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Estrogen Receptor Binding to a DNA Response Element in Vitro Is Not Dependent upon Estradiol†

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ABSTRACT: Gel shift assays were employed to distinguish between the contribution of 17β-estradiol (E_2) and a short heating step to the ability of the rat uterine cytosolic estrogen receptor (ER) to bind to the estrogen response element (ERE) from the vitellogenin A2 gene (vitERE). Despite the popularity of models in which the ER is a ligand-activated DNA-binding protein, these studies find that estrogen does not significantly contribute to receptor–DNA complex formation. An avidin–biotin complex with DNA (ABCD) assay was utilized to obtain quantitative measurement of the affinities of the ER for the vitERE and a mutant sequence. Scatchard analysis gave a dissociation constant of 390 ± 40 pM for the E_2-occupied, heated ER to the vitERE. The data fit a one-site model and evidence for cooperativity was not observed. A dissociation constant of 450 ± 170 pM was obtained for the unoccupied, heated ER, leading to the conclusion that estrogen was not necessary for specific binding to DNA. The percentage of ER capable of binding vitERE varied with each cytosol preparation, ranging from 60 to 100% and estrogen did not appear to affect this variation. Competition against the vitERE with a 2-bp mutant sequence showed a 250-fold lower relative binding affinity of the receptor for the mutant over the vitERE sequence. This ability of the ER to discriminate between target and nonspecific DNA sequences was also not dependent on the presence of estrogen.

It has been widely proposed that estrogens effect gene transcription by inducing their receptor protein to bind to specific DNA sequences in target genes. This attractive model of steroid receptors as ligand-activated DNA-binding proteins has been commonly presented as established consensus in the literature (Baulieu, 1989; Evans, 1988; Hunt, 1989; Picard et al., 1988, 1990) and even in a recent textbook (Alberts et al., 1989). This model traces its origin to the fact that a characteristic of the in vitro transformed estrogen receptor (ER) is its ability to bind to DNA–cellulose, albeit with only micromolar affinity and a modest increase in affinity induced by steroid (Skafar & Notides, 1985). A number of treatments, including heating or high salt, lead to receptor transformation and this process was generally agreed to also require the presence of hormone (Grody et al., 1982). The receptor would be expected to display a 1000- to 10000-fold increase in binding affinity to target DNA sequences in response to hormone according to theoretical calculations, if, indeed, hormone confers the ability of the receptor to select target sequences over the mass of DNA in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989).

One such target sequence for estrogen action has been identified in the 5′-flanking region of the vitellogenin genes of *Xenopus* and chicken (Klein-Hitpass et al., 1986; Walker et al., 1984). Transcription of these genes in vivo is strongly dependent on the presence of estrogen (Wahli, 1981). The

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Xenopus A2 gene has a single copy of the perfect palindrome GGTCACAGTGACC located 330-bp upstream of the transcriptional start site (Walker et al., 1984). This vitellogenin estrogen response element (vitERE) is sufficient to confer estrogen responsiveness to a heterologous promotor in gene-transfer studies (Klein-Hitpass et al., 1986). Base pair changes in this sequence have been identified that rendered the heterologous promotor glucocorticoid responsive, as well as a 2-bp change that failed to activate transcription in response to either hormone (Klock et al., 1987). Several groups have demonstrated the ability of ER to bind to this small, defined sequence of DNA. Klein-Hitpass et al. (1988) used a DNA–cellulose competition assay to demonstrate that the receptor bound to DNA fragments with a rather modest 5- to 10-fold greater relative affinity if the fragments contained the vitERE. These experiments were performed with occupied receptor, thus the dependence of relative DNA-binding affinity on hormone was not evaluated. Peale et al. (1989) used a gel filtration assay to obtain an estimate of receptor affinity of 0.5 nM for the vitERE. Again, these studies were conducted with occupied receptor only. Kumar and Chambon (1988) and Martinez and Wahli (1989) used the gel mobility shift assay to qualitatively demonstrate the binding of the ER to the vitERE and to assess the dependence upon hormone. These studies concluded that receptor must be occupied with a ligand in order to bind the specific DNA fragment in vitro and that heating alone was not sufficient. Together these studies provide considerable evidence for specific binding of the receptor to the vitERE. However, studies to quantitate the effect of estrogen on receptor binding affinity for the vitERE in comparison to nonresponsive DNA sequences have not been reported.

In order to determine the thermodynamic binding parameters (K_d, dissociation constant; B_{max}, receptor in DNA binding form) for the ER to specific sequences of DNA, we adopted avidin–biotin–nucleic acid methodology developed by the Sharp group (Grabowski & Sharp, 1986) and later applied to thyroid hormone receptor–DNA binding studies (Glass et al., 1987). Modification of this avidin–biotin complex with DNA (ABCD) assay to work with a filtration apparatus has allowed collection of thermodynamic binding data from numerous reactions with relative ease. We chose the *Xenopus* vitellogenin A2 gene ERE and the 2-bp mutant of this sequence as described by Klock et al. (1987) to obtain a measure of the receptor's ability to select a sequence known to confer estrogen responsiveness on a heterologous promotor over a sequence that does not.

Occupation of the receptor is often coupled with some treatment such as salt, heat, or even dilution that is known to promote transformation. This study separately evaluated the relative contributions of estrogen and heat to induction of receptor–DNA binding activity. Our results show that the occupied ER displays sequence selectivity since its relative binding affinity for the mutant is 250-fold less than the 390 pM dissociation constant observed for binding to the vitERE. This is despite the fact that both sequences are perfect palindromes. Surprisingly, we find that DNA binding activity is induced solely by heating and that estrogen has no significant effect on receptor–DNA complex formation in vitro.

Materials and Methods

Estrogen Receptor Preparation. Uteri were dissected from 19–20 day-old, female, Sprague-Dawley rats and collected in TED (10 mM Tris-HCl, pH 7.5 at 25 °C, 1.5 mM EDTA, 0.5 mM DTT) on ice. All subsequent procedures were performed on ice or at 4 °C unless indicated otherwise. Uteri were stripped of fat, minced into 1–2-mm pieces, and rinsed several times with TED. Uteri were homogenized by hand in a Duall glass–glass homogenizer in TSDG [10 mM Tris-HCl, pH 7.5 at 25 °C, 50 mM NaCl, 1 mM DTT, 10% (vol/vol) glycerol] at 3 uteri/mL. Sodium molybdate (MoO_4^{2-}) at 10 mM was added to TED and TSDG for the preparation of molybdate stabilized cytosol. Homogenates were centrifuged at 435680*g* for 10 min at 4 °C in a Beckman TLA-100 ultracentrifuge. The supernatant fraction was designated the cytosol. Cytosols generally contained 2–4 mg/mL protein as determined by the method of Bradford (1976) and 2–3 nM ER as determined by a modification of the hydroxylapatite assay (Erdos et al., 1970). Typically, an aliquot of cytosol (5–40 μL) was occupied with 10 nM [^{3}H]-17β-estradiol (^{3}H-E_2, 90–110 Ci/mmol, New England Nuclear) for 1–3 h at 4 °C. Samples were then mixed with 1 mL of T buffer (50 mM Tris-HCl, pH 7.5 at 25 °C) and 0.25 mL of a 70% slurry of hydroxylapatite (equilibrated with T buffer, BioRad) for 30 min at 4 °C with periodic vortexing. Two milliliters of T buffer was added and the hydroxylapatite was pelleted by centrifugation at 1000*g* for 3 min at 4 °C. Pellets were washed five times with 2 mL of T buffer and then extracted with 1 mL of 100% ethanol for 30 min at room temperature. One-half milliliter of the ethanol sample was added to 3.5 mL of Ready-safe scintillation cocktail (Beckman) and subjected to liquid scintillation counting. Nonspecific binding was estimated by including a 200-fold molar excess of diethylstilbestrol (DES) over ^{3}H-E_2 to duplicate samples. Specific binding was calculated as total minus nonspecific.

Occupation and Heating of Cytosols. The four forms of the ER used in these studies were generated by the following treatments of cytosol prior to use in DNA binding assays: (1) *unoccupied, unheated + MoO_4^{-2} ER*, molybdate-stabilized cytosol was kept on ice for 2–4 h; (2) *unoccupied, heated ER*, cytosol was kept on ice for 1–3 h, then heated at 30 °C for 45–60 min, and returned to ice; (3) *occupied, unheated + MoO_4^{2-} ER*, molybdate-stabilized cytosol was incubated with 10–40 nM E_2 (^{3}H-E_2 for ABCD assays) for 2–4 h on ice; and (4) *occupied, heated ER*, cytosol was incubated with 10–40 nM E_2 (^{3}H-E_2 for ABCD assays) for 1–3 h on ice, then heated at 30 °C for 45–60 min, and returned to ice.

Restriction Fragment DNA. Plasmids pERE15 and pERE15mT contain the ERE derived from the *Xenopus* vitellogenin A2 gene and a 2-bp mutant of that sequence, respectively (Klock et al., 1987). A fragment containing these sequences was generated by *Bam*HI–*Hin*dIII digestion of each plasmid as shown in Figure 1A. These restriction fragments were labeled by an end-filling reaction with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dCTP (800 Ci/mmol, New England Nuclear) by standard protocols (Maniatis et al., 1982). A typical reaction mix contained 200 μg of digested plasmid, 200 μM dTTP, 200 μM dATP, 200 μM dGTP, 1 μM [α-^{32}P]dCTP, and 20 units of enzyme in a final volume of 100 μL. Reactions were allowed to proceed for 60 min at room temperature, and then the labeled 55-bp restriction fragment was purified away from plasmid and free nucleotide by electrophoresis on 15% polyacrylamide gels. The purified restriction fragment was quantitated by ethidium bromide dot assay (Maniatis et al., 1982). Specific radioactivity ranged from 3×10^{17} to 7×10^{17} dpm/mol restriction fragment.

Synthetic Oligonucleotides. Synthetic oligonucleotides (21 bases) for each strand of the consensus vitERE sequence and the 2-bp mutant were obtained from the Biotechnology Center

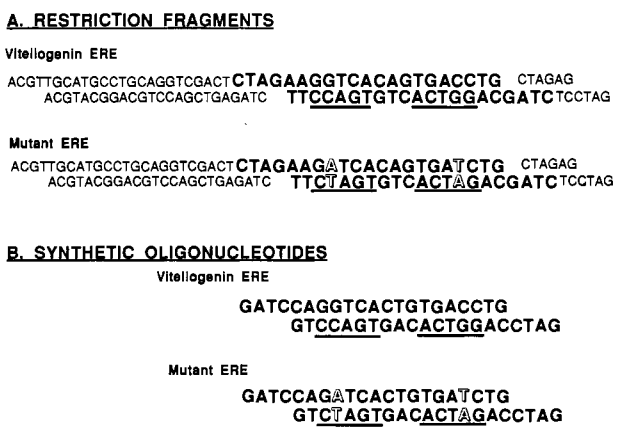

FIGURE 1: Defined DNA sequences used in DNA binding assays. (A) Sequence of the *Bam*HI–*Hin*dIII restriction fragment from the pERE15 plasmid (Vitellogenin ERE) or from the pERE15mT plasmid (Mutant ERE). The bases in bold type represent the synthetic oligonucleotide inserted into the XbaI site of the pBLCAT2 plasmid (Klock et al., 1987). The base sequence underlined for the Vitellogenin ERE fragment represents the palindromic ERE from the Xenopus vitellogenin A2 gene (Walker et al., 1984). The two base pair changes in the mutant ERE are outlined. (B) Synthetic oligonucleotide sequences derived from (A).

at the University of Wisconsin—Madison. In addition to the core palindromic sequence, each oligonucleotide was designed with *Bam*HI linkers. Complementary strands were mixed in 10 mM Tris-HCl (pH 8 at 25 °C), 1 mM EDTA, and 200 mM NaCl, heated to 95 °C, allowed to cool to room temperature over 2–3 h to promote annealing, and then stored at 4 °C. The double-stranded oligonucleotides with *Bam*HI 5′-overhangs are shown in Figure 1B. Double-stranded oligonucleotides were dialyzed against the appropriate buffer immediately prior to use in competition assays. For use in the ABCD assay, oligonucleotides were labeled with biotin by an end-filling reaction with the Klenow fragment of DNA polymerase and biotin-16-dUTP (Enzo Diagnostics) or biotin-14-dATP (BRL). A typical reaction mix contained 150 μg of double-stranded oligonucleotide, 50 μM biotin-nucleotide, 200 μM each of the other three nucleotides (unlabeled), and 100 units of enzyme in a final volume of 1 mL. The reactions were allowed to proceed for 60 min at room temperature and the labeled oligonucleotides (25 bp) were purified by electrophoresis on 20% polyacrylamide gels. Oligonucleotide solutions were quantitated spectrophotometrically at 260 nm. The extinction coefficient for each oligonucleotide was obtained by summation of the extinction coefficients for each component nucleotide and multiplied by 0.6 to correct for the loss in absorbance upon base pairing (Bush, 1974). Analytical 20% polyacrylamide gel electrophoresis was performed on each batch of biotin-labeled oligonucleotide before and after incubation with streptavidin–agarose beads (BRL) to ensure quantitative precipitation.

DNA Gel Shift Assays. Cytosols treated as indicated in the figure legends were preincubated with 0.1 mg/mL poly(dI-dC)-(dI-dC) (Sigma) at 4 °C before addition of ^{32}P-labeled restriction fragment DNA in a final volume of 20 μL in TSDG buffer. Various concentrations of unlabeled vitERE oligonucleotide were included in this incubation for competition studies. The binding reaction was allowed to proceed for up to 18 h at 4 °C. Protein–DNA complexes were resolved from free DNA on low ionic strength polyacrylamide gels as described with minor modifications (Carthew et al., 1985). Samples were loaded onto low ionic strength 4% polyacrylamide gels (0.15 × 16 cm; acrylamide:bisacrylamide weight ratio of 30:1), which had been preelectrophoresed for 1–2 h at 30 mA in buffer consisting of 6.7 mM Tris-HCl, pH 7.5 at 25 °C, 3.3 mM NaOAc, and 1 mM EDTA. Buffer was recirculated between compartments and the electrophoresis apparatus was cooled to 19 °C during the sample run. Gels were electrophoresed at 60 mA for approximately 1.5 h. Upon completion of electrophoresis, gels were transferred to Whatman 3MM paper, dried, and autoradiographed by using either flashed Kodak XAR-5 or XRP-5 film at −80 °C with a Du Pont Kronex screen. Following autoradiography, the free and protein-bound ^{32}P-labeled restriction fragments were cut from the dried gel and placed into vials with 4 mL of Beckman Ready-safe scintillation cocktail, and radioactivity was quantitated by liquid scintillation counting.

Gel Supershift Assay. Rabbit immune serum raised against a peptide from the rat ER (ER712 antisera, Furlow et al., 1990) was added to the binding reaction at a final dilution of 1:1000 to retard (or "supershift") the receptor–DNA complexes in the standard gel shift assay as described above.

ABCD Assays. Cytosols treated as indicated in the figure legends were incubated with biotin-labeled vitERE oligonucleotide in TSDG at 4 °C for 0.5–22 h in a final volume of 50 or 100 μL. Various concentrations of non-biotin-labeled oligonucleotide were included in this incubation for competition assays. Twenty microliters of a 50% slurry of streptavidin–agarose in TSDG containing 1 mg/mL BSA was added to each sample at the end of the binding reaction. Samples were rotated on a orbital shaker at 100 rpm for 1 h at 4 °C to absorb biotin–oligonucleotide to the resin. Samples were filtered under low vacuum into econocolumns (BioRad) and the resin was rapidly washed 9 × 3 mL with ice-cold TT buffer [20 mM Tris-HCl, pH 7.5 at 25 °C, 0.01% (vol/vol) Tween-20, BioRad]. In the case of the ^{3}H-E_2 occupied receptor, resin was dried under vacuum and then eluted with 0.5 mL of 100% ethanol into scintillation vials. Econocolumns containing unoccupied receptor were capped, and 100 μL of a 10% fast-flow hydroxylapatite (Calbiochem) slurry in TSDG was added to bind any receptor dissociating from the DNA during the subsequent estrogen occupation step. ^{3}H-E_2 was added to a final concentration of 1 nM and samples were allowed to incubate on ice for 1–2 h. Samples were then rapidly washed under low vacuum with 8 × 3 mL of ice-cold TT buffer, dried, and eluted with 0.5 mL of 100% ethanol into scintillation vials. Three and one-half milliliters of Beckman Ready-safe cocktail was added and radioactivity was quantitated by liquid scintillation counting.

Background due to ^{3}H-E_2 was determined by including a 200-fold excess of DES over the ^{3}H-E_2 concentration in the receptor occupation step. Nonspecific DNA binding in binding curve experiments was determined by including 500 nM non-biotin-labeled vitERE in duplicates for each biotin-labeled vitERE concentration. Specific binding was calculated by subtraction of binding in the presence of 500 nM non-biotin-labeled vitERE from total binding. Only one or the other of these two types of corrections was made in each experiment.

Control experiments were performed to determine the optimum conditions for the assay, including the amount of streptavidin–agarose per binding reaction, time of incubation with the streptavidin–agarose, composition of the wash buffer, and the number of washes to efficiently separate bound and free receptor. Experiments were conducted in which molybdate was added after heating the cytosol to ensure that molybdate did not interfere with the assay itself. In addition, experiments were conducted to determine the amount of carrier DNA [poly(dI-dC)-(dI-dC) or pUC 18] required to obtain optimal binding. In fact, addition of 10–120 mg/mL pUC

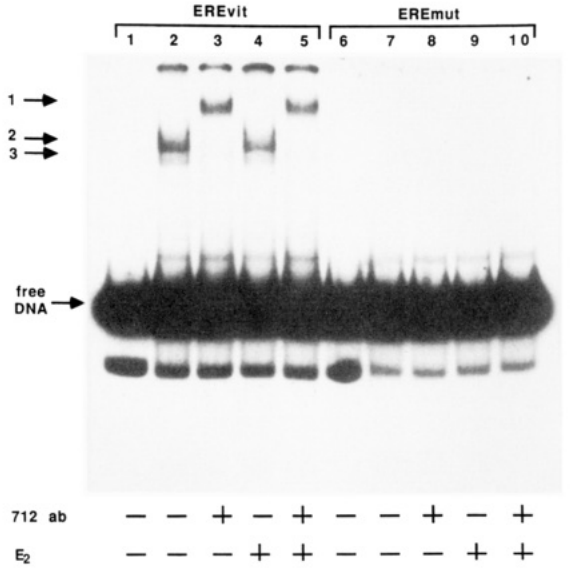

FIGURE 2: Effect of E_2 on gel mobility shift assay of uterine cytosol with vitERE or mutant ERE restriction fragments. Cytosol from immature rat uteri was heated in the presence of 10 nM E_2 (lanes 4, 5, 9, and 10) or ethanol vehicle (lanes 2, 3, 7, and 8) as indicated. Samples containing 0.4 nM ER were incubated with 3 nM of either the ^{32}P-labeled vitERE restriction fragment (EREvit) or the ^{32}P-labeled mutant ERE restriction fragment (EREmut) for 19.5 h at 4 °C. Lanes 3, 5, 8, and 10 had antisera ER712 against the ER included during this incubation. Lanes 1 and 7 are controls without cytosol. Free and protein-bound DNA was separated by nondenaturing polyacrylamide gel electrophoresis as described in Materials and Methods. ^{32}P-labeled DNA is visualized by autoradiography.

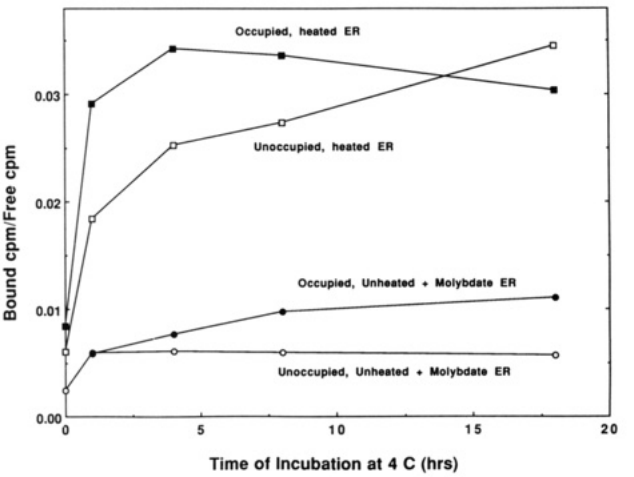

FIGURE 3: Time course of ER binding to the vitERE restriction fragment: effects of heat, molybdate, and E_2. Cytosols were prepared in the presence (open and closed circles) or absence (open and closed squares) of 10 mM sodium molybdate. Cytosol samples were then treated as follows: molybdate cytosol plus ethanol vehicle, 1.5 h on ice (unoccupied, unheated + MoO_4^{2-}: open circles); molybdate cytosol plus 10 nM E_2, 1.5 h on ice (occupied, unheated + MoO_4^{2-}: closed circles); cytosol plus ethanol vehicle, 45 min on ice, then 45 min at 30 °C (unoccupied, heated: open squares); cytosol plus 10 nM E_2, 45 min on ice, then 45 min at 30 °C (occupied, heated: closed squares). Each cytosol sample contained 0.4 nM ER and was incubated with 3 nM ^{32}P-labeled vitERE restriction fragment for the times indicated. Free and protein-bound DNA were separated by nondenaturing polyacrylamide gel electrophoresis and bands sliced out of the gel and quantitated by liquid scintillation counting.

18 to the binding reaction did not appreciably affect the receptor binding as compared to reactions done in the absence of any carrier DNA. Similar results were obtained with poly(dI-dC)-(dI-dC). Therefore, carrier DNA was not included in ABCD assays performed with rat uterine cytosol.

RESULTS

Estrogen Receptor Binds to the ERE in the Absence of Estrogen. The ability of the ER from rat uterine cytosol to bind selectively to the ERE from the vitellogenin A2 gene was examined by the gel shift assay. Cytosol samples were heat-treated in the presence or absence of E_2 and then incubated with a ^{32}P-labeled, 55-bp restriction fragment containing either the wild-type vitERE or a restriction fragment containing 2-bp mutations (mutant ERE; sequences shown in Figure 1A). The gel shift assay was employed to visualize DNA–protein complexes. As can be seen in Figure 2 (lanes 2 and 4), a pair of shifted bands, or a doublet, is formed when the vitERE is incubated with heat-treated cytosol. Qualitatively and quantitatively the protein–DNA complexes are similar for both the estrogen-treated (lane 4) and -untreated (lane 2) cytosol. Addition of the ER712 antipeptide antisera specific for the ER to the incubation results in the quantitative shift of both bands of the doublet to a slower migrating species (lanes 3 and 5), indicating the presence of the ER in the complex. This supershift was observed whether ER712 antisera incubation with the cytosol was performed before or after the addition of DNA (data not shown). These shifted bands were not observed when the mutant ERE restriction fragment was incubated with cytosol (lanes 7–10).

Effects of Heat and Time on Receptor Binding to DNA. The dramatic increase in receptor binding to the vitERE restriction fragment after heating cytosol for 1 h at 30 °C is illustrated in Figure 3. Uterine cytosols were prepared in the presence or absence of 10 mM sodium molybdate. Cytosol samples were treated as described in Materials and Methods to yield the four forms of the receptor: (1) unoccupied, unheated + MoO_4^{2-}; (2) unoccupied, heated; (3) occupied, unheated + MoO_4^{2-}; and (4) occupied, heated. All cytosol samples were then incubated with ^{32}P-labeled vitERE restriction fragment at 4 °C for the times shown. Gel shift assays were performed to assess the extent of receptor–DNA complex formation. Heating of the cytosol resulted in an approximately 4-fold increase in receptor–DNA complex formation. The presence of E_2 was not necessary for complex formation, though it appeared to have a small effect on maximizing complex formation. Complex formation approached its maximum in 2–3 h of incubation with DNA and was stable to 18 h (the limit of the experiment). Molybdate was employed to stabilize the unheated form of the ER (Shyamala & Leonard, 1980). We did observe activation of DNA binding in cytosol samples that were not stabilized with molybdate and were incubated at 4 °C overnight; a condition known to cause receptor transformation (Hansen & Gorski, 1989). This was independent of the presence of estrogen (data not shown).

To demonstrate that the ER was binding to the vitERE palindrome in the 55-bp restriction fragment, competition studies were carried out with a synthetic oligonucleotide consisting of the vitERE flanked by *Bam*HI linkers (Figure 1B). An increasing concentration of the unlabeled vitERE oligonucleotide was added to a set concentration of ^{32}P-labeled vitERE restriction fragment and the binding reaction was initiated with a constant volume of either occupied, heated or unoccupied, heated cytosol. The extent of receptor–DNA complex formation was determined by gel shift assay. As shown in Figure 4, total complex formation was inhibited by 50% at approximately 60 and 130 nM of unlabeled vitERE oligonucleotide for the unoccupied, heated and occupied, heated ER, respectively. These results were consistent with ER binding to the vitERE palindrome in the 55-bp resriction

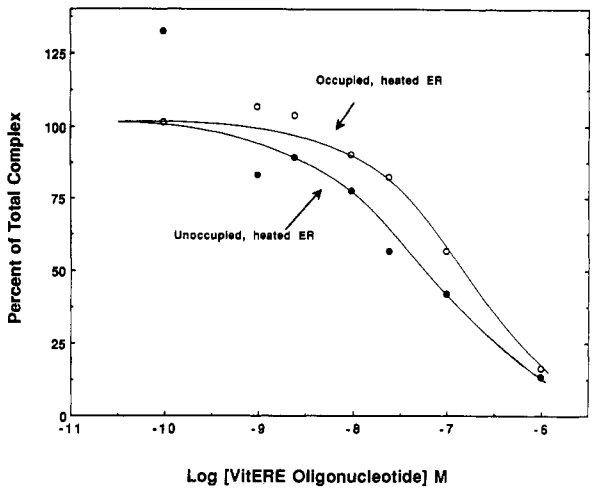

FIGURE 4: Competition with vitERE synthetic oligonucleotide. Occupied, heated cytosol (open circles) or unoccupied, heated cytosol (closed circles) containing 0.4 nM ER was incubated with 5 nM ^{32}P-labeled vitERE restriction fragment in the presence of various concentrations of a synthetic oligonucleotide of the vitERE (Figure 1B). Free and protein-bound DNA complexes were separated by nondenaturing polyacrylamide gel electrophoresis and quantitated by liquid scintillation counting.

fragment. However, since the absolute binding affinity of the ER to the 55-bp restriction fragment was not known, calculation of the affinity of the ER for the vitERE could not be made. Furthermore, the data in Figures 3 and 4 clearly demonstrate that E_2 is not required for DNA binding activity, although the reproducibility of the small E_2 effect observed required further study. The following experiments were designed to address these questions directly.

Determination of Thermodynamic Binding Parameters of the Estrogen Receptor to Synthetic Oligonucleotides. In order to determine the thermodynamic binding parameters (K_d, equilibrium dissociation constant, and B_{max}) for the ER to specific sequences of DNA and to evaluate the role of estrogen on these parameters, a more quantitative assay than gel shifts was required. An ABCD assay was employed as described in Materials and Methods. This assay had the advantages over the gel shift assay of allowing fast separation of bound complexes from free, the ER itself was directly measured, and large numbers of binding reactions could be run in a single experiment. All of these characteristics of the ABCD assay contributed to the ease and precision with which quantitative data were collected. These experiments were performed with the synthetic oligonucleotides shown in Figure 1B.

To obtain valid binding parameters it was necessary to demonstrate that the DNA–receptor binding reaction had reached equilibrium at the low concentrations of DNA and was stable at the higher concentrations of DNA employed in a binding experiment. Figure 5 represents a series of experiments in which the time required for the receptor to form a stable complex with the vitERE at 4 °C was measured. The duration of that stable complex at a low and high concentration of DNA was also determined for all four forms of the cytosolic ER. Less than 10% of the ER from cytosols prepared in the presence of molybdate bound to vitERE regardless of the presence of estrogen or the length of incubation at 4 °C (Figure 5A,C). The concentration of vitERE was raised as high as 200 nM with an 18-h incubation and still no appreciable binding was observed with the occupied, unheated + MoO_4^{2-} cytosol (data not shown). These results indicated that this form of the receptor either could not bind DNA or had an extremely low affinity for DNA. A notable observation was that heated cytosols achieve a similar maximum of binding

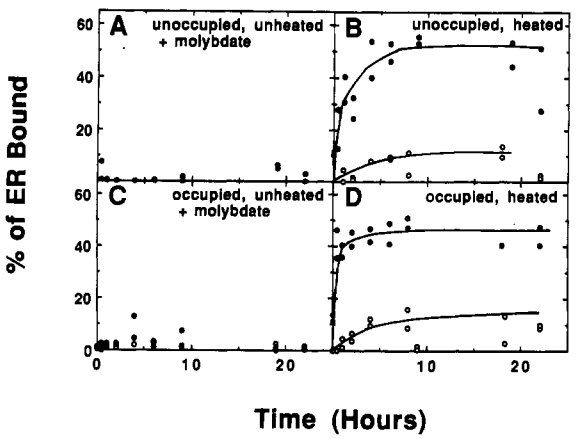

FIGURE 5: Time to stable maximum binding of the four forms of the ER to the vitERE oligonucleotide. Cytosol was prepared in the presence (A, C) or absence (B, D) of molybdate, occupied with 20 nM ^{3}H-E_2 (C, D; final [^{3}H-E_2] in the binding reaction was 4 nM), and heated for 60 min at 30 °C (B, D). Binding reactions containing either 20 nM biotin-labeled vitERE oligonucleotide (closed circles) or 2 nM biotin-labeled vitERE oligonucleotide (open circles) and 0.5–1 nM ER were incubated at 4 °C for the times indicated. Receptor–DNA complex formation was quantitated by the ABCD assay as described in Materials and Methods. ^{3}H-E_2 background was determined by addition of 200-fold excess of DES to duplicate samples.

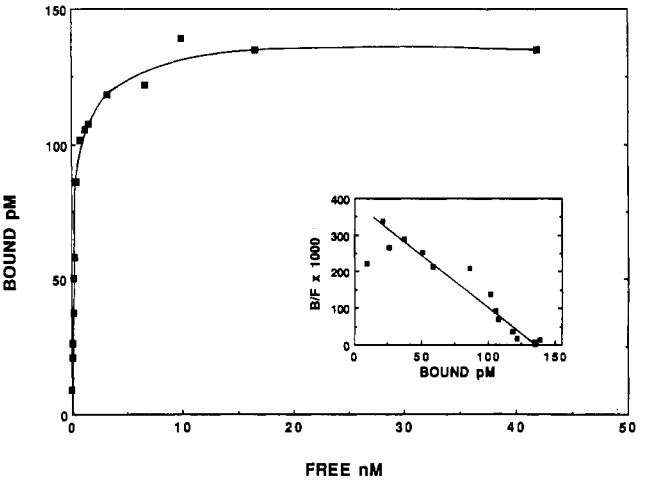

FIGURE 6: Quantitation of occupied, heated ER binding affinity for vitERE oligonucleotide. Cytosol was occupied with 40 nM ^{3}H-E_2 (final [^{3}H-E_2] in binding reaction was 4 nM) and heated at 30 °C for 1 h. Binding reactions containing 0.21 nM ER and various total concentrations (0.01–25 nM) of biotin-labeled vitERE oligonucleotide were incubated at 4 °C for 16.5 h. Receptor–DNA complex formation was quantitated by the ABCD assay as described in Materials and Methods. Nonspecific DNA binding was determined by the addition of 500 nM non-biotin-labeled vitERE oligonucleotide to duplicate binding reactions. Data are plotted as a saturation curve with the linear transformation of Scatchard (1949) shown as an inset.

regardless of the presence of E_2 (Figure 5B,D). The stable maximum binding for the heated cytosols was approached within 4–5 h for the high concentration of vitERE and within 10 h for the lower concentration. The complexes were stable for at least 18 h. On the basis of these experiments, overnight incubations of 12–18 h at 4 °C were chosen for thermodynamic binding experiments.

Saturation binding curves for the receptor binding to the vitERE oligonucleotide were generated by incubating a constant volume of cytosol with an increasing concentration of biotin-labeled oligonucleotide. Nonspecific DNA binding was subtracted from each point by inclusion of 500 nM non-biotin-labeled vitERE in duplicate binding reactions. The saturation curves with their linear transformation by the

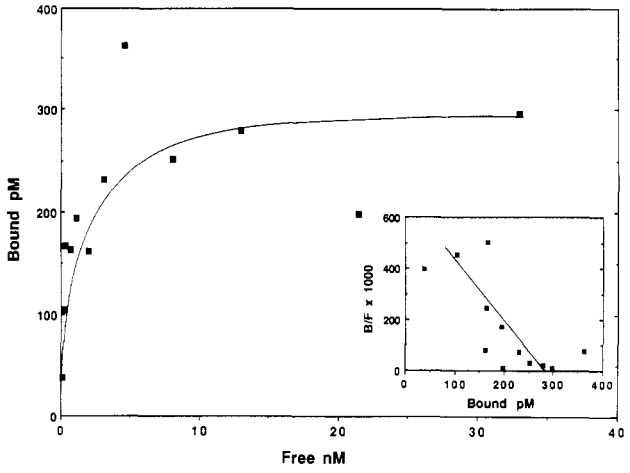

FIGURE 7: Quantitation of unoccupied, heated ER binding affinity for vitERE oligonucleotide. Experiment was performed as described in Figure 6, but with the unoccupied, heated form of the receptor. Reactions contained 0.43 nM ER and were incubated at 4 °C for 16 h.

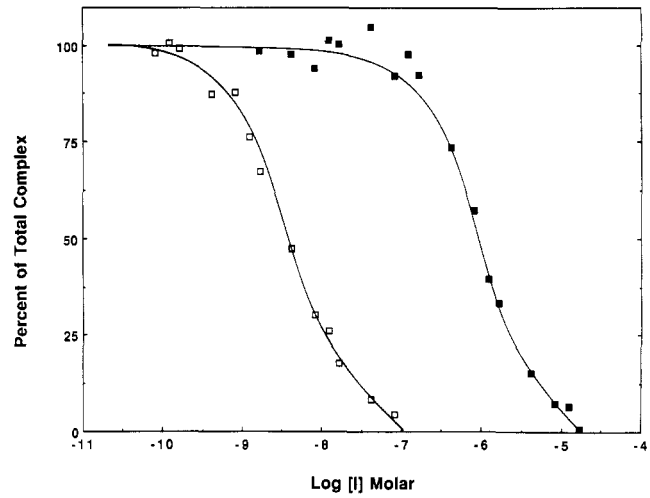

FIGURE 8: Competition of the vitERE oligonucleotide–ER complex with the mutant ERE oligonucleotide. Cytosol was occupied with 40 nM ^{3}H-E_2 (final [^{3}H-E_2] in binding assay was 4 nM) and heated at 30 °C for 1 h. Reactions containing 0.27 nM ER, 1.7 nM biotin-labeled vitERE oligonucleotide, and various concentrations of non-biotin-labeled vitERE oligonucleotide (0.08–83 nM, open squares) or mutant ERE oligonucleotide (1.7 nM–17 μM, closed squares) were incubated at 4 °C for 16 h. Receptor bound to the biotin-labeled vitERE oligonucleotide was quantitated by the ABCD assay as described in Materials and Methods. Data are plotted as percent of total bound versus log [I], where I is the unlabeled oligonucleotide. Total bound was determined as total ER bound to the biotin-labeled vitERE, less receptor bound in the presence of 100-fold excess unlabeled vitERE.

method of Scatchard (1949) are shown in Figure 6 for the occupied, heated receptor and Figure 7 for the unoccupied, heated receptor. The thermodynamic parameters (K_d and B_{max}) were estimated from the linear transformation of the data by a nonlinear least-squares method using the computer program LIGAND (McPherson, 1985; Munson & Rodbard, 1980). K_d values of 390 ± 40 pM for the occupied, heated receptor and 450 ± 170 pM for the unoccupied, heated receptor were calculated from the data shown. The B_{max} values, or total receptor capable of binding to DNA, was 67% of the total ER in the assay for both the occupied, heated receptor and the unoccupied, heated receptor. The percentage of total receptor that can bind the vitERE varied with each cytosol preparation, ranging up to 100%, although 60–80% was the value usually observed.

Figures 6 and 7 are typical of a number of experiments in which no significant effect of estrogen on the K_d or B_{max} of the receptor preparation was observed. However, the precision with which the dissociation constant was determined for the unoccupied, heated ER was considerably less than that obtained for the occupied, heated ER. We believe that this was a function of the technical difficulty of quantitating the unoccupied, heated ER by radioligand binding assay at the end of the ABCD assay. A ^{3}H-E_2 solution was added directly to the plastic econocolumns in which the unoccupied ER/biotin–oligonucleotide/streptavidin–agarose complex had been isolated. This was necessary in order to occupy and quantitate the ER in the complex; however, a large and somewhat variable amount of background due to ^{3}H-E_2 association with the plastic resulted. Analysis of data from three independent experiments gave dissociation constants for the occupied, heated ER and the unoccupied, heated ER binding to the vitERE oligonucleotide that were the same within experimental error. Evidence for cooperativity or multiple sites was not observed in the data analysis for either form of the receptor.

In addition to binding to an ERE, the receptor must be able to select that target sequence in the presence of a great excess of nontarget DNA in the nucleus. The gel shift assay did not show complex formation between the mutant ERE sequence and the ER. However, this is probably due to the gel shift assay's inability to detect lower affinity interactions when the DNA binding protein is a low percentage of the total protein, as is the case for the ER in a cytosol. The receptor has been demonstrated to bind to nonspecific DNA, albeit with low affinity (Skafar & Notides, 1985). In order to quantitate the difference in affinity of the ER for the vitERE over the mutant ERE, competition studies were conducted by using the ABCD assay. Figure 8 shows the results of a competition experiment with non-biotin-labeled vitERE or mutant ERE against biotin-labeled vitERE. The biotin-labeled vitERE was set at a constant, nonsaturating concentration of 1.7 nM while the non-biotin-labeled oligonucleotide (I) concentration was varied. A 50% inhibition (IC_{50}) of receptor binding to the biotin-labeled vitERE was observed at 990 ± 80 nM mutant ERE and 4 ± 0.3 nM vitERE. Therefore, the relative binding affinity for the mutant sequence was 250, indicating a measurable though considerably lower affinity of the receptor for this sequence. An estimate of the actual affinity of the receptor for the mutant sequence can be calculated from the following equation:

$$K_i = \frac{IC_{50}}{1 + (F/K_d)}$$

K_i is the equilibrium dissociation constant for the mutant ERE. K_d is the equilibrium dissociation constant for the vitERE, equal to 390 pM. IC_{50} is the [mutant ERE] to inhibit binding 50%, equal to 990 nM. F is the [biotin-labeled vitERE] in assay, equal to 1.7 nM. By this method a value of 185 nM for the binding constant of the receptor to the mutant sequence is obtained. The data were also analyzed by the LIGAND computer program, which gave a value of 190 ± 20 nM. The binding curves showed no evidence for cooperativity or multiple sites.

DISCUSSION

These studies clearly demonstrate that a short heat treatment of the rat uterine cytosolic ER is sufficient to activate high affinity and sequence specific DNA binding. Estrogen occupation of the receptor is not required and has no substantial effect on the character of this DNA binding activity.

Our data do not rule out a less than 2-fold effect of E_2 on ER–DNA complex formation; however, we do not believe that such a small difference in complex formation can represent the point of regulation of estrogen-responsive genes. Unexpectedly, the receptor's ability to discriminate between a target sequence (vitERE) and a mutant is also not dependent on estrogen. The 250-fold difference in the ER's affinity for specific and nonspecific DNA sequences is still below the 1000- to 10 000-fold difference that is predicted to be necessary in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989). However, our own observations suggest that changes in buffer conditions such as salt concentration may increase this discrimination (F. E. Murdoch, D. Hoeffel, and J. Gorski, unpublished results). It is also possible that DNA topology plays an important role in sequence discrimination since a recent report suggests that the ER has a higher affinity for its specific sequence when it is single stranded (Lannigan & Notides, 1989). The receptor has also been reported to prefer non-B-form DNA (Lannigan & Notides, 1989; Thomas & Kiang, 1988; Thomas & Thomas, 1989). However, the estrogen dependence of these observations has not been determined.

Our results document the lack of an effect of estrogen on the receptor's affinity for DNA in a quantitative study. While this may be surprising, the qualitative observation of unoccupied steroid hormone receptors binding to DNA has been reported. Willmann and Beato (1986) reported specific binding of the cytosolic glucocorticoid receptor to elements in the mouse mammary tumor virus (MMTV) in the absence of hormone, using DNA–cellulose and footprinting assays. Work by another group has demonstrated calf uterine cytosolic ER binding to defined DNA sequences in the absence of hormone by gel shift assay (Klein-Hitpass et al., 1989). However, this study did not pursue this observation and the authors suggested that only a minor population of the receptor, perhaps exposed to endogenous estrogens, was forming the complex with DNA. The quantitative nature of the studies in this paper have allowed us to determine that, in fact, the majority of receptor binds to vitERE with similar affinity regardless of the presence of hormone. The agreement between receptor concentration obtained by a standard ^3H-E_2 binding assay and an exchange assay (Katzenellenbogen et al., 1973) has confirmed our assumption that our cytosolic ER preparations do not contain a significant population of ER occupied with endogenous estrogens (data not shown). Results similar to those of Klein-Hitpass et al., also utilizing the gel shift assay, have recently been reported for the mouse uterine ER, but in nuclear extracts instead of cytosols (Curtis & Korach, 1990).

Even in studies in which an effect of hormone on receptor–DNA complex formation has been quantitated, review of the data reveals only small changes in the fold affinity induced by ligand. Rodriguez et al. (1989) demonstrated that hormone increased the affinity of the progesterone receptor for a defined DNA sequence by only 1.5-fold. In the case of ER binding to bulk calf thymus DNA sequences, only a 2- to 5-fold effect of estrogen on the affinity of the receptor for DNA was found (Skafar & Notides, 1985). These observations have remained controversial because they have been made in vitro, and the functional significance of hormone-independent DNA binding in vivo has not been accommodated by present models of hormone action. Work from the Beato group has suggested that the kinetics of receptor interaction with DNA may be accelerated by hormone, but the rate constants changed only 2- to 5-fold for the association and 10- to 20-fold for the dissociation rates (Schauer et al., 1989).

A study that demonstrated a strong dependence on estrogen for receptor binding to defined DNA sequences in vitro was reported by Kumar and Chambon (1988). The authors performed gel shift assays using the vitERE with whole cell extracts from MCF-7 cells or HeLa cells transfected with an ER expression vector. They showed that dramatically more protein–DNA complex was observed in the presence of estrogen. A similar study with transfected HeLa cells was performed by Martinez and Wahli (1989), who also showed a strong effect of E_2 on protein–DNA complex formation. These results are in sharp contrast to ours and those of Klein-Hitpass et al. (1989) and Curtis and Korach (1990) discussed above. It is difficult to reconcile our results with the two studies showing hormone dependence of protein–DNA complex formation. However, there are a number of technical differences including the assay conditions of time and temperature, the source of ER (the human MCF-7 cell line or human HeLa cells transfected with the human ER versus uterine preparations from rat), and preparation of whole cell extracts instead of cytosols. It is possible that nuclear proteins may participate in the formation of a receptor–DNA complex in a hormone-dependent manner. Such a complex might exhibit a much greater stability than the receptor–DNA complex alone. However, the study by Curtis and Korach discussed above employed nuclear extracts from mouse uterus and assay conditions similar to those of Kumar and Chambon, and although three ER–DNA complexes were observed, hormone dependence was not (Curtis & Korach, 1990).

The ability of the unoccupied receptor to bind DNA in vitro raises questions about the interaction of the receptor with DNA in the cell when ligand is absent. Recent work from the Evans' group suggests that the unoccupied thyroid hormone receptor can bind DNA in the nucleus (Damm et al., 1989). They were able to demonstrate that the unoccupied thyroid hormone receptor was capable of repressing the transcription of an estrogen-responsive construct by using transient expression assays in whole cells. This repression apparently was mediated by the unoccupied thyroid hormone receptor competing for binding to the ERE, which had been previously demonstrated in vitro (Glass et al., 1988). Picard et al. (1990) recently suggested that the degree to which the different receptors of the steroid/thyroid hormone family is dependent upon hormone for function varies. On the one extreme is the glucocorticoid receptor, which requires hormone for all functions from nuclear localization to enhancement of gene transcription. At the other extreme is the thyroid hormone receptor, which is localized in the nucleus, binds DNA, and can even repress gene expression in the absence of hormone. The only function that apparently requires hormone is enhancement of gene expression. These authors place the ER in a middle class on the basis of the assumption that estrogen is required for DNA binding but not for nuclear localization. Our results and those cited above provide considerable evidence that the ER is more similar to the thyroid hormone receptors since the only function that requires hormone is enhancement of gene transcription.

The validity and relevance of receptor–DNA binding affinities measured in vitro must be evaluated in the context of the concentrations observed under conditions of physiological function. Is an affinity of 400 pM for the ER binding to specific DNA, or 200 nM for nonspecific DNA, reasonable in terms of the physiological behavior of both the occupied and unoccupied receptors? Although the observed affinities are 2 orders of magnitude lower than reported for DNA binding proteins in bacteria such as the *lac* repressor (Lin & Riggs, 1975, and references therein), these affinities are consistent

with observed receptor behavior during homogenization. It is well established that the ER is localized to the nucleus in the absence of ligand (King & Greene, 1984; McClellan et al., 1984; Picard et al., 1990; Welshons & Gorski, 1986; Welshons et al., 1984). However, upon homogenization in low-salt buffer the unoccupied receptor is recovered in the cytosol whereas the occupied receptor remains tightly associated with the nucleus (Gorski et al., 1984). Assuming a radius of 5 μm for a uterine cell nucleus and 10000 receptors/cell, a nuclear concentration of 30 nM ER can be calculated. Such a high concentration of ER would drive unoccupied, as well as occupied, receptor binding to specific DNA sequences in the nucleus, assuming affinities in the range reported here. Upon dilution of unoccupied ER during homogenization with low-salt buffers, receptor–DNA complexes would be expected to dissociate, allowing unoccupied ER to be extracted into the cytosol. Extraction of the unoccupied ER into the cytosol would be dependent upon the volume of dilution with at least a 10-fold dilution predicted to be required for significant solubilization of the unoccupied ER. This is in fact quite consistent with the results of Sheridan's group on the volume of low-salt buffer required for efficient extraction of the unoccupied receptor into the cytosol (Martin & Sheridan, 1982; Sheridan et al., 1979, 1981). On the basis of this reasoning, we propose that the ER is bound to its specific DNA target sequences regardless of the hormonal status of the cell. This is consistent with the work of Sakai and Gorski (1984), which suggests that the ER in the cell is always associated with a nuclear component. Furthermore, we suggest that the tight nuclear binding observed upon occupation with ligand is not due to receptor–DNA interactions alone but must be mediated by interaction with another nuclear component.

What then is the role of hormone in the cell? The unoccupied, nontransformed ER recovered in cytosols behaves as a large complex with a sedimentation coefficient of 8 S on sucrose density gradients (Grody et al., 1982). Careful characterization of this complex by many investigators has revealed other proteins, the 90-kDa heat shock protein (hsp90) in particular, associated with the receptor (Grody et al., 1982; Redeuilh et al., 1987). One view suggests that the role of estrogen is to promote dissociation of this complex, allowing receptor to bind DNA and thereby elicit changes in gene expression (Baulieu, 1989; Picard et al., 1988). Indeed, heating the cytosolic ER in vitro does result in conversion of the 8S form of the receptor to a 4–5S form (Grody et al., 1982; Moncharmont et al., 1982; M. Fritsch and J. Gorski, unpublished observations), and this may well account for the heat activation of DNA binding we observe in vitro. However, our studies find that neither the activation of DNA binding nor transformation of the ER from an 8S to a 4–5S form is dependent upon estrogen in vitro. Furthermore, the antiestrogen monohydroxytamoxifen does promote ER binding to an ERE without activating gene transcription in whole cells (Webster et al., 1988). Therefore, the role of ligand is not merely to unmask the DNA binding domain. Although interesting roles for hsp90 in receptor transport or stabilization in the cytoplasm after synthesis have been proposed (Pratt et al., 1989), there is limited evidence that the ER is associated with hsp90 in the nucleus. We propose that the association of ER with hsp90 occurs when the normally nuclear localized ER is exposed to the cytoplasmic environment during homogenization and that this does not reflect the normal state of the unoccupied receptor in the intact nucleus. Following this reasoning, we suggest that heating actually restores the ER to its normal activity and that the ER is always associated with DNA in the nucleus. We speculate that estrogen induces the formation of a larger complex, presumably between the ER and other proteins, in the highly ordered environment of the intact nucleus. Hormone-dependent formation of such a complex would be the mechanistic basis for the tight nuclear binding observed for the occupied receptor and changes in transcriptional activity, which lead to the physiological responses to estrogens.

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^{1}H NMR Dipolar Echo Decay Spectroscopy: A Sensitive Probe of Membrane Structure†

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ABSTRACT: The structural and motional properties of membrane lipids in various physical states and macroscopic organizations are elucidated by ^{1}H NMR dipolar echo decay spectroscopy (DECODE). Multilamellar lipid dispersions in the gel ($L_{\beta'}$) and liquid-crystalline (L_α) states and a nonbilayer, hexagonal (H_{II}) phase are readily distinguished, a dynamic profile within these phases is identified, and dipolar order parameters are obtained in the fluid phases. The method is suitable for any pulsed NMR spectrometer. DECODE provides the first depth-dependent assay of lipid order that does not rely on isotopic labeling or exogenous probe.

The architecture of lipids in biological membranes is vital to cellular function. Studies of lipid organization have traditionally utilized techniques such as electron spin resonance and fluorescence polarization, which rely on the behavior of exogenous perturbing reporter molecules. Of the nonperturbing techniques, nuclear magnetic resonance (NMR)[1] is most widely used. The high abundance, sensitivity, and ubiquity of protons in lipid systems suggested a promising avenue for ^{1}H NMR. Efforts to realize this potential were largely abandoned in the 1970s, mostly due to difficulties posed by broad, largely featureless line shapes induced by dipolar couplings. Subsequent studies by ^{2}H NMR proved valuable but sacrificed broad utility because of the requirement for isotopic substitution and more sophisticated instrumentation. Ultrasonic disruption preserved the advantages of ^{1}H NMR in the liquid-crystalline state but sacrificed dipolar information.

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; CL, 1′,3′-di-*O*-(3-*sn*-phosphatidyl)-*sn*-glycerol; DECODE, dipolar echo decay spectroscopy; DFT, delayed Fourier transform spectroscopy; NMR, nuclear magnetic resonance.

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