Estrogen Receptor Binding to DNA: Affinity for Nonpalindromic Elements from the Rat Prolactin Gene†

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ABSTRACT: The estrogen receptor (ER) binds with high affinity to the nonclassical estrogen response elements (ERE) found in the rat prolactin gene. There are two putative EREs in this gene; at −1582 and −1722 upstream of the transcriptional start site. We used DNA binding assays based on the immunoprecipitation of receptor with bound DNA to quantitate the binding of ER to these two elements. ER bound each element with significantly higher affinity than it bound to nonspecific DNA, but with 10−100-fold less affinity than that for the classical ERE sequence derived from the vitellogenin A2 gene. These comparisons rank the prolactin gene sequences as weak EREs. We also observed a 1:1 ratio of ER to ERE in the bound complexes, indicating that these high-affinity interactions were not dependent upon an ER homodimer. These data support the role of these sequences in mediating estrogen regulation of the prolactin gene.

Estrogens bind with high affinity to the estrogen receptor (ER)[1] protein localized in the cell nucleus. ER contains a DNA binding domain capable of binding to specific, *cis*-acting sequences of DNA within target genes, termed estrogen response elements (ERE). Various investigators have studied the difference in affinity between ER binding to the ERE and ER binding to bulk DNA, required to accomplish occupancy of the ERE. In order for the ER to distinguish an ERE from the mass of nontarget DNA sequences, an affinity difference of three to four orders of magnitude is predicted by theoretical calculations, given the concentrations of ER and DNA in the mammalian nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989). Quantitative binding studies from this laboratory (Murdoch et al., 1990, 1991) demonstrated this degree of discrimination. Our experiments compared ER binding to the perfectly palindromic ERE from the *Xenopus* vitellegenin A2 gene (AGGTCA half-site with 3-bp spacer, VitERE), with the 2-base-pair (bp) mutation of this sequence that fails to mediate an estrogen response (Klock et al., 1987; MutantERE), and with nontarget DNA in the form of the pUC18 plasmid. Little quantitative information is available about the interaction of ER with EREs that deviate from the classical palindromic sequence. This is despite the fact that nonclassical ERE sequences predominate in endogenous genes. The contribution of DNA structure to the binding discrimination of ER to nonclassical EREs has also received little attention.

The rat prolactin gene (Prl) is regulated by estrogens (Shull & Gorski, 1986; Maurer, 1981; Lieberman et al., 1978, 1981) and contains two nonclassical sequences that have been proposed to act as EREs. A sequence at −1582 bp upstream of the transcriptional start site (PrlERE-2) deviates from the classical half-site of AGGTCA by two bases in the 5′ half-site and by one base in the 3′ half-site. Prl gene fragments containing PrlERE-2 have been shown to mediate estrogen responses in transfection assays (Nelson et al., 1986; Maurer & Notides, 1987; Somasekhar & Gorski, 1988a,b) A second putative ERE (PrlERE-1) was identified at −1722 of Prl by sequence analysis and DNA binding studies (Lannigan & Notides, 1989). PrlERE-1 has a perfect 5′ half-site, but the 3′ half-site deviates from the classical by four out of six bases. The PrlEREs also differ from the VitERE in that they are found over 1500 bp upstream of the transcriptional start site and are 140 bp apart (Maurer et al., 1981). The single VitERE is only 330 bp upstream in the A2 gene (Walker et al., 1984). Naturally occurring, nonclassical EREs in genes such as *Xenopus* vitellogenin B1, as well as half-sites in genes such as chicken ovalbumin, have been observed to synergize in response to estrogens (Martinez & Wahli, 1989; Tora et al., 1988; Kato et al., 1992). It is therefore reasonable to speculate that the PrlEREs might synergize *in vivo*, but this has not been studied to date. Biologically, the responses of the vitellogenin A2 and Prl genes to estrogen are also quite different. Vitellogenin A2 is dramatically up-regulated 1000-fold from a very low basal level of transcription within minutes of estrogen exposure (Brock & Shapiro, 1983). In contrast, Prl displays a significant basal level of transcription that is up-regulated only 3−6-fold after several hours of estrogen exposure (Shull & Gorski, 1985).

Several mechanisms for the different qualities of estrogen-regulated gene transcription observed with the vitellogenin

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[1] Abbreviations: bp, base pair; ER, estrogen receptor; ERE, estrogen response element; VitERE, vitellogenin ERE; Prl, prolactin; ABCD, avidin−biotin complex with DNA; DRIP, DNA−receptor immunoprecipitation; RIPD, receptor-immunoprecipitation DNA; RBA, relative binding affinities, K_d, equilibirium dissociation constant.

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A2 and Prl genes can be proposed. First, the PrlEREs, individually or together, may simply fail to bind ER with the same degree of high affinity observed for VitERE. The data in this report address this mechanism. Second, estrogen-induced changes in chromatin structure may be rate-limiting for the Prl transcriptional response. Cullen et al. (1993) have reported estrogen-induced changes in the Prl chromatin that support a looping model. Finally, the topology of the PrlEREs may play a role in regulating the timing and strength of the transcriptional response by affecting ER binding.

The data in this report demonstrate high affinity interactions between ER and the double-stranded forms of the PrlEREs. ER bound to the double-stranded PrlEREs with significantly higher affinity than to nonproductive sites, but with 10−100-fold less affinity than to the VitERE. ER bound to PrlERE-1 with a 10-fold higher affinity than PrlERE-2. We have previously reported that a single ER molecule is capable of high affinity interaction with VitERE without homodimerization (Furlow et al., 1993). We observed the same 1:1 stoichiometry of ER binding to the PrlEREs. Given that the 3′ half-site of PrlERE-1 deviates considerably from the perfectly palindromic classical ERE, an ER heterodimer could accomplish the specificity and relatively high affinity that we observe. We also observed severe interference of ER binding to the PrlEREs (but not VitERE) in the presence of cytosolic proteins prepared from rat uterus. Our data suggest that the interaction of ER with the PrlEREs, but not VitERE, is influenced by the presence of other proteins in cytosols *in vitro*.

MATERIALS AND METHODS

Estrogen Receptor Preparation. Cytosols were prepared from 19-day-old, female Sprague-Dawley rats as previously described (Murdoch et al., 1990), with the exception that all steps were performed in TDG (10 mM Tris-HCl, pH 7.5, at 25 °C, 1 mM dithiothreitol, 10% v/v glycerol). 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 as previously described (Murdoch et al., 1990).

Occupation and Heating of Cytosols. The occupied, heated form of the ER was used in all experiments. Cytosol was incubated with 10−40 nM [^{3}H]17β-estradiol (^{3}H-E$_2$, 90−110 Ci/mmol, New England Nuclear) in ABCD assays or unlabeled 17β-estradiol in other assays, for at least 2 h on ice, and then heated at 25 or 30 °C for 60 min and returned to ice.

Single-Stranded Synthetic Oligonucleotides. Synthetic oligonucleotides were obtained from the University of Wisconsin, Department of Biochemistry, Oligonucleotide Synthesis Facility. In addition to the core palindromic sequence, each oligonucleotide was designed with *Bam*HI linkers. Oligonucleotides were designed as complimentary strands of 21 or 31 bases, with 5′ overhangs for labeling with either biotin-labeled nucleotide, [^{32}P]nucleotide, or [^{35}S]-nucleotide using the Klenow fragment of DNA polymerase as previously described (Murdoch et al., 1990, 1991). The sequences of the oligonucleotides, without ends filled in, are shown in Figure 1. Complementary single strands were mixed and hybridized to produce double-stranded elements for labeling. Biotin-labeled oligonucleotides (25 bp) were purified by electrophoresis on 20% polyacrylamide gels. The

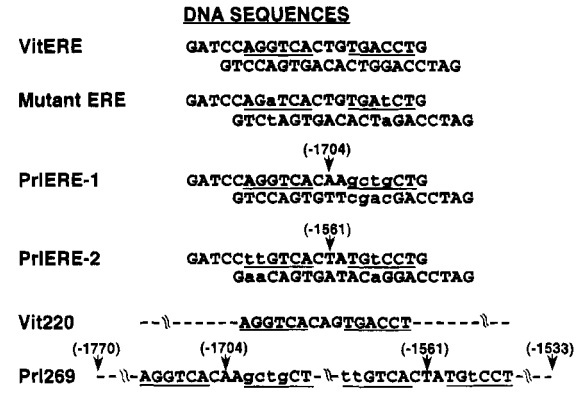

FIGURE 1: DNA sequences. Oligonucleotides and restriction fragments used in DNA binding assays are shown. The oligonucleotides are shown in the double-stranded form prior to labeling. The numbers correspond to the location of the base indicated relative to the transcriptional start site in the rat Prl gene. The half-sites for the EREs are underlined, and deviations from the VitERE sequences are shown in lowercase letters.

^{32}P-labeled or ^{35}S-labeled oligonucleotides (25 or 35 bp) were purified on NENsorb columns (New England Nuclear). Oligonucleotide solutions were quantitated spectrophotometrically at 260 nm. The extinction coefficient for each oligonucleotide was calculated 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). Unlabeled double-stranded oligonucleotides were used as competitors against labeled, double-stranded oligonucleotides in the DNA binding assays.

Plasmid DNA. Plasmid was prepared by the alkaline-SDS lysis method and banded twice on CsCl gradients by standard protocols (Maniatis et al., 1982). DNA was quantitated by absorbance at 260 nm using an extinction coefficient of 13 200 M/cm. This is an average extinction coefficient for a single base pair; thus the concentration of base pairs was estimated by this method. The pBLCAT-ERE plasmid construct was generously provided by Dr. Gerd Klock (Klock et al., 1987). A 220 bp insert containing the VitERE was released from the pBLCAT-ERE plasmid by restriction enzyme digestion and labeled with [^{32}P]nucleotide using the Klenow fragment of DNA polymerase, and the labeled fragment was purified by gel electrophoresis. A 269 bp restriction fragment containing the −1770 to −1533 region of the rat prolactin gene (i.e., both PrlEREs) was released from the Prl269 plasmid (F. E. Murdoch and J. Gorski, unpublished results) by restriction enzyme digestion, purified by HPLC on a Gen-Pak Fax column (Waters), and labeled as above.

Avidin−Biotin Complex with DNA (ABCD) Assays. Occupied, heated cytosols were incubated with biotin-labeled VitERE oligonucleotide in TDG plus 100 mM KCl at 4 °C for 16−18 h in a final volume of 100 μL. Various concentrations of unlabeled oligonucleotide were included in this incubation step for competition assays. Twenty microliters of a 50% streptavidin-agarose slurry in TDG containing 1 mg/mL bovine serum albumin was added to each sample at the end of the binding reaction. Samples were rotated on an orbital shaker at 100 rpm for 1 h at 4 °C to absorb biotin−oligonucleotide on the resin. Filtration of the resin and quantitation of bound ER was performed as previously described (Murdoch et al., 1990). Background

^{3}H-E_2 binding was determined in duplicate tubes without DNA and was subtracted from total ER binding.

DNA–Receptor Immunoprecipitation (DRIP) Assays. These assays were conducted as previously described with the following modifications (Furlow et al., 1993). Briefly, cytosols were incubated with affinity purified antisera against ER (Furlow et al., 1990; ER712, 1:20 final dilution) or rabbit IgG as control, protein-A agaraose, and 10 nM E_2 in TDG at 4 °C for a minimum of 2 h. This step allowed the ER to be occupied with E_2 and to form a complex with the antibody on protein-A agarose. This reaction mix was then heated at 25 °C for 60 min to transform the ER to its DNA binding form. An aliquot of this reaction mix was used as the source of ER for incubation with ^{35}S-labeled VitERE in TDG plus 100 mM KCl at 4 °C overnight (15–20 h). Various concentrations of unlabeled oligonucleotide were included in this incubation for competition assays. The ER–^{35}S-VitERE complex on protein-A agarose was collected by filtration on econocolumns (Bio-Rad) under slow vacuum, and the complex was washed and quantitated by liquid scintillation counting.

Receptor Immunoprecipitation (RIPD) Assays. This was a modification of the DRIP assay protocol in which the immunoprecipitated ER was washed prior to incubation with DNA in order to reduce the concentration of cytosolic proteins in the DNA binding assay. Cytosols were incubated at 4 °C for 2–4 h with the same concentrations of affinity-purified antibody against ER or rabbit IgG as control, protein-A agarose beads, and salts as for the DRIP assay described above. The heat transformation of ER was also performed as in the DRIP protocol. However, before incubation with DNA, the agarose beads were collected by centrifugation and washed once with TDG, once with 20 mM Tris-HCl (pH 7.5 at 25 °C) containing 0.1% (v/v) Tween-20, and finally with TDG, all at 4 °C. The beads were resuspended in TDG containing 0.3 mg/mL bovine serum albumin and kept at 4 °C. These ER–antibody–protein-A beads were then incubated with DNA in reactions that exactly matched the DRIP assays and were processed the same. We have termed this DRIP assay modification as the RIPD assay (receptor immunoprecipitation, DNA).

Gel Shift Assays. Protein–DNA binding reactions were performed in TDG buffer containing 150 mM KCl, 0.2 mg/mL poly[dI-dC]-[dI-dC], and 0.3 mg/mL bovine serum albumin. Each reaction included 10–20 μg of cytosolic protein and 10 nM ^{32}P-labeled DNA molecule as indicated in the legend for Figure 5. Incubations were at 4 °C overnight (at least 16 h). Complexes were separated on 4% polyacrylamide gels in 0.5× TBE buffer at 4 °C and visualized by autoradiography.

Data Analysis. Equilibrium dissociation constants for ER binding to DNA were estimated by a nonlinear least-squares method using the computer program LIGAND (Munson & Rodbard, 1980; McPherson, 1985).

RESULTS

ER Binds to the Prl EREs with High Affinity. We determined the binding affinity of the putative PrlEREs for the rat uterine ER by a competition assay against the VitERE. The DNA ligands used in these studies are shown in Figure 1. Each of the two 21 bp oligonucleotides derived from the putative EREs of the Prl gene was used as an unlabeled competitor against ^{35}S-labeled VitERE in the RIPD assay. The inhibition curves for PrlERE-1, PrlERE-2, and VitERE are shown in Figure 2. In competition experiments we used unlabeled VitERE as the internal control for the determination of relative binding affinities (RBA values). The RBA was calculated as

$$\text{RBA} = \frac{K_d \text{ for test oligonucleotide}}{K_d \text{ for VitERE oligonucleotide}}$$

where K_d is the equilibrium dissociation constant for ER binding. In our experience, the experimental determination of the K_d for ER-oligonucleotide binding can vary between experiments. The RBA values normalize the data for comparison of binding affinities between experiments.

The data shown in Figure 2 yielded RBA values of 42 for the PrlERE-1 and 358 for the PrlERE-2 compared to ER binding to the VitERE. This indicates that ER bound to the PrlEREs with lower affinity than to VitERE, and PrlERE-1 bound ER with higher affinity than did PrlERE-2. These data are summarized in Table 1, where all the quantitative data from the studies in this report are tabulated. Also shown

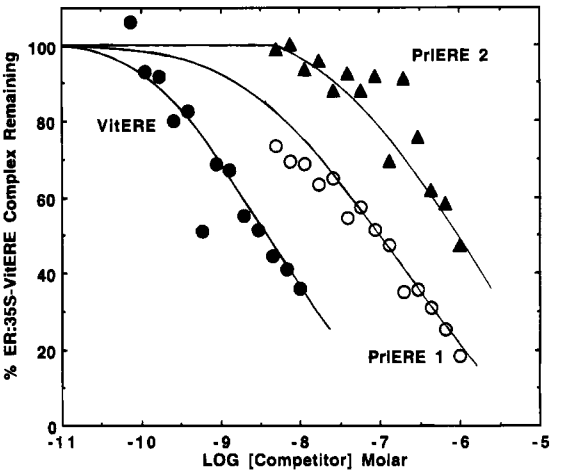

FIGURE 2: RIPD assay: competition against [^{35}S]VitERE for ER binding. Rat uterine cytosol was occupied with E_2 at 4 °C and then heat-transformed at 25 °C for 1 h. The ER was immunoprecipitated and incubated with a subsaturating concentration of ^{35}S-labeled VitERE35 (1 nM) and a range of unlabeled 21 bp oligonucleotide concentrations at 4 °C. Final ER concentration in the assay was 166 pM. ER bound to [^{35}S]labeled VitERE35 was isolated, and data were analyzed by the RIPD assay method as described in Materials and Methods. Each inhibition curve was replicated at least two times.

Table 1: Relative Binding Affinities of Estrogen Receptor for Prolactin DNA

DNA ligand	data source	type of assay	K_d (nM)	RBA to VitERE
Mutant ERE	Murdoch (1991)	ABCD		2600
pUC18 plasmid	Murdoch (1991)	ABCD		190000
VitERE	Figure 2	RIPD	1.7 ± 0.24	1
PrlERE-1	Figure 2	RIPD	70.2 ± 22	42
PrlERE-2	FIgure 2	RIPD	602 ± 444	358
VitERE	Figure 3	DRIP	1.2 ± 0.07	1
PrlERE-1	Figure 3	DRIP	222 ± 40	180
PrlERE-2	Figure 3	DRIP	1730 ± 490	1406
VitERE	Figure 4	ABCD	2.3 ± 0.3	1
PrlERE-1	Figure 4	ABCD	350 ± 29	152
PrlERE-2	Figure 4	ABCD	7382 ± 720	3196

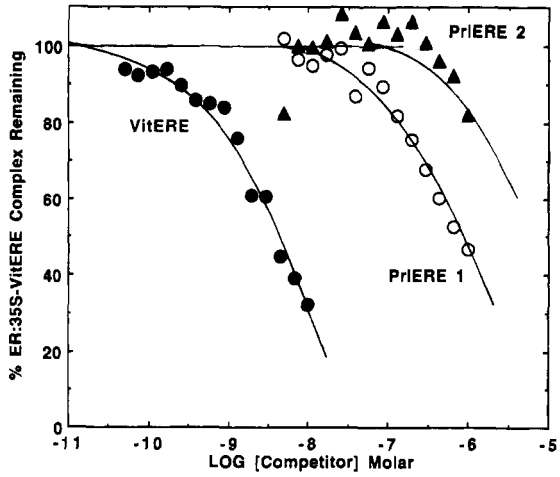

FIGURE 3: DRIP assay: competition against [^{35}S]VitERE for ER binding. This experiment was matched with the experiment shown in Figure 2. The same reagents were used, but the DRIP assay method was used (i.e., ER was not immunoprecipitated prior to the incubations with DNA). Final ER concentration in the assay was 212 pM. Each inhibition curve was replicated at least three times.

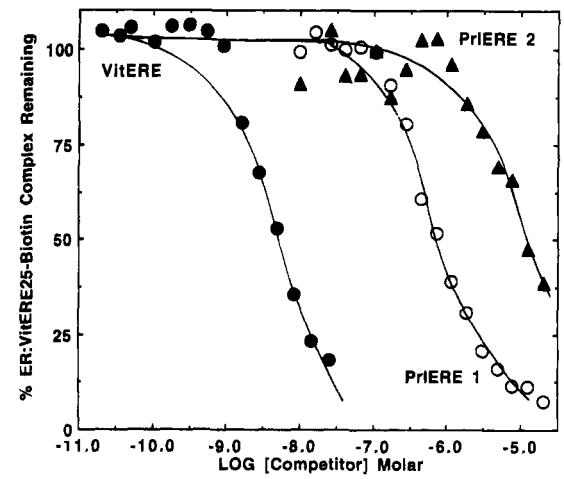

FIGURE 4: ABCD assay: competition against biotin-VitERE for ER binding. Rat uterine cytosol (2.4 nM ER) was occupied with ^{3}H-E_2 at 4 °C for 6.5 h and heat-transformed at 30 °C for 1 h. This cytosol was incubated with a subsaturating concentration of biotin-labeled VitERE (1 nM) and a range of unlabeled 21 bp oligonucleotide concentrations at 4 °C. Final concentration of ER in the assay was 0.49 nM with 8 nM ^{3}H-E_2. ER bound to biotin-VitERE was isolated, and data were analyzed as described in Materials and Methods. Each inhibition curve was replicated at least three times.

are the RBA values for a VitERE mutant that does not mediate an estrogen response (Klock et al., 1987) and for nonspecific DNA in the form of the pUC-18 plasmid. These data were previously reported by our laboratory and, although determined by a different assay method (ABCD), they illustrate the tremendous difference between ER binding to VitERE or nonspecific DNA (Murdoch et al., 1991). One of our major points in this previous report was that a functional ERE must display 1 000−10 000-fold higher affinity than nontarget DNA for the ER. The RBA values for both of the PrlERE oligonucleotides in the present study indicate a higher affinity than that expected for nontarget DNA. The comparison of ER binding to the PrlEREs with ER binding to VitERE leads us to the conclusion that the PrlEREs are relatively high affinity binding sites for ER.

Cytosolic Proteins Interfere with ER Binding to the Prl EREs. Figure 3 shows the results of competition assays with unlabeled oligonucleotides against a 35 bp ^{35}S-labeled VitERE by the DRIP protocol in which the whole cytosol is used as a source of ER. The experiment shown was conducted with the same reagents used to produce the data shown in Figure 2. The RBA values obtained for ER binding the PrlEREs are dramatically different between Figures 2 and 3. The RBA values obtained in the DRIP assay (Figure 3) were 180 for the PrlERE-1 and 1406 for the PrlERE-2. The difference between the two assay methods was the decreased concentration of cytosolic proteins in the RIPD assay. This was accomplished by incubating the protein-A agarose beads with cytosol and antibody to ER and then washing the beads, prior to incubation with DNA. As described in Materials and Methods, both the DRIP and RIPD assays were set up such that the ER was bound to antibody on the protein-A agarose during the incubation with DNA. The significant increases in the RBA values for the PrlEREs, obtained in the DRIP assay, were not due to a major change in the affinity of ER for VitERE. The calculated K_d for VitERE by the DRIP assay was 1.2 ± 0.07 nM versus 1.7 ± 0.24 nM by the RIPD assay (Table 1). This was only a 0.7-fold difference, whereas the fold differences were 3.2 for PrlERE-1 and 2.9 for PrlERE-2. The increase in PrlERE RBA values in the DRIP assay represented a decrease in the apparent affinity for ER. Therefore, the presence of cytosolic proteins interfered with the binding of ER to the PrlEREs, but not to the VitERE.

We obtained similar results using the ABCD assay in which the ER was radioactively labeled with ^{3}H-E_2 (Figure 4). This assay used whole cytosol as the source of ER. Each of the unlabeled 21 bp oligonucleotides was used as a competitor against biotin-labeled VitERE. Quantitative analysis of these data yielded K_d values of 2.3, 350, and 7382 nM for VitERE, PrlERE-1, and PrlERE-2, respectively. The RBA values were 152 for PrlERE-1 and 3196 for PrlERE-2. These values are consistent with those obtained by the DRIP assay as shown in Figure 3. The RBA values for PrlERE-2 did vary 2−3-fold between replicate experiments. The precise determination of this RBA value was difficult due to the very low apparent affinity for this site in the presence of cytosolic proteins. Comparison of RBA values for the PrlEREs by ABCD assay with the RBA value for MutERE by ABCD assay (Table 1) support the conclusion that PrlERE-1 binds ER with high affinity. However, by ABCD assay PrlERE-2 appears to be equivalent in affinity for ER with the MutERE which is a nonresponsive sequence. The data in Figures 2, 3, and 4 did not allow us to characterize the interaction of cytosolic proteins with Prl DNA sequences nor potential interactions between the two PrlEREs.

Binding of Cytosolic Proteins to Prl DNA Sequences. We performed gel shift assays to qualitatively assess the interaction of the two PrlEREs and the pattern of complexes formed with ER and cytosolic proteins. We used a 269 bp restriction fragment from −1770 to −1533 of the Prl gene (Figure 1). This fragment contains both of the putative PrlEREs. Figure 1 also shows a 220 bp restriction fragment of the pBLCAT-ERE vector that contains one copy of the *Xenopus* VitERE, which we used as a positive control (Klock et al., 1987). Figure 5 shows an autoradiograph of a gel shift experiment in which ^{32}P-labeled DNA (the VitERE 25-bp oligonucle-

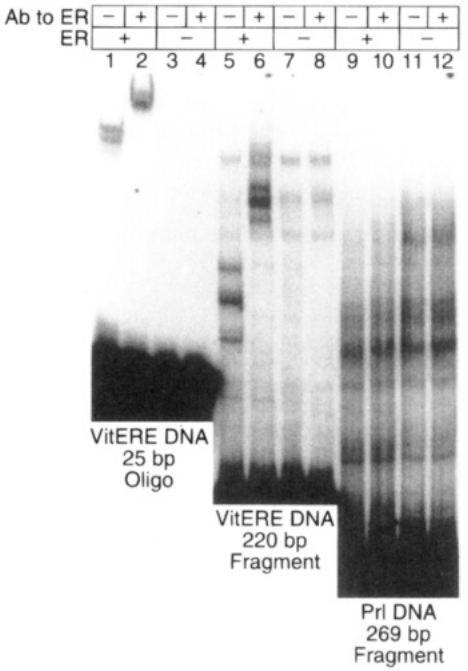

FIGURE 5: Gel shift assay of cytosolic proteins binding DNA. The autoradiograph of the gel is shown. The reactions loaded into lanes 1−4 contained 10 nM ^{32}P-labeled VitERE 25 bp oligonucleotide, lanes 5−8 contained 10 nM ^{32}P-labeled VitERE 220 bp restriction fragment, and lanes 9−12 contained 10 nM ^{32}P-labeled Prl 269 bp restriction fragment. Reactions in lanes 1, 2, 5, 6, 9, and 10 contained 9.5 μg of protein from an ER-containing rat uterine cytosol. Reactions in lanes 3, 4, 7, 8, 11, and 12 contained 10.5 μg of protein from an ER-depleted rat uterine cytosol. Antisera specific for ER (Furlow et al., 1990; ER-712) were included at a final dilution of 1:133 in the indicated reactions. Reactions for each ^{32}P-labeled DNA probe without cytosolic protein did not produce retarded bands (data not shown). The data shown for each reaction are representative of at least three independent experiments.

otide, the VitERE 220 bp fragment, or the 269 bp Prl gene fragment) was incubated with rat uterine cytosols. Lanes 1, 5, and 9 show the pattern of protein−DNA complexes obtained with each of the DNA probes and an ER-containing rat uterine cytosol.

Three methods were used to identify complexes that contained ER. First, an antibody (ER-712) that specifically recognizes the ER was added to the reaction in lanes 2, 6, and 10 (Furlow et al., 1990). The doublet of bands observed with the VitERE oligonucleotide (lane 1) was quantitatively supershifted by antibody (lane 2) as previously reported, confirming the presence of ER in these complexes (Murdoch et al., 1990). The three bands observed with the VitERE 220 bp fragment (lane 5) were quantitatively supershifted by antibody (lane 6), indicating the presence of ER in these complexes. These data also validated the utility of this gel shift system with DNA restriction fragments. In contrast, the pattern of shifted bands observed with the 269 bp Prl fragment was not altered by the addition of antibody (lanes 9 and 10). This result suggested either that ER was not present in any of the Prl269−protein complexes or that antibody did not have access to ER in these complexes. Second, to examine the latter possibility, we prepared a rat uterine cytosol that was depleted of ER. This was accomplished by incubation of freshly dissected uteri with 10 nM 17β-estradiol for 1 h prior to homogenization and cytosol preparation. Under these conditions the ER was occupied and remained tightly associated with the nuclear pellet during centrifugation and was thus depleted from the cytosol. Depletion of ER from the cytosol was confirmed by a ^{3}H-E_2 exchange assay (Katzenellenbogen et al., 1973; data not shown). This preparation of cytosol was used in lanes 3, 4, 7, 8, 11, and 12. ER containing complexes with VitERE 25 bp oligonucleotide and the VitERE 220 bp fragment were clearly depleted, as seen in lanes 3, 4, 7, and 8. However, none of the complexes with the 269 bp Prl fragment were depleted (lanes 11 and 12). Third, we conducted competition experiments with the VitERE or PrlEREs against the ^{32}P-labeled VitERE oligonucleotide complexes seen in lane 1 of Figure 5 (data not shown). A 5-fold molar excess of the VitERE containing plasmid completely competed the [^{32}P]-VitERE-ER doublet band, whereas competition was not complete with a 200-fold molar excess of the PrlEREs containing plasmid. A 10-fold molar excess of unlabeled VitERE oligonucleotide completely competed the [^{32}P]-VitERE-ER doublet band, whereas a 100-fold molar excess of either PrlERE-1 or PrlERE-2 oligonucleotide gave no competition. We conclude that ER can form a specific complex with the VitERE 220 bp fragment, but not the 269 bp Prl fragment under these experimental conditions. We did observe a number of reproducible protein−DNA complexes with Prl, which is consistent with the possibility that cytosolic proteins interfered with ER binding to the putative PrlEREs. These observations are consistent with the data in Figures 2 and 3.

We did observe ER binding to the Prl269 fragment upon depletion of the cytosolic proteins. We wanted to use the Prl269 fragment in the RIPD assay in order to address the possible interaction of the two PrlEREs. Unfortunately, we observed very high background binding of the DNA restriction fragments to the sepharose beads and were unable to obtain quantitative data. The DRIP and RIPD assays were developed to obtain highly quantitative data and employ a number of very short washes to separate bound and free DNA ligand. A qualitative form of this immunoprecipitation assay approach was used for progesterone receptor (Edwards et al., 1989) and adapted for ER (Furlow et al., 1993). In our modification of the assay, ER was partially purified from cytosol by ammonium sulfate precipitation, incubated with radiolabeled DNA restriction fragments, antibody, and then immunoprecipitated with Pansorbin cells. The cells were washed extensively, and the radiolabeled DNA was extracted and analyzed by agarose electrophoresis. We found, using this method, that ER preferentially bound to the Prl 269 bp restriction fragment as compared with the plasmid DNA restriction fragment (data not shown). However, due to the unknown amount of dissociation during the extensive washes, quantitative information was not obtained and interactions between EREs could not be assessed. These data demonstrate the ability of ER to specifically bind to one or both of the PrlEREs in the presence of nonspecific DNA.

One ER Molecule Is Bound per ERE. The DRIP and RIPD assays allowed the number of molecules of ER bound to each molecule of ERE to be determined directly. The B_{max} parameters derived from competition curves shown in Figures 2 and 3 and additional binding experiments (curves not shown) gave the molar concentration of DNA bound to ER in the immunoprecipitated complexes under conditions of saturating DNA. We performed parallel immunoprecipitation reactions containing ^{3}H-E_2 in order to measure the molar concentration of ^{3}H-E_2 in the immunoprecipitated

Table 2: Molar Ratio of Estrogen Binding to EREs in ER−DNA Complexes[a]

DNA ligand	assay type	E2 bound (pM ± SEM)	DNA bound (pM) (B_{max} ± SE)	ER/DNA ratio in complex	mean ratio ± SE
VitERE	DRIP	284.0 ± 11.8	302.0 ± 13.6	0.94 ± 0.06	
VitERE	DRIP	581.0 ± 7.1	459.0 ± 17.0	1.27 ± 0.02	1.01 ± 0.06
VitERE	RIPD	323.0 ± 30.2	262.0 ± 23.8	1.23 ± 0.16	
VitERE	RIPD	323.0 ± 30.2	283.0 ± 35.0	1.14 ± 0.15	
VitERE	RIPD	323.0 ± 30.2	252.0 ± 30.0	1.28 ± 0.17	1.22 ± 0.28
PrlERE-1	DRIP	212.0 ± 15.8	194.0 ± 11.0	1.09 ± 0.12	
PrlERE-1	RIPD	166.0 ± 27.0	169.0 ± 12.0	0.98 ± 0.23	
PrlERE-2	DRIP	160.0 ± 13.5	260.0 ± 2.0	0.62 ± 0.07	
PrlERE-2	RIPD	166.0 ± 27.0	146.0 ± 54.0	1.14 ± 0.26	

[a] The B_{max} values for each DNA ligand binding to ER were estimated using the LIGAND computer program from binding curves in Figures 2 and 3 and additional binding curves not shown. The B_{max} value represents the concentration of DNA ligand in the immunoprecipitated complex at saturation. The concentration of E_2 bound was determined by performing immunoprecipitation reactions containing ^3H-E_2 in parallel with the DNA-binding immunoprecipitation reactions (RIPD or DRIP). Each ER protein contains a single site for ^3H-E_2 binding; therefore, the concentration of E_2 bound represents the concentration of ER in the immunoprecipitated complex. SE: standard error from the nonlinear least-squares fit of the data to the binding curve. SEM: standard error of the mean.

complexes. Since 1 mol of ^3H-E_2 binds 1 mol of ER protein, this gave the maximum molar concentration of ER that was available to be in the complex with DNA. These data and their associated errors are summarized in Table 2. The molar ratio of ER to DNA at saturation in the complex for each type of DNA is calculated. We observed an ER to DNA molar ratio between 0.62 and 1.28 for any individual experiment regardless of DNA ligand. These data are most consistent with a model in which one molecule of ER is bound per ERE. This model predicts a ratio of 1.0. The slightly higher ratios we observed may be due to the presence of a small population of ER capable of binding estrogen and antibody, but not DNA. The 0.62 ratio observed in one experimental set may be due to an underestimation of ER concentration. These data do not support a model of an ER homodimer binding to each ERE, which predicts a minimum ratio of 2.0.

DISCUSSION

The studies in this report provide quantitative data on the thermodynamic binding parameters of ER with the nonpalindromic PrlEREs. We observed a specific, high affinity binding of ER with each of the putative PrlEREs. Viewed in the context of ER binding affinities for the classical ERE, a nonresponsive mutant ERE, and nontarget plasmid DNA, the PrlEREs rank as weak EREs. ER displayed a relative affinity for PrlERE-1 of 42-fold less than for VitERE and for PrlERE-2 of 358-fold less than for VitERE.

We extrapolate a three orders of magnitude difference in the affinity of ER for the PrlERE-1 over plasmid DNA based on our earlier work with VitERE (Murdoch et al., 1991). That report measured a RBA of 190 000 for ER binding plasmid DNA compared to VitERE. We estimate the fold difference in affinity of ER for PrlERE-1 over plasmid DNA by dividing the RBA of 190 000 by the RBA for PrlERE-1 of 42 to give a value of 4524. This is an extrapolated value based on comparison of independently collected sets of data using different assays. However, these data suggest that the PrlERE-1 has the degree of specificity we expect for an independently functional ERE (Murdoch et al., 1991). Experiments to test the ability of PrlERE-1 to independently activate transcription in response to estrogen have not been reported. If transcriptional activation is simply correlated to the ER's binding affinity for an element in a simple reporter gene construct, then we predict that PrlERE-1 will be transcriptionally active. The case with PrlERE-2 is more complex. A similar calculation extrapolates this element to have less than three orders of magnitude binding affinity discrimination over plasmid DNA, and therefore the PrlERE-2 may not have sufficient specificity to successfully compete for ER binding in the nucleus. However, work from several laboratories strongly supports the role of PrlERE-2 in estrogen-induced transcription (Nelson et al., 1986; Maurer & Notides, 1987; Somasekhar & Gorski, 1988a,b; Waterman et al., 1988). In those experiments, some Prl sequence flanking the PrlERE-2 seemed to be required, indicating that this element may not function independently (Day & Maurer, 1989; Maurer & Notides, 1987).

Our experimental goal was to measure the inherent affinity of the ER for each of the PrlEREs individually as small oligonucleotides and together in the context of a 269 bp restriction fragment from the Prl gene. Our experiments were complicated by the presence of nonspecific DNA-binding proteins in our ER preparations. One of the experimental advantages of ER is that it can be isolated in a cytosolic preparation, generally free of the high concentrations of competing DNA-binding proteins found in a nuclear extract. However, cytosols do contain some DNA-binding proteins in addition to the ER. It is possible that our cytosolic preparations of ER simply contained large concentrations of a fairly nonspecific DNA-binding protein. The observation that these cytosolic proteins preferentially interfered with ER binding to Prl gene sequences, but not ER binding to the VitERE oligonucleotide or the Vit220 DNA restriction fragment, suggests some specificity that warrants future studies. We overcame the problems with the interfering cytsolic proteins by partially purifying the ER by immunoprecipitation. On the basis of the data shown here, we do not draw specific conclusions about the importance of the interfering cytosolic proteins in ER function. Rather, we think the importance of these data is in highlighting the complications of performing DNA-binding assays, particularly by the gel shift method, with cellular extracts.

We measure a 1:1 stoichiometric relationship between ER and both VitERE and PrlERE oligonucleotides in binding complexes. We have previously reported this observation with VitERE oligonucleotides (Furlow et al., 1993). This observation is not consistent with a model in which ho-

modimer formation of ER is required for high affinity DNA binding. Although our DNA binding experiments are conducted with low concentrations of ER (below 1 nM) and a molar excess of DNA, the E_2-occupation and heat-transformation steps were conducted with ER concentrations above 1 nM. These conditions have been observed to promote ER homodimerization (Notides et al., 1981). However, the equilibrium complex that we observe at the end of the DNA-binding reaction contains a single ER bound at a single ERE. These data do not preclude the possibility that ER homodimers also bind EREs under different experimental conditions. Nor do our studies examine the structure of the putative heterodimer. Rather, our data preclude a model in which homodimerization is a requirement for high affinity binding of ER to an ERE.

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