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Articles

Marked Effects of Salt on Estrogen Receptor Binding to DNA: Biologically Relevant Discrimination between DNA Sequences†

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ABSTRACT: Avidin-biotin complexed with DNA (ABCD) assays were employed to determine the binding affinity of estrogen receptor (ER) to DNA under various salt conditions. Type and concentration of salt in the reaction buffer dramatically affected the ability of the ER to discriminate between DNA sequences. Under appropriate salt conditions, ER was able to bind to the estrogen response element from the *Xenopus* vitellogenin A2 gene with at least 3 orders of magnitude greater affinity than a two base pair mutant sequence, and 5 orders of magnitude greater affinity than plasmid DNA. In these studies, the best discrimination was observed under conditions of salt type and concentration that more closely approximated intracellular conditions, i.e., 100-150 mM potassium salts. Analysis of the binding affinities for ER to all three types of DNA over a range of KCl concentrations indicated that the ionic interactions upon ER binding were the same for the three DNA molecules tested. Therefore, the additional stability of ER binding to target DNA sequences was contributed by nonionic interactions.

Estrogens bind with high affinity to a specific receptor protein localized in the cell nucleus. The estrogen receptor (ER)[1] contains a DNA binding domain capable of binding to specific sequences within a target gene termed estrogen response elements (ERE). This model for ER action predicts that the ER is capable of distinguishing an ERE from the mass of DNA sequences in the eukaryotic nucleus. Given the concentrations of ER and DNA in the mammalian nucleus, theoretical calculations predict a difference of 3-4 orders of magnitude in the binding affinities between target and nontarget DNA sequences is needed for the ER to find its ERE amidst the mass of DNA in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989). Recent studies of ER-DNA interactions have focused on target sequences, particularly the ERE derived from the *Xenopus* vitellogenin A2 gene (VitERE; Klein-Hitpass et al., 1986, 1988; Walker et al., 1984), with only an occasional comparison with nontarget DNA sequences. Qualitative comparisons between ER binding to the VitERE and other sequences in gel shift assays clearly indicate an ability of the ER to distinguish between sequences (Darwish et al., 1991; Klein-Hitpass et al., 1989; Kumar & Chambon, 1988). However, these studies did not determine the relative binding affinity between sequences. Peale et al. (1988) obtained an equilibrium dissociation constant (K_d) of 0.5 nM for ER binding the VitERE and a K_d of 0.2 μM for the plasmid containing the VitERE. Quantitative binding studies from this laboratory (Murdoch et al., 1990) employed the VitERE and the 2 bp mutation of this sequence that failed to mediate an estrogen response as described by Klock et al. (1987, MutERE). The difference in ER affinity for these two sequences was 250-fold in that study. The studies to date indicate an ability of ER to discriminate between sequences, but quantitatively do not show the required degree of discrimination predicted. There has been considerable controversy in the literature regarding the role of hormone in steroid receptor-DNA interactions, but recent work has shown that both the occupied and unoccupied ER can bind DNA with the same high affinity and specificity in vitro [Bagchi et al., 1990; see Murdoch et al. (1990) and references cited therein].

Protein-DNA binding interactions are highly dependent upon salt concentrations, with the general observation that binding affinities decrease with increasing salt concentration [see Record et al. (1976) and references cited therein]. The key question to be addressed here is not the absolute affinity

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[1] Abbreviations: ER, estrogen receptor(s); ERE, estrogen response element(s); VitERE, ERE derived from the *Xenopus* vitellogenin A2 gene; MutERE, 2 bp mutation of VitERE; KGlu, potassium glutamate; Tris-HCl, tris(hydroxymethyl)aminomethane titrated with hydrochloric acid to the indicated pH; DTT, dithiothreitol; ^{3}H-E$_2$, 17β-[2,4,6,7-^{3}H]-estradiol; bp, base pair(s); HPLC, high-performance liquid chromatography; ABCD assay, avidin-biotin complexed with DNA assay; RBA, relative binding affinity.

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FIGURE 1: Synthetic oligonucleotides used in DNA binding assays. The sequence underlined for the vitellogenin ERE oligonucleotide (VitERE) represents each arm of the palindromic ERE from the *Xenopus* vitellogenin A2 gene (Walker et al., 1984). The 2 bp changes in the mutant ERE oligonucleotide (MutERE) are outlined. Sites of incorporation of biotin-14-dATP (BRL) for labeled DNA are indicated by an asterisk.

of ER for any particular sequence of DNA but the magnitude of the discrimination between target and nontarget DNA sequences. Therefore, we considered the possibility that altering salt conditions might optimize the differences in ER binding to DNA.

The studies presented in this paper demonstrate that under appropriate salt conditions the ER can discriminate between the VitERE and a 2 bp mutant with 3 orders of magnitude difference in affinity. The ER was able to discriminate between VitERE and plasmid DNA with 5 orders of magnitude difference in affinity. These results indicate that in the nucleus the ER should be able to stably bind an ERE preferentially over the mass of nonspecific DNA sites available. Furthermore, these data provide evidence that the ER makes similar ionic interactions with all three of these types of DNA and that sequence-specific binding must therefore be mediated by the addition of nonionic contacts between residues on the ER and accessible functional groups on the bases of DNA.

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 DTT, and 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 approximately 40 nM 17β-[^{3}H]estradiol (^{3}H-E_2, 90–110 Ci/mmol, New England Nuclear) for at least 2 h on ice, then heated at 30 °C for 60 min, and returned to ice.

Synthetic Oligonucleotides. Synthetic oligonucleotides for each strand of the consensus VitERE sequence and a 2 bp mutant (Klock et al., 1987) were obtained from the Biotechnology Center at the University of Wisconsin—Madison. In addition to the core palindromic sequence, each oligonucleotide was designed from *Bam*HI linkers. Oligonucleotides were either fully complimentary strands of 25 bases or only 21 bases, to leave 5′ overhangs for labeling with biotin as previously described (Murdoch et al., 1990). The sequences of the oligonucleotides used are shown in Figure 1. Biotin-labeled oligonucleotides (25 bp) were purified by electrophoresis on 20% polyacrylamide gels. Unlabeled oligonucleotides used for competition experiments were purified by ion-exchange HPLC on a Waters Gen-Pak Fax column. 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).

Plasmid DNA. Determination of the affinity of ER for completely random DNA was made with the pUC18 plasmid. 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 the absorbance at 260 nm using an extinction coefficient of 13 200 M^{-1} cm^{-1}. This is an average extinction coefficient for a single base pair; thus, the concentration of base pairs was estimated by this method. Experiments with pUC18 plasmid DNA were conducted at very low binding density of ER on the plasmid, such that each base pair defined a new binding site and the best estimate of the concentration of binding sites was the concentration of base pairs (McGhee & von Hippel, 1974).

ABCD Assays. Occupied, heated cytosols were incubated with biotin-labeled VitERE oligonucleotide in TDG plus the indicated concentration of various salts at 4 °C for 16–18 h in a final volume of 100 μL. Various concentrations of non-biotin-labeled oligonucleotide were included in this incubation 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 and then were filtered. Filtration and quantitation of ER bound to the biotin-labeled VitERE were performed as previously described (Murdoch et al., 1990). Background ^{3}H-E_2 binding was determined in duplicate tubes without DNA for each salt condition and was subtracted from total ER binding.

Data Analysis. Equilibrium constants for ER binding to DNA were estimated by a nonlinear least-squares method using the computer program LIGAND (McPherson, 1985; Munson & Rodbard, 1980). Equality of the slopes in Figure 5 was tested by an analysis of covariance (SAS Institute Inc., 1988) with the assistance of the CALS and VETMED Statistical Consulting Facility at the University of Wisconsin—Madison.

Results

Salt Dramatically Affects the Formation of ER–DNA Complexes. The effect of type and concentration of salt on the ability of the ER to form complexes with either the VitERE or MutERE was examined using the ABCD assay. As shown in Figure 2, the concentration of NaCl, KCl, or a combination of KCl and KGlu all dramatically affected the percentage of ER–DNA complex formation with qualitatively similar patterns. The ER–MutERE complex was considerably less stable than the ER–VitERE complex at higher concentrations of all the salt types. The greatest discrimination appeared to be between 100 and 125 mM salt for NaCl, between 125 and 150 mM salt for KCl, and between 150 and 200 mM salt for KCl + KGlu. In addition, the VitERE–ER complex displayed considerable stability over a wide range of salt concentrations in KCl + KGlu.

The other striking qualitative observation seen in both the NaCl and KCl panels of Figure 2 was that less than 20% of maximum ER–DNA complex formed in the absence of the salt. We were prompted to investigate the effects of KGlu on ER–DNA complex formation by the work of Leirmo et al. (1987) that showed a dramatic enhancement of protein–DNA interactions when glutamate was substituted for chloride anions. However, when ER–DNA complexes were titrated with KGlu in the absence of any chloride ion, although the pattern

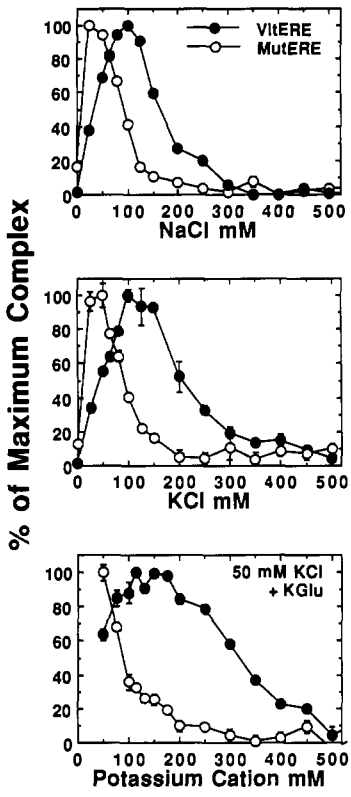

FIGURE 2: Effect of salt concentration on the formation of ER complexes with VitERE or MutERE. A fixed, nonsaturating concentration of biotin-labeled DNA [0.5 nM VitERE (●) or 100–200 nM MutERE (○)] was incubated with a fixed concentration of cytosolic ER (0.47–0.59 nM) with increasing concentrations of the indicated salts. The formation of complexes between ER and the biotin-labeled DNA was determined by the standard ABCD assay as described under Materials and Methods. Data are shown as the percent of maximum complex that formed at a particular salt condition with that DNA. Error bars represent the standard error of the mean.

was qualitatively similar to the KCl + KGlu panel in Figure 2, only about 15% of ER was complexed to DNA at the maximum (data not shown) as compared to 30–40% of ER complexed to DNA at the maximum in all three panels of Figure 2. Maximum ER of 30–40% was reasonable because a nonsaturating concentration of DNA was chosen such that about half of the ER would be complexed to DNA under optimal conditions. In the absence of other variables, decreasing salt concentrations are expected to promote protein–DNA interactions [see Record et al. (1976) and references cited therein]. Therefore, the decrease in ER–DNA complex formation observed at concentrations of chloride salts below 50 mM probably reflected an effect on the functional conformation of the ER protein itself whereas the loss of complex formation at higher salts was probably due to a titration of ionic interactions between ER and DNA, which was the phenomena of interest in these studies.

Effects of Salt on the Affinity of ER for VitERE and MutERE. The affinity of ER for the VitERE and MutERE was determined by competition experiments at various salt conditions as shown in Figure 3. The concentration of unlabeled oligonucleotide required to reduce ER complex formation with the biotin-labeled VitERE by 50% (IC_{50}) is proportional to the affinity of ER for the unlabeled oligonucleotide as indicated by the equation (Bylund, 1980):

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

K_I is the equilibrium dissociation constant for the unlabeled

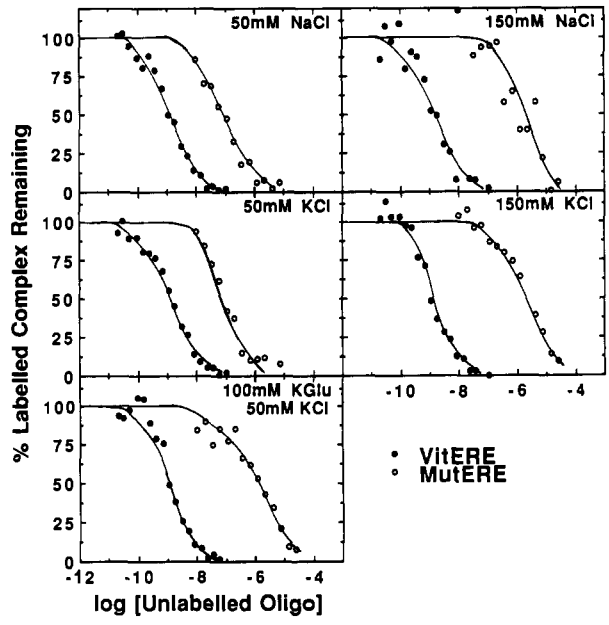

FIGURE 3: Effect of salt type at 50 or 150 mM on the relative binding affinity (RBA) of ER for VitERE over MutERE. Cytosolic ER (0.41 nM) was incubated with a nonsaturating concentration of biotin-labeled VitERE (0.5 nM) with the concentration and type of salts indicated in each panel. Increasing concentrations of unlabeled VitERE or MutERE oligonucleotide were added to the binding reaction to compete with biotin-labeled VitERE for ER. Determination of ER–VitERE–biotin complex formation was made by the standard ABCD assay as described under Materials and Methods. The binding parameters calculated from these data are shown in Table I.

Table I: Equilibrium Dissociation Constants for ER Binding DNA from Figure 3[a]

salt conditions	K_d(Vit-ERE) (pM)	K_d(Mut-ERE) (nM)	RBA
50 mM NaCl	580 ± 85	60 ± 14	100
150 mM NaCl	700 ± 182	800 ± 460	1100
50 mM KCl	700 ± 86	50 ± 12	70
150 mM KCl	320 ± 63	900 ± 138	2800
100 mM KGlu + 50 mM KCl	360 ± 77	900 ± 170	2500

[a] The data from Figure 3 were subjected to analysis by the LIGAND computer program to generate the equilibrium dissociation constants and their associated standard errors (McPherson, 1985). The RBA was calculated as the ratio of the K_d for MutERE over the K_d for VitERE.

oligonucleotide. K_d is the equilibrium dissociation constant for the biotin-labeled VitERE. F is the concentration of biotin-labeled VitERE in the assay. When the competition was performed with unlabeled VitERE, $K_i = K_d$.

The data in Figure 3 clearly demonstrated that raising the salt concentration resulted in a substantial increase in the IC_{50} for the MutERE, indicative of a decreased affinity of the ER for this sequence. Quantitative analyses of the data in Figure 3 were performed and are presented in Table I. The data were fitted to both a one-site and a two-site model. In all cases for ER binding to VitERE or MutERE oligonucleotides, a one-site model produced statistically the best and most appropriate fit of the data. Thus, in the range of experimental conditions performed here, the oligonucleotides behaved as homogeneous ligands for the ER, and only one population of ER capable of binding DNA was detected. At 50 mM NaCl, the RBA was 100, and increasing the NaCl concentration to 150 mM increased the RBA to 1100. At 50 mM KCl, the RBA was 70, and increased to 2800 or 2500 in the presence of 150 mM KCl or 50 mM KCl + 100 mM KGlu, respectively. These

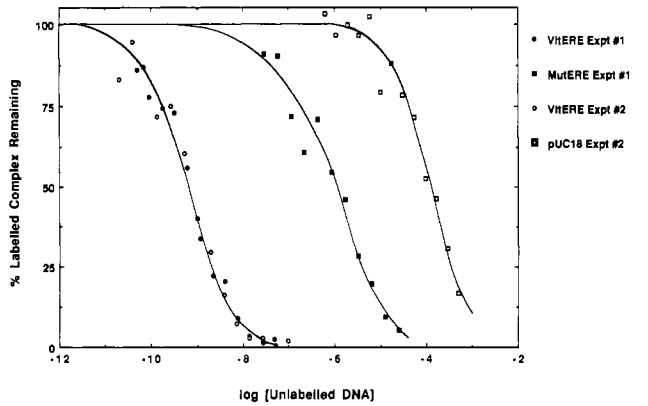

FIGURE 4: Competition against biotin-labeled VitERE for ER by unlabeled VitERE, MutERE, or pUC18 at 100 mM KCl. Experiment 1: A nonsaturating concentration of biotin-labeled VitERE (0.5 nM) was incubated with cytosolic ER (0.41 nM) and increasing concentrations of unlabeled VitERE (●) or MutERE (■) oligonucleotide. Determination of ER–VitERE–biotin complex formation was made by the standard ABCD assay as described under Materials and Methods. Experiment 2: Performed as experiment 1; biotin-labeled VitERE concentration was 0.5 nM, ER concentration was 0.58 nM, and competitions were with unlabeled VitERE (○) or pUC18 (□).

data demonstrate the dramatic effects of salt concentration and type on ER affinity for DNA.

ER Discriminates between VitERE and Plasmid DNA with 5 Orders of Magnitude Difference in Affinity. Although the ER displayed the predicted minimum 3 orders of magnitude difference in affinity between the VitERE and MutERE at 150 mM salt concentrations, an even greater discrimination might be expected between VitERE and completely random DNA. In order to determine the affinity of ER for completely random DNA, competition studies were conducted with pUC18 plasmid DNA as shown in Figure 4. The IC_{50} for pUC18 DNA was 2 orders of magnitude greater than the IC_{50} for MutERE, and the IC_{50} for MutERE was 3 orders of magnitude greater than for VitERE. In experiment 1, the equilibrium dissociation constant for ER binding to VitERE was 150 ± 66 pM, and for the MutERE, it was 390 ± 91 nM. The ratio of these affinities gave an RBA of 2600. In experiment 2, the equilibrium dissociation constant for ER binding to VitERE was 120 ± 43 pM, and for pUC18 DNA, it was 23 ± 6 μM. The ratio of these affinities gave an RBA of 1.9×10^5. These data demonstrate the tremendous influence of sequence on ER affinity for DNA.

Experiments similar to those shown in Figure 4 were carried out over a wide range of KCl concentrations. The quantitative results of these studies are shown in Table II. The change in binding affinities for ER to VitERE and MutERE as a function of KCl concentration closely paralleled the qualitative results shown in Figure 2. ER binding to VitERE was optimal between 90 and 130 mM KCl, whereas the optimum for ER binding MutERE or pUC18 DNA was at 50 mM KCl. At higher KCl concentrations, the binding affinities decreased. In these experiments, the percentage of ER capable of binding VitERE (B_{max}) varied between 60 and 100% for salt concentrations between 50 and 170 mM (data not shown). In this range of salt concentrations, a consistent effect of salt on the B_{max} value was not observed. In addition, the B_{max} routinely varied from 60 to 90% of ER in each different cytosol preparation assayed under the same salt conditions (data not shown). In assays above 170 mM KCl, the B_{max} values were lower, which may have been due to an effect of salt on the functional conformation of the ER. However, the predominant effect of salt was always on affinity rather than binding capacity for the concentrations studied here. These data strongly

Table II: Equilibrium Dissociation Constants for ER Binding DNA at Various [KCl][a]

[KCl] (mM)	K_d(Vit-ERE) (pM)	K_d(Mut-ERE) (nM)	RBA	K_d-(pUC18) (μM)	RBA
50	620 ± 79	50 ± 12	80		
	700 ± 86	50 ± 12	70		
	450 ± 70			3.2 ± 1	7.0×10^3
60	450 ± 98	90 ± 20	200		
	300 ± 42			5.6 ± 1.8	1.9×10^4
70	640 ± 65	230 ± 38	360		
	200 ± 74			11 ± 3	5.5×10^4
80	510 ± 96	230 ± 38	450		
	220 ± 108			18 ± 4	8.2×10^4
90	280 ± 42	300 ± 42	1100		
	190 ± 92			19 ± 4	1.0×10^5
100	150 ± 66	390 ± 91	2600		
	120 ± 43			23 ± 6	1.9×10^5
110	220 ± 75			60 ± 14	2.7×10^5
120	180 ± 31			60 ± 19	3.3×10^5
130	210 ± 20				
150	320 ± 63	900 ± 140	2800		
160	700 ± 170				
170	490 ± 70				
185	840 ± 82				
200	600 ± 240				
225	1500 ± 290				

[a] Equilibrium dissociation constants for ER binding to VitERE, MutERE, or pUC18 DNA at various concentrations of KCl. Constants for ER binding to VitERE at 130–225 mM KCl (except 150 mM KCl, data were from Figure 4) were determined from saturation binding curves in which 0.2 nM ER was incubated with increasing concentrations of biotin-labeled VitERE at each concentration of KCl. Constants for ER binding to VitERE at all other KCl concentrations as well as the parameters for ER binding to MutERE and pUC18 were determined in competition experiments as described in Figure 4.

support the proposal that the salt-dependent decrease in ER–DNA complexes seen in Figure 2 was due to a titration of ER–DNA ionic interactions.

The analysis of ER binding to pUC18 plasmid was more complex than ER binding to the VitERE or MutERE synthetic oligonucleotides in that the data could be fit to a two-site model as well as to a one-site model. However, the error on the binding parameters estimated from a two-site model ranged as high as 100%. This observation could be due to either multiple sites on ER for DNA binding, overlap of binding sites on the plasmid, or heterogeneity of the DNA ligand. Given that we have never been able to define more than a single population of ER when a homogeneous DNA ligand (i.e., an oligonucleotide) was used, we do not interpret the plasmid data as indicative of more than one type of binding site on ER. Overlap should be insignificant at the very low binding densities at which the ER occupied the pUC18 plasmid (Kowalczykowski et al., 1986). Rather, because pUC18 plasmid is a heterogeneous sequence and therefore represents a heterogeneous population of binding sites for the ER, we conclude that the ER is binding to variable, low-affinity sites. The parameters presented in Table II for ER binding pUC18 plasmid were derived from a fit of the data to a one-site model. Therefore, these values represent the average K_d for ER binding to any site in the pUC18 plasmid.

Binding of ER to Specific or Nonspecific DNA Sequences Results in the Same Amount of Ionic Displacement. Binding of a protein to DNA is accompanied by the release of cations from the DNA, and the entropic effect of this ionic displacement is a driving force for the binding reaction (deHaseth et al., 1977). Thus, cations (as well as anions bound to the DNA binding site on the ER) are stoichiometric participants in the DNA–ER binding interaction, and the affinity of the protein–DNA interaction is predicted to decrease with in-

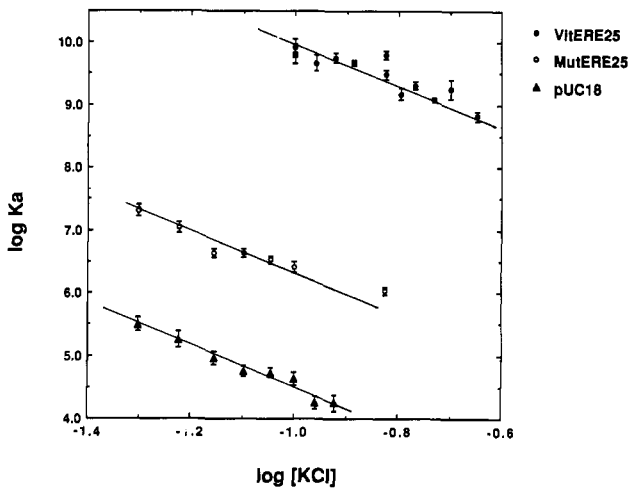

FIGURE 5: log–log graph of the binding affinity of ER to DNA as a function of [KCl]. The equilibrium dissociation constants in Table II were converted to equilibrium association constants, and log K_a was plotted against log [KCl].

creasing salt concentration [see Record et al. (1991) for a review of the theory of salt effects on protein–DNA interactions]. An analysis of the change in DNA–ER binding affinity as a function of salt concentration gives a measure of ionic displacement as illustrated by the equation (Record et al., 1976):

$$-\frac{\partial \log K_a}{\partial \log [\text{cation}]} = k + m'\psi$$

K_a is the equilibrium association constant for ER binding DNA. k is the displacement of anions, and m' is the displacement of cations upon ER–DNA association. ψ is a measure of counterion binding to the DNA, which is 0.88 for double-helical native DNA.

The slope of a log–log plot of ER affinity for a particular DNA versus salt concentration is predicted to be a measure of the ionic displacement resulting from the ER–DNA association. Data from Table II over the range of [KCl] where the affinity was decreasing for VitERE, MutERE, and pUC18 were plotted (Figure 5). An analysis of covariance testing for the equality of the slopes of each line was performed, giving an F value = 0.01 (2, 23 degrees of freedom) and a p value = 0.9894. This analysis strongly supported the conclusion that all three sets of data had the same slope, with the pooled estimate being -3.40 ± 0.28. Therefore, the ionic displacement upon ER association with all three of these DNA ligands was essentially the same.

Discussion

The data presented in this study demonstrate that the ER is capable of distinguishing between target and nontarget DNA sequences with 3–5 orders of magnitude difference in affinity under appropriate salt conditions. The VitERE and MutERE sequences differ by only 2 bp, but the MutERE fails to mediate an estrogen response in gene transfer studies (Klock et al., 1987). At 150 mM KCl, the affinity of ER for the VitERE is (2.8×10^3)-fold greater than for the MutERE. A difference in affinity of 10^3–10^4-fold between target and nontarget sequences is expected for ER to be able to select target sequences over the mass of DNA in the eukaryotic nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989). ER displays this level of discrimination for VitERE over MutERE in these studies. Furthermore, with an estimated concentration of ER in the nucleus of 10–30 nM (Murdoch et al., 1990), these affinities predict saturation of the VitERE site (assuming the concentration of VitERE is below the concentration of ER) with little occupation of the MutERE site even in the absence of bulk DNA. Therefore, the failure of the MutERE sequence to mediate an estrogen response can be attributed solely to its decreased affinity for ER.

The ability of ER to discriminate between VitERE and bulk DNA is even more dramatic as evidenced by the binding studies using pUC18 plasmid. At 120 mM KCl (the highest concentration for which data were obtained), the affinity of ER for VitERE is (3.3×10^5)-fold greater than for pUC18 plasmid. The interaction of ER with bulk DNA is therefore of low affinity, but in the nucleus, the mass of DNA makes this a high-capacity interaction. This low-affinity, high-capacity interaction between ER and bulk DNA might contribute to the accumulation of ER in the nucleus in the absence of hormone.

The affinities of ER for bulk DNA reported here compare reasonably well with previous reports given the tremendous variation in conditions and assay methodologies. Yamamoto and Alberts (1974) reported an affinity of 300 μM for ER binding calf thymus DNA at 150 mM NaCl by sedimentation partition chromatography. Peale et al. (1988) reported an affinity of 0.2 μM for ER binding to the pGEM-1 plasmid at 100 mM KCl using a gel filtration binding assay. This affinity was calculated on the basis of a nonspecific binding site being the same length as a specific site—38 bp for their construct. However, at low occupancy of nonspecific sites on the plasmid by ER, each base pair can define the start of a nonspecific binding site (McGhee & von Hippel, 1974). Therefore, the affinity of 0.2 μM could be underestimated by as much as 38-fold. Skafar and Notides (1985) used DNA–Sepharose chromatography at a constant salt concentration to obtain a binding affinity for occupied ER to calf thymus DNA of 0.1 μM at 160 mM KCl. This rather high affinity may be due to the assay employed since the kinetics of absorption chromatography will be different than an assay performed in solution.

It is striking how well the affinities of ER for specific versus nonspecific DNA reported here agree with the thoughtful predictions made by Yamamoto and Alberts (1975) over 16 years ago. On the basis of an analysis of data then available in the literature and drawing an analogy to the *E. coli lac* repressor protein, whose specific DNA binding site (the *lac* operator) had been identified, these authors proposed a model for ER interaction with DNA. Long before the first ERE was identified, their model predicted that the genome contained up to 1000 specific binding sites for ER with affinities in the range of 0.1–10 nM that were masked by ER binding to bulk DNA with micromolar affinity. Although experiments to determine the number of ERE per genome remain to be reported, the affinities reported here for specific and nonspecific DNA fit well into this old model. A major point of their model which is not supported by our data is the notion that hormone acts as an allosteric effector on the ER, thereby inducing DNA binding. We have seen no dependence upon hormone for ER binding to DNA (Murdoch et al., 1990).

The affinities of ER for all three types of DNA tested in this study were highly dependent on salt conditions. The best discrimination of ER between target and nontarget sequences was seen under salt conditions that more closely approximated physiological conditions than the others tested. Although the precise ionic composition in the eukaryotic cell and the nucleus

is not known, it is generally agreed that the ionic strength is about 150 mM, potassium is the major cation, and polyanions of DNA and RNA as well as amino acid residues provide a large portion of counteranions. Potassium salts in the range of 125–150 mM gave the best discrimination in our studies. Although we did not directly investigate salt effects on ER conformation and function, it is known that this protein's tendency to aggregate can be affected by salt (Giannopoulos & Gorski, 1971). This may be a factor in the decreased ER–DNA complex formation at low salt or in KGlu.

An analysis of the change in equilibrium binding constants for ER to each of the three DNAs tested over a range of KCl concentrations gives us information on the relative ionic displacement when each of these complexes is formed. The equality of slopes from the plots of log K_a versus log [KCl] shown in Figure 5 for ER complexed with either VitERE, MutERE, or pUC18 plasmid DNA is evidence that the ionic displacement upon complex formation is the same for all three of these complexes. Therefore, the ionic interaction of ER with DNA, regardless of sequence, is similar, and the additional stability of ER–DNA complexes of particular sequence is provided by additional nonionic contacts between amino acid residues on ER and functional groups on bases accessible to ER in the DNA grooves. Formation of these hydrogen bonds or nonpolar interactions between ER and specific bases on the DNA apparently do not interrupt the non-sequence-specific ionic interactions between ER and DNA for the DNA molecules we have tested.

Skafar and Notides (1985) reported studies on the ionic interaction of ER with calf thymus DNA that showed a much greater change in K_a as a function of [KCl] than did our studies. These investigators also reported an effect of estradiol on these thermodynamic parameters. The many differences between their assay conditions and ours—higher salt concentrations, use of an isocratic elution from DNA–Sepharose assay method, and partially purified ER—may account for differences in estimates of thermodynamic parameters. Although these investigators and others (Rodriguez et al., 1989) have calculated numbers of salt bridges between protein and DNA from such studies, we have not. The structures of several prokaryotic protein–DNA complexes have been solved in recent years, and the number of salt bridges observed have differed from predictions made from thermodynamic studies. However, ionic interactions of a weaker character are no doubt important (Harrison & Aggarwal, 1990).

In summary, the ER is capable of recognizing the VitERE, which mediates a strong estrogen regulation of its promoter, with a difference in affinity of 5 orders of magnitude over bulk DNA and 3 orders of magnitude over a 2 bp mutant that does not mediate an estrogen response. Therefore, we conclude that in this model system, the affinity of ER for the VitERE is sufficient to confer specificity of response. However, most ERE are not the perfect palindromic sequence represented by the VitERE (Berry et al., 1989; Darwish et al., 1991; Deharo et al., 1990; Lannigan & Notides, 1989; Maurer & Notides, 1987; Walker et al., 1984), and the affinities of ER for these sequences are probably lower. Studies ongoing in this laboratory indicate that the affinities of ER for the putative ERE of the rat prolactin gene are closer to those observed for the MutERE than for the VitERE (F. E. Murdoch, L. M. Byrne, E. A. Ariazi, and J. Gorski, unpublished results). Lannigan and Notides (1989) have reported very low affinity binding of ER to double-stranded ERE from prolactin and suggest that the ER must actually bind single-stranded DNA in the regulation of the prolactin gene. Certainly, if the binding of ER to these nonpalindromic ERE sequences is considerably lower than that reported for the VitERE, more complex mechanisms for conferring specificity must be operating.

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Gene Structure and Expression of the Rat Cytochrome P450IIC13, a Polymorphic, Male-Specific Cytochrome in the P450IIC Subfamily[†,‡]

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ABSTRACT: The male-specific CYP2C13 gene has been isolated from two independent rat genomic libraries. This gene spans more than 50 kb and contains eight introns which are subject to the GT-AG rule. Two allelic forms of the CYP2C13 gene were identified. Determination of the exonic sequences revealed that one of them encodes cytochrome P450(+g) and the other encodes cytochrome P450(-g). Using allele-specific restriction enzyme sites, a good correlation between the genotype and the phenotype of CYP2C13 was shown. Nucleotide substitutions between the (+g) and the (-g) genes exist not only in the exons but also in the introns and the 5'-flanking region. Although five nucleotide differences were identified within 287 base pairs of the (+g) and (-g) 5'-flanking regions, the transcription initiation sites were identical. In addition to a canonical TATA box located 31 base pairs upstream of the start site of transcription, putative binding sites for the liver-enriched and liver-specific transcription factors HNF1/LF-B1/APF, HNF3, HNF4/AF-1, C/EBP, LAP, and eH-TF/TGT3 and the ubiquitous factors NF-1 and OTF-1 were identified.

Cytochrome P450IIC13 (P450g)[1] is a constitutive male-specific form of cytochrome P450 in rat liver, which, in a reconstituted system, catalyzes the hydroxylation of testosterone at the 6β- and 15α-positions (Ryan et al., 1984). Cytochrome P450IIC13 is known to possess a phenotypic polymorphism indicated by the high, low, or intermediate protein levels found in livers of outbred strains of male Long Evans or Sprague-Dawley rats. On the other hand, inbred ACI rats express only high levels, while inbred Fischer rats express only low levels of P450IIC13 (Bandiera et al., 1986; McClellan-Green et al., 1987; Rampersaud et al., 1987). In contrast to the protein levels which apparently differ from strain to strain, the amount of P450IIC13 mRNA is found to be almost identical (McClellan-Green et al., 1987, 1989a). Genetic experiments demonstrated additive inheritance for the P450IIC13 phenotype (Rampersaud et al., 1987; Rampersaud & Walz, 1987a,b). Two different groups have isolated and sequenced cDNAs for P450IIC13 from male Sprague-Dawley rats (McClellan-Green et al., 1989a; Zaphiropoulos et al., 1990a; Yeowell et al., 1990). Although the 5'-noncoding leader sequence and the 3'-noncoding region of the cDNA isolated from the (-g) phenotype (low protein levels) were identical to that of the (+g) phenotype (high protein levels), the coding sequence differed by nine bases resulting in seven amino acids changes (Yeowell et al., 1990).

In our laboratory the growth hormone (GH) effects on the expression of sex-specific cytochrome P450s have been extensively investigated. For example, the female-specific P450IIC12 (P45015β) requires continuous GH exposure for expression (Zaphiropoulos et al., 1988). On the other hand, P450IIC13 is dramatically suppressed by continuous GH treatment in inbred ACI rats (McClellan-Green et al., 1989b). Since P450IIC12 and -IIC13 have highly similar primary structures, the mechanisms underlying their dramatically opposed GH regulation are under intense investigation.

In the present work, we used genomic libraries from outbred Sprague-Dawley rats to isolate the genes encoding the (+g) and the (-g) forms of P450IIC13 as a means to study regulation of expression at the gene level. This allowed the dem-

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[1] Abbreviations: P450, cytochrome P450; kb, kilobase pair(s); bp, base pair(s); GH, growth hormone; SDS, sodium dodecyl sulfate; 1× SSC, 0.015 M sodium citrate/0.15 M NaCl (pH 7.0)

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