size; Bolabs, Lake Havasu City, AZ). Bed volumes were 8 ml. The columns were pumped at a flow rate of 15–20 ml per hr. Thirty to 50 fractions of 0.5 ml were collected per column. Plasmid DNA was eluted in the void volume; ER was included in the column; and E_2 was partially adsorbed to the column, eluting as a peak well resolved from the ER protein. Fractions were counted in 3.5 ml of aqueous scintillation fluid. Counting efficiency was 20%.

To determine ER-specific $[^3H]E_2$ binding for each column fraction, radioactivity in samples of ER labeled with $[^3H]E_2$ in the presence of excess E_2 was subtracted from radioactivity in samples of ER labeled with $[^3H]E_2$ alone. To determine amounts of DNA-bound ER, radioactivity in ER samples lacking added DNA was subtracted from radioactivity in ER samples containing DNA.

RESULTS

Demonstration of Specific Binding to the Consensus Sequence. To test the 38-bp sequence for specific binding of ER, we assayed the elution of ER from Sephacryl S-1000 gel filtration columns in the presence and absence of plasmid DNA containing the sequence. With this resin, plasmid DNA and ER bound to plasmid DNA were eluted in the void volume, whereas ER was included in the column volume. Plasmid DNA containing no insert (pGEM-1 control) bound a small amount of ER nonspecifically, resulting in elution of a small shoulder of ER at the leading edge of the profile seen with ER alone (Fig. 2). When equal amounts of plasmid DNA bearing one or four copies of the 38-bp sequence were added to ER, a distinct increase in the quantity of ER eluted in the void volume could be seen. Gel filtration was performed after a 30-min incubation of reaction components. Time-course analysis demonstrated that binding reached a maximum at 10 min and remained relatively constant for 2 hr (not shown). Fig. 2*B* shows the elution profiles of DNA-bound ER obtained by subtracting the values for ER alone from the values for ER plus DNA. The area under each of these peaks was proportional to the number of copies of the consensus sequence in the respective plasmids. These re-

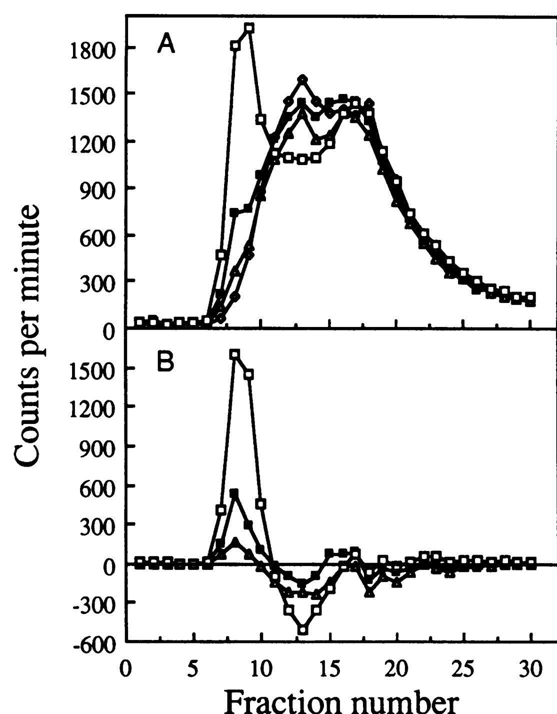

FIG. 2. Gel filtration elution profiles of ER demonstrating specific binding to the consensus sequence. ER was incubated with DNA in TDP buffer containing 100 mM KCl (pH 7.5 measured at 22°C). (*A*) Elution profiles of ER alone (◇), ER in the presence of pGEM-1 (△), pGEM-1 with a single inserted sequence (■), and pGEM-1 with four tandem inserted sequences (□). Plasmid DNA was eluted in fractions 6–10, ER was eluted in fractions 11–15, radioactivity in fractions 16–20 represents E_2 bound nonspecifically to other proteins, and free E_2 was eluted in fractions 30–40 (not shown). (*B*) The same data with values for ER alone subtracted from values for ER plus the pGEM-1 plasmids.

sults clearly indicate that the consensus sequence is responsible for efficient binding of ER to the plasmid DNA.

Optimization of Binding Conditions. Conditions of ionic strength and pH that maximize ER binding were determined. The effect of ionic strength is seen in Fig. 3. Binding of

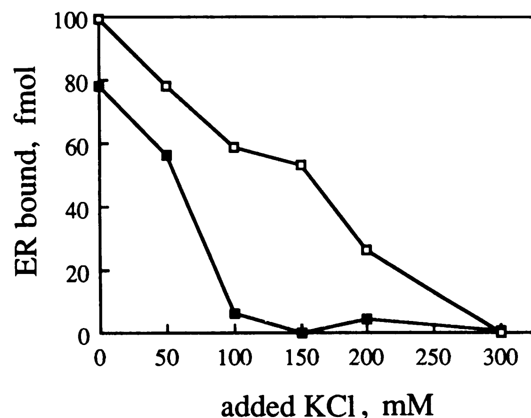

FIG. 3. Effects of ionic strength on ER binding to DNA. Standard conditions were used except that ER was equilibrated with E_2 in TDP buffer without KCl. Aliquots of ER (100 μl) were mixed with 80 μl of TDP containing the required amount of KCl (2.5 times the final concentration) and 20 μl of DNA in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and then were incubated at 25°C for 30 min. Gel filtration was performed in TDP buffer containing the indicated concentration of KCl. Binding of ER to plasmid DNA was determined from the area under curves as in Fig. 2*B*. The quantity of ER bound to either pGEM-1 DNA (■) or pGEM-1 with four tandem inserted sequences (□) is shown.

[^{3}H]ER to both control plasmids and plasmids bearing four tandem copies of the sequence decreased with increasing ionic strength. Although control plasmids bound very little ER when added salt exceeded 100 mM, plasmids bearing the consensus sequence continued to display significant binding ability when added salt reached 200 mM. Maximum specificity was achieved in the range of 100–150 mM added salt.

The effect of pH is seen in Fig. 4. The nonspecific binding capacity of control plasmids was consistently low at all pH values, whereas binding of [^{3}H]ER to the specific site increased from pH 6.5 to 8.0 and then decreased abruptly at pH 8.5. Binding of E_2 to ER was not significantly altered over this range of pH (not shown) and, thus, does not account for the observed effects. Column elution profiles indicated that marked aggregation of ER had occurred at pH 6.5 and 7.0. This is consistent with the acidic isoelectric point (pH 5.4) reported for the ER protein (12). Hepes buffer rather than Tris buffer was used in this experiment for better control of pH over the range of pH values tested. The results obtained at pH 7.5 were essentially identical for either buffer.

Measurement of the Dissociation Constant. To determine the affinity constant of the binding reaction, the amount of ER binding to pGEM-1 containing a single inserted sequence was measured over a range of DNA concentrations while a fixed ER concentration was maintained. Fig. 5 *Inset* shows the binding profile of ER to pGEM-1 and to pGEM-1 containing the sequence, along with the subtracted values indicating net specific binding to the sequence. Fig. 5 shows the binding data for pGEM-1 containing the sequence, analyzed by the method of Scatchard (13). The shape of the curved line on this plot indicates that the interaction of receptor with the entire plasmid has both high- and low-affinity components, as expected. Data points representing specific binding to the sequence, shown on the same graph, form a straight line. From the slope of this line, we calculated an apparent K_d value of 5×10^{-10} M. Calculation of the binding of ER to pGEM-1 alone (not shown) yielded an apparent K_d value of 2×10^{-7} M for binding to an average region of plasmid DNA equivalent in length to that of the specific sequence.

Effect of Linearization of the Plasmid on Binding. Our experiments were routinely performed with 90% supercoiled plasmid DNA. To test the effects of quantitative linearization, plasmid DNA containing a single specific sequence was digested either with *Bgl* I, which cuts 1.3 kilobases downstream of the point of insertion of the sequence, or with *Pst* I, which cuts 8 nucleotides upstream from the insertion site,

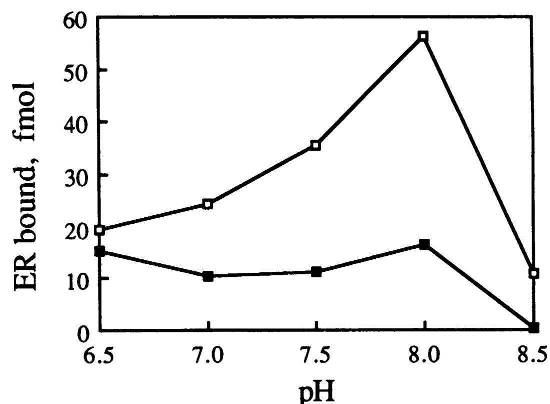

FIG. 4. Effects of pH on ER binding to DNA. Standard conditions were used except that 40 mM Hepes was used in place of 40 mM Tris. The pH indicated is the pH at 25°C. Binding of ER to either pGEM-1 (■) or pGEM-1 with four tandem inserted sequences (□) was determined as in Fig. 3.

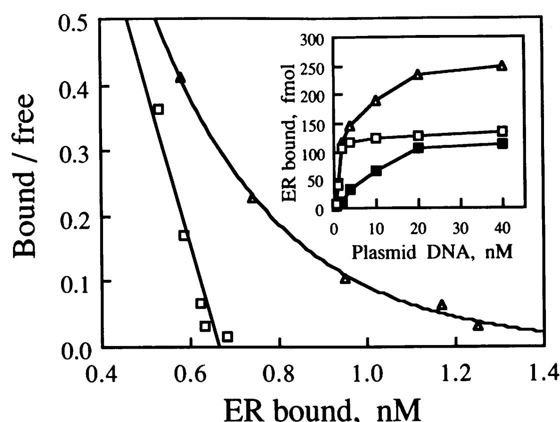

FIG. 5. Scatchard analysis of binding data. A fixed quantity of ER (4.2 nM) was titrated with increasing amounts of DNA in TDP buffer containing 100 mM KCl (pH 7.5 at 22°C). (*Inset*) Binding of ER to pGEM-1 (■) and to pGEM-1 with a single inserted specific sequence (△). ER binding to the specific sequence (□) was derived by subtracting data for ER bound to pGEM-1 from data for ER bound to the pGEM-1 with the inserted specific sequence.The data from *Inset* for ER binding to pGEM-1 with a single inserted site (△) and for ER binding to the specific sequence (□) is plotted according to Scatchard (13).

or with *Bam*HI, which cuts 14 nucleotides downstream of the insertion site. ER (2.5 nM) and plasmid DNA (10 nM) were incubated under standard binding conditions and analyzed by gel filtration. All linearized plasmid DNA, regardless of sequence or site of cutting, bound a similar additional quantity of ER (15 fmol of ER per pmol of DNA molecule). In the case of the plasmid containing the specific site, this increase equaled 15% of the amount bound by the original supercoiled plasmid (100 fmol of ER per pmol of plasmid DNA). Because of the lower initial binding of ER to the supercoiled pGEM-1 plasmid (12 fmol of ER per pmol of plasmid DNA), the same increase approximately doubled the amount of ER bound to pGEM-1 DNA. Therefore, the increase in ER binding resulting from linearization of DNA can be attributed to the presence of free DNA ends rather than to improved access to the specific binding site.

Contribution of Regions of the Consensus Sequence to Binding Specificity. The specific sequence was cleaved approximately in the center with *Alu* I (see Fig. 1), and each half was introduced into pGEM-1 for binding measurements. Plasmids containing the upstream half with the inverted repeat demonstrated 49% of the specific binding observed with plasmids containing the complete sequence. Plasmids containing the downstream A/T-rich half of the sequence displayed no additional ER binding beyond that of pGEM-1 alone.

DISCUSSION

In the earliest demonstration of ER binding to a specific DNA sequence, Jost *et al.* (5) showed that cleavage of the 3′ pentamer in the sequence 5′ G-G-T-C-A-N-N-N-T-G-A-C-C 3′ eliminated specific binding. More recently, a 35-nucleotide-long fragment containing the same inverted repeat was shown to confer estrogen responsiveness to a linked gene (6). Consequently, we were encouraged to test related sequences for specific ER binding *in vitro*. We have shown that a sequence of 38 nucleotides, representing a consensus of sequences flanking estrogen-responsive genes and containing the inverted repeat, binds ER with high affinity and specificity. pGEM-1 plasmids bearing a single copy of the sequence bound 3 times as much ER at 100 mM KCl as did control plasmids lacking the sequence. Therefore, on a relative length basis, this sequence is hundreds of times more effective in binding ER than is plasmid DNA. Plasmids bearing four copies of the sequence in tandem bind correspondingly more ER. We also have demonstrated that the upstream half of the specific sequence containing the inverted repeat binds ER, although only half as efficiently as the full-length sequence. In contrast, the downstream A/T-rich half of the specific sequence is inactive in binding ER. Therefore, the two halves of the sequence act in a synergistic manner. It is possible that the inverted repeat represents the primary binding site for ER, while the A/T-rich region facilitates stable binding by allowing a conformational change in the DNA structure or by providing additional contact points for ER. Klein-Hitpass *et al.* (6) showed that a DNA fragment of 18 nucleotides containing only the inverted repeat can confer estrogen responsiveness *in vivo*, although less efficiently than the entire 35-bp sequence. Indeed, the correlation of their findings with our results suggests that the binding assay described is capable of reliably estimating the functional effectiveness of different sequences *in vivo*.

Gel filtration assays were thought to be inadequate for measuring ER binding to DNA because ER aggregates and, thus, appears to move as though bound to DNA (14). However, under our assay conditions, ER alone migrates in a distinct position from DNA. Also, impurities in DNA preparations could potentially induce aggregation of ER, again giving the false impression that DNA binding has occurred (14). However, the use of plasmids lacking the specific binding site measures nonspecific ER aggregation. Furthermore, the fact that plasmids bearing additional numbers of inserts display proportionately greater binding capacity for ER clearly confirms that the sequence itself is responsible for the binding of ER.

A consistent decrease in ER binding was observed with increasing ionic strength. However, specific binding to the inserted sequence was more resistant to increasing ionic strength than was nonspecific binding. Indeed, significant binding occurred when added KCl exceeded 100 mM only with plasmids containing the inserted sequence. Binding of ER to the sequence increases as pH increases from 6.5 to 8.0. The Hepes buffer used in this experiment contributes increasingly to ionic strength, adding about 27 mM as the pH increases from 6.5 to 8.0. The fact that ER binding to DNA increases over this range is the opposite of what could result if the ionic strength contribution of the buffer, rather than pH, were responsible. The higher range of pH facilitated both binding of ER to DNA and measurement by gel filtration, since ER aggregation was minimized above pH 7.5. Our observed pH optimum of 7.5–8.0 contrasts with reports by Skafar and Notides (15), who observed a sharp binding optimum of ER to DNA at pH 7.4, and by Compton *et al.*, who noted higher binding of the progesterone receptor to DNA at pH 6.5 than at 7.5 (16).

Binding data analyzed by the method of Scatchard yielded an apparent K_d of 5×10^{-10} M for the dissociation of ER bound to the specific sequence. This value was about 1/400th of the corresponding value for receptor binding to an equivalent length of pGEM-1 DNA, clearly indicating a capacity of ER for selective association with a discrete sequence. A K_d of 5×10^{-10} M is consistent with the value measured for the high-affinity component of ER binding to nuclei *in vitro* (9, 17–19).

Linearization of plasmid DNA did not significantly change binding efficiency to the specific sequence, demonstrating that supercoiling is not necessary for specific binding. The exact conformation of the specific sequence most favorable for binding remains to be elucidated.

Cooperativity of ER binding was not tested. It has been reported that ER, glucocorticoid receptor, and progesterone receptor all bind to DNA in a cooperative manner (1, 15, 20). This property could be detected by using plasmids bearing

more than one binding site because binding of receptor to one site should affect binding to adjacent sites. One receptor may facilitate the binding of others to neighboring sites. Alternatively, steric factors may prevent binding of two receptors to immediately adjacent sites. Such a finding could then allow the measurement of the effective "footprint" of the ERs by insertion of increasingly larger DNA fragments between two binding sites until interference is no longer detected.

It is notable that the consensus sequence binding site used in these experiments, derived from frog and chicken sequences, efficiently binds calf ER. This result was anticipated from the functionality of the *Xenopus* sequence in human MCF-7 cells (6). These results suggest that the binding sites for ERs are strongly conserved among species.

Clearly the details of the interaction of ER with the binding site are not revealed in our experiments. It is formally possible, though not likely, that the receptor is bound indirectly to the DNA by another protein, which itself recognizes the sequence of interest. This possibility can be tested, since DNA binding should always copurify with E_2 binding if ER itself is active.

With this system, we also can test the functionality of other estrogens and antiestrogens on ER–DNA interaction. For example, the ability of the 4-hydroxytamoxifen–ER complex to bind to DNA can now be characterized.

Finally, the effect of ER on the local structure of the DNA can now be studied in detail. For example, DNA unwinding or helicase activity could be detected by established methods.

We have described a system that allows measurement of ER binding to a high-affinity site derived from the sequences of known estrogen-responsive genes. The assay is simple and relatively insensitive to contaminating proteins and buffer components. It offers an opportunity to examine additional consequences of ER binding that could explain its functions *in vivo*.

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