Estrogen Receptor α Interaction with Estrogen Response Element Half-Sites from the Rat Prolactin Gene†

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ABSTRACT: Estrogen regulation of the rat prolactin gene requires sequences within the DNase I hypersensitive site II (HSII). We have used overexpressed mouse estrogen receptor α (ERα) protein to study interactions of ERα with an imperfect estrogen response element (ERE) and four ERE half-site sequences from HSII. We confirmed that ERα has higher affinity for ERE half-sites than for the imperfect ERE. As expected, the imperfect ERE formed a complex with ERα similar to that between mERα and a consensus ERE in gel shift assays. The ERα complex with half-sites, however, had faster mobility on a 4% polyacrylamide gel than the ERα complex with a consensus ERE, indicating that the complexes had different compositions. Ferguson analysis revealed that the ERα/half-site complex had a larger molecular weight and higher negative charge than the ERα/consensus ERE complex. Similar results were observed with purified human ERα, showing that the ERα/half-site complex contained only ERα and oligonucleotides. These results are best explained by a model in which a dimer of ERα is bound to two half-site oligonucleotides. We propose that two ERα dimers may interact with the four ERE half-sites in HSII to influence estrogen regulation of this gene.

Estrogens are important regulators of mammalian growth and metabolism, accomplishing these functions through the regulation of expression of specific genes (*1*−*3*). Estrogens regulate the activities of the transcription factors estrogen receptor (ER)[1] α and ER*β*, which bind to specific DNA sequences known as estrogen response elements (EREs) in the vicinity of estrogen-regulated genes. The consensus ERE, the DNA sequence with highest affinity for ERα, is an inverted repeat of the sequence AGGTCA separated by three base pairs (*4*). Although this sequence has been used for most in vitro studies of ERα/DNA interaction, its natural occurrence is rare (*5*, *6*), and many EREs instead contain weaker ERα binding sequences. Imperfect EREs are similar to consensus EREs but have mutations which reduce their affinity for ERα (for examples, see *7*−*9*). Some EREs consist only of half-sites, in which unpaired AGGTCA sites are present (*10*−*12*). The ERs stimulate transcription through the recruitment of coactivator complexes which contain histone acetylase and methylase activities (*13*, *14*).

Estrogens have been known for some time to be important regulators of transcription of the prolactin gene in the lactotroph cells of the anterior pituitary. The estrogen-responsive sequences are found within DNase I hypersensitive site II (HSII), 1.5−1.8 kbp from the promoter (reviewed in *15*). Recent results from mice with a deletion of the ERα gene (ERKO mice) have confirmed the importance of estrogens in prolactin production: Female ERKO mice have 20-fold lower prolactin mRNA levels than wild-type mice, reduced lactotroph cell density, and smaller anterior pituitaries overall (*16*). Another important regulator of the prolactin gene is the transcription factor Pit-1, which binds to four sites close to the promoter and four sites within HSII (*17*). In heterologous cell lines, both Pit-1 and ERα must be present for estrogen regulation of the prolactin gene to occur (*18*, *19*).

The DNA sequences mediating estrogen responsiveness of the rat prolactin gene have been studied in some detail. An imperfect ERE close to Pit-1 binding site 1D was identified by its ability to bind to purified ERα (*20*). This sequence was shown to be essential for estrogen regulation in reporter gene assays (*21*, *22*); however, in some studies, additional upstream (*22*) or downstream (*23*) sequences were also required for an estrogen response. Lannigan and Notides (*24*) identified another DNA sequence within HSII to which ER bound with high affinity; the secondary structure of the DNA was important for ER binding to this sequence, but the exact site of ER binding was not identified.

Murdoch et al. (*25*) identified a sequence close to Pit-1 binding site 4D which bound ER with higher affinity than the imperfect ERE identified earlier. This sequence contained one perfect half-site of the consensus ERE. Intriguingly, the ratio of ER to ERE appeared to be 1:1, suggesting that ER may bind to this sequence as a monomer, and we have been interested in characterizing this interaction further. Unfortunately, the interaction was too weak for detection in gel shift assays when rat uterine cytosol was the source of ER

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[1] Abbreviations: ER, estrogen receptor; ERE, estrogen response element; ERKO, estrogen receptor knockout; PRL, prolactin; HSII, DNase I hypersensitive site II; E_2, 17*β*-estradiol; MHT, 4-hydroxytamoxifen; MW, molecular weight; DBD, DNA binding domain.

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(*25*); however, using mouse ERα overproduced in a baculovirus system (*26*), we have now been able to detect ERα binding to this sequence and other ERE half-sites from HSII in gel shift assays. We have found that the complex between ERα and half-sites is distinct from that between ERα and a consensus ERE. The ERα/half-site complex forms a faster-migrating complex on a 4% polyacrylamide gel, which would usually indicate a smaller protein/DNA complex. In this case, however, Ferguson analysis indicated that the ERα/half-site complex has a higher molecular weight and higher negative charge than that of ERα with a consensus ERE, and we attribute this to one ERα dimer interacting with two half-site oligonucleotides. In vivo the four ERE half-sites in the rat prolactin HSII region may interact with two ERα dimers and contribute to estrogen regulation of this gene. In this scenario, each ERα dimer would interact with two widely separated half-sites, forming a bridge between them.

EXPERIMENTAL METHODS

Oligonucleotides. Oligonucleotides were ordered from Research Genetics. Two 31-base oligonucleotides were annealed to form a 35 bp double-stranded oligonucleotide with a 4-base overhang on each end. Oligonucleotides were labeled with the Klenow fragment by filling in the ends with [α-^{32}P]dATP and unlabeled dCTP, dGTP, and dTTP as previously described (*26*). Labeled oligonucleotides were purified over DuPont NENSORB 20 columns.

Estrogen Receptor. Purified human ERα protein was from Panvera Corp. (Madison, WI). Mouse ERα was produced in Sf21 cells using a baculovirus system described previously (*26*). The preparation of cell extract from Sf21 cells has also been described previously (*26*).

Gel Shift Assays. Gel shift samples were prepared in TDGK buffer (10 mM Tris, pH 7.5, 1 mM dithiothreitol, 10% glycerol, 0.1 M KCl) with 0.1 mg/mL sheared herring sperm DNA, 40 000 cpm of labeled oligonucleotide, and Sf21 cell extract containing mERα or purified hERα. Some samples contained antibody ER712 (*27*) at 1:10 final dilution. The storage solution for ER712 contained 50% glycerol and 10 mg/mL bovine serum albumin. The samples were incubated on ice for 4 h before being loaded onto the gel. Orange G dye was loaded into one lane to monitor the progress of the electrophoresis. The running buffer was low ionic strength TAE (6.7 mM Tris, pH 8.0, 3.3 mM sodium acetate, 1.0 mM EDTA). The gels contained 1× running buffer with 2.0% glycerol and 4.0% acrylamide (acrylamide:bisacrylamide ratio of 37.5:1). After this solution was degassed, 450 μL of 10% ammonium persulfate and 50 μL of TEMED were added to initiate polymerization; polymerization proceeded for at least 45 min. Gels were run at 60 mA constant current with buffer recirculation. After 30 min pre-electrophoresis, samples were loaded and run for about 90 min, until the Orange G dye was near the gel bottom. The gel was then dried onto Whatman 3M paper and exposed to film.

Ferguson Analysis. A series of eight nondenaturing gels from 3.0 to 6.5% polyacrylamide (acrylamide:bisacrylamide ratio of 19:1) were subjected to electrophoresis, with protein standards and ER/ERE samples on the same gels. The gel shift sample conditions were the same as those given above, except that each sample contained antibody ER712 or nonspecific IgG at 1:10 final dilution. Both antibody stocks contained 50% glycerol and 10 mg/mL bovine serum albumin. The protein standards were thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome *c* (12 kDa). Protein standards were prepared individually in TDGK buffer; 50 μg of each protein was loaded on each gel. The running buffer was high ionic strength Tris−glycine (50 mM Tris base, 390 mM glycine, 1.0 mM EDTA). Electrophoresis was carried out at 45 mA constant current without buffer recycling until the orange dye neared the bottom, about 90 min. After electrophoresis, the half of each gel containing the protein standards was removed and transferred to Coomassie blue staining solution (45% ethanol, 10% acetic acid, 0.1% Coomassie brilliant blue dye). After overnight staining, the gels were incubated with 2−3 batches of destaining solution (30% ethanol, 5% acetic acid), and then dried on Whatman 3M paper. The half of each gel containing the gel shifts was transferred directly to Whatman 3M paper, and the top and bottom of the gel were marked on the paper. The gels were then dried and exposed to film. The migration distances of the protein standards and gel shift bands from the top of the gel were measured, and the relative mobility (R_f) was calculated by dividing these by the migration distance of the orange dye from the top of the gel. The slopes and *y* intercepts of the Ferguson plots were determined by linear regression using the computer program Cricket Graph III.

RESULTS

Overexpressed mERα Binding to ERE Half-Sites Can Be Detected by Gel Shift Assays. In this study we compared mERα binding to sequences from the rat prolactin gene with mERα binding to the consensus ERE from the *Xenopus* vitellogenin A2 gene (vit ERE) (*4*). The rat prolactin sequences are all found within DNase I hypersensitive site II (HSII), which contains the sequences required for estrogen regulation (*20*, *21*, *28*). The sequences were prepared as 35 bp oligonucleotides with the sequences given in Figure 1. The numbers in parentheses after the prolactin (PRL) oligonucleotide names refer to their locations relative to the transcription initiation site. The coordinates used are those reported by Kladde et al. (*29*), which differ somewhat from the sequence reported by Maurer (*30*). PRL1 is the imperfect ERE identified by Maurer and Notides (*20*). PRL2 is the half-site studied by Murdoch et al. (*25*). Since the PRL2 half-site had high affinity for ER, we also tested three other half-sites found within HSII for ER binding: PRL3, PRL4, and PRL5. One of these half-sites (PRL5) was found to be essential for estrogen regulation of the rat prolactin gene (*23*). PRL6 was selected as a negative control which contains no known ERE sequence. We also included a two base pair mutant of the vit ERE (mut ERE) which has about 1000-fold lower affinity for ER than the consensus vit ERE (*31*).

As a first step, we studied whether overexpressed mERα at two different concentrations could bind to these sequences on gel shift (Figure 2). While 1.0 nM mERα protein was enough to shift vit ERE, binding to PRL1−3 could not be detected at this concentration; however, with 20 nM mERα present, binding to all of these sequences could be detected. Addition of antibody ER712 produced supershifts, showing that these shifted bands definitely contained mERα. The

vit ERE
AGCTTCGAGG**AGGTCA**CAG**TGACCT**GGAGCGGATC

mut ERE
AGCTTCGAGG**AGATCA**CAG**TGATCT**GGAGCGGATC

PRL1 (-1600 to -1573)
AGCTGCATTT**TTGTCA**CTA**TGTCCT**AGAGTGGATC

PRL2 (-1740 to -1714)
AGCTAACTAA**AGGTCA**CAAGCTGCTTCAGATGATC

PRL3 (-1547 to -1521)
AGCTATAGATCATG**AGGTCA**TAACGATTTATGATC

PRL4 (-1786 to -1760)
AGCTAGAACC**AGGTCA**TCTGTCAGTCCAAATGATC

PRL5 (-1573 to -1547)
AGCTGCTTTG**GGGTCA**GAAGAGGCAGGCAGAGATC

PRL6 (-1631 to -1608)
AGCTCATGAGTGGAACTTTGGAGTGCATTAAGATC

FIGURE 1: Oligonucleotides used in this study. All oligonucleotides were 35 base pairs in length and double-stranded. Vit ERE is the consensus ERE from the Xenopus vitellogenin A2 gene (*4*); mut ERE is similar to vit ERE, but contains a mutation in each half-site (*31*). The numbers in parentheses after the prolactin (PRL) oligonucleotides refer to the locations of the sequences relative to the transcription start site. The numbers are based on the sequence of Kladde et al. (*29*).

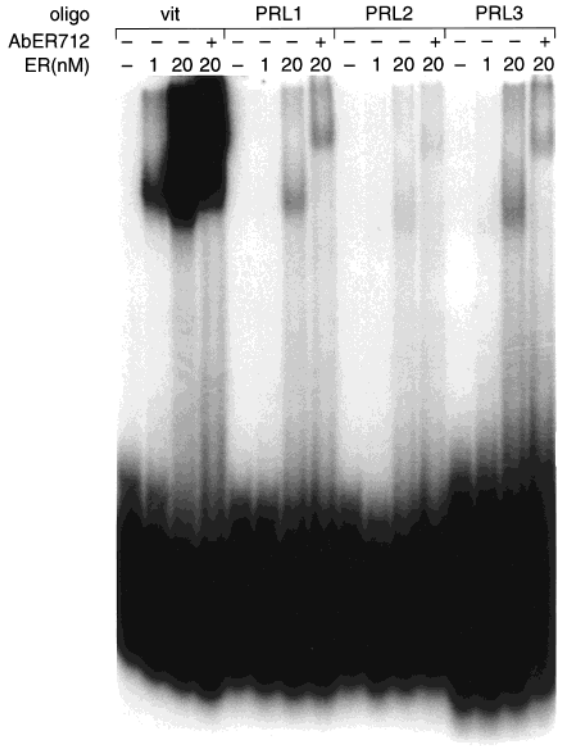

FIGURE 2: Gel shift assay with two concentrations of mERα protein. Gel shift assays were performed with Sf21 cell extract containing either 1 or 20 nM mERα protein. For each oligonucleotide, the leftmost lane contains no ER protein, while the rightmost lane contains antibody ER712, which causes a supershift of the mERα/DNA complex.

prolactin EREs show less binding at 20 nM mERα than vit ERE at 1.0 nM mERα, suggesting that the differences between affinities are greater than 20-fold. This is in agreement with previous studies with ER from rat uterus (*25*).

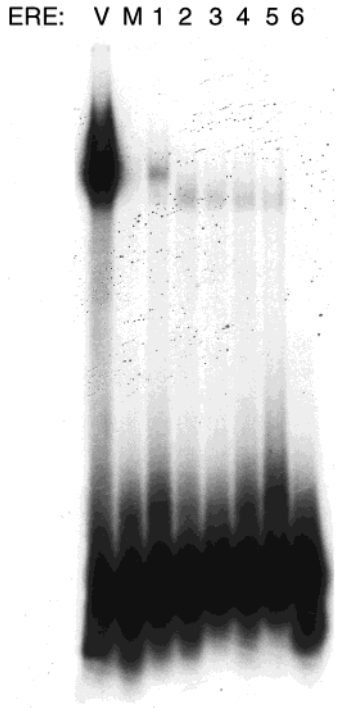

FIGURE 3: Gel shift assay of sequences from the rat prolactin gene. Each lane contains Sf21 cell extract with 20 nM mERα protein. V, vit ERE; M, mut ERE; 1−6, PRL1−PRL6.

In Figure 2 the mobility of mERα bound to PRL2 and PRL3 half-sites appeared to be greater than that of the mERα complex with vit ERE or PRL1. This can be seen more clearly in Figure 3, in which two other half-site sequences from HSII were tested for ER binding (PRL4 and PRL5). Also in this gel, mut ERE and PRL6 were run as negative controls. In this figure, it can clearly be seen that mERα bound to all of the oligonucleotides containing a half-site (PRL2−5), and the mobility of the complex was the same regardless of which half-site oligonucleotide was bound. Also the mobility of the mERα/half-site complex is higher than that for mERα bound to vit ERE or PRL1. PRL1 and vit ERE form complexes with mERα with similar mobilities. Similar results have been found for ER interacting with other imperfect EREs (*7*−*9*). There is a very faint band present in the lane containing mut ERE, but no complex is seen between mER and PRL6. Therefore, mER is binding to the PRL1−5 oligonucleotides through specific binding interactions.

Previously Murdoch et al. (*25*) found that the PRL2 half-site sequence bound ER from rat uterine cytosol with higher affinity than the PRL1 imperfect ERE. We wanted to determine whether the same was true for overexpressed mouse ERα from Sf21 insect cells. To compare the relative affinities of mERα for PRL1 versus PRL2 and PRL3, we used unlabeled oligonucleotides to compete with ^{32}P-labeled vit ERE in a gel shift assay with overexpressed mERα (data not shown). The amounts of cold oligonucleotides were 50 times and 500 times as much as the labeled vit ERE. The PRL1 oligonucleotide did not reduce the ER/vit ERE band at all, even at 500-fold excess, while PRL2 and PRL3 at 500-fold excess reduced the ER/vit ERE band by 45% and 33%, respectively. The results of this assay showed that, similar to ER from rat uterine cytosol, overexpressed mouse ERα had higher affinity for a half-site than for the imperfect ERE from HSII.

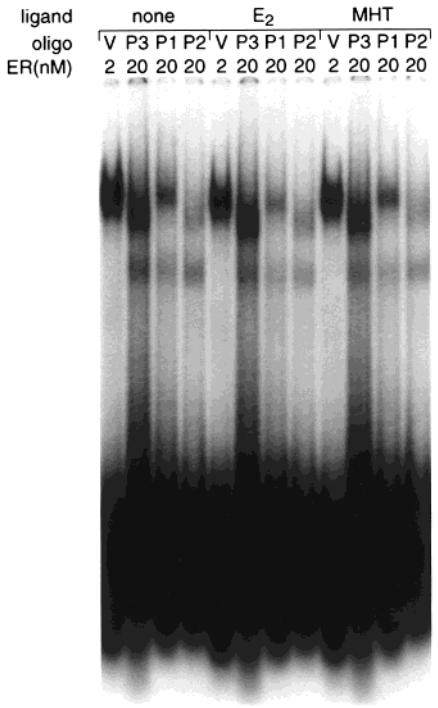

FIGURE 4: Effects of ligand occupancy on binding of mERα to DNA sequences from the rat prolactin gene. Gel shifts were performed with the oligonucleotides vit ERE (V), PRL3 (P3), PRL1 (P1), and PRL2 (P2). Sf21 cell extract containing either 2 nM mERα (for vit ERE) or 20 nM mERα (for PRL oligonucleotides) was present. The samples were incubated either with no ligand, with 20 nM 17β-estradiol (E_2), or with 4-hydroxytamoxifen (MHT). Each sample contained 0.2% ethanol.

ER binds to a consensus ERE with the same affinity regardless of whether it is bound to an estrogen, an antiestrogen, or no ligand (*32*). We studied whether binding of the estrogen 17β-estradiol (E_2) or the antiestrogen 4-hydroxytamoxifen (MHT) to mERα affected its interactions with the PRL1 ERE or the PRL2 and PRL3 half-sites (Figure 4). We found that these same complexes formed in approximately equal amounts regardless of occupancy of the receptor by E_2, MHT, or no ligand; however, we cannot rule out small effects of these ligands on the affinity of the mER for half-sites.

Characterization of the Difference between mERα/vit ERE and mERα/Half-Site Complexes by Ferguson Analysis. The difference in mobility between mERα complexes with vit ERE and half-sites could be due to a difference in size, charge, or conformation of the protein/DNA complexes. To further characterize this difference, we performed gel shift assays on a range of polyacrylamide gels and subjected the results to Ferguson analysis (*33*). Two values can be determined from a Ferguson plot: the retardation coefficient (K_R) is the negative of the slope, and the log mobility at 0% T (log Y_0) is the *y* intercept. The K_R is related to molecular size and shape, while log Y_0 depends on the charge and, to a lesser extent, the size of the complex. In a previous study, we showed that the overexpressed mouse ERα bound to vit ERE as a dimer with one oligonucleotide present, but Ferguson analysis of gel shift data resulted in an overestimate of the molecular weight of the complex (*26*). When we compared the ERα/vit ERE complex with a supershifted complex containing ERα, vit ERE, and antibody ER712, we found that the difference in MW predicted by Ferguson analysis was very close to the molecular weight of one antibody. Thus, in this system Ferguson analysis appears to be more effective for determining the differences between similar protein/DNA complexes than for determining the exact molecular weight of a complex.

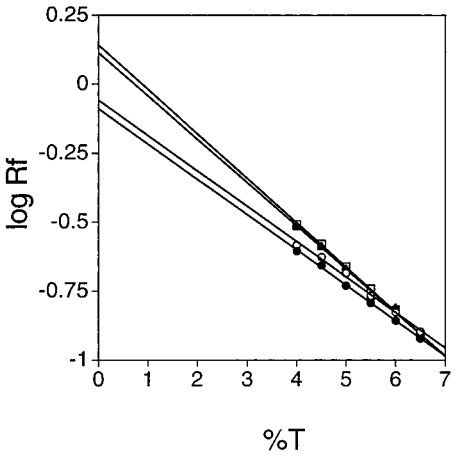

FIGURE 5: Ferguson plots of mERα and purified hERα binding to vit ERE and PRL2 oligonucleotides. The relative mobility (R_f) of each protein/DNA complex was measured on a series of gels with different percentages of acrylamide (%T), and the data are presented as a Ferguson plot. mERα/vit ERE, black circles; hERα/vit ERE, open circles; mERα/PRL2, black triangles; hERα/PRL2, open squares. For mERα/vit ERE, $K_R = 0.128$, log $Y_0 = -0.090$, $r^2 = 0.999$; for hERα/vit ERE, $K_R = 0.128$, log $Y_0 = -0.058$, $r^2 = 0.994$; for mERα/PRL2, $K_R = 0.157$, log $Y_0 = 0.114$, $r^2 = 0.994$; for hERα/PRL2, $K_R = 0.161$, log $Y_0 = 0.142$, $r^2 = 0.999$.

Ferguson plots of mERα complexes with vit ERE and PRL2 half-site are shown in Figure 5. At lower polyacrylamide concentrations (%T), there is a difference between the mobilities of mERα with vit ERE or with PRL2 ERE. At higher %T, however, the difference is less pronounced, and this results in Ferguson plots which cross at around 7.0% T. The Ferguson plots for these complexes have different slopes and *y* intercepts, providing strong evidence that mERα is forming a complex with half-sites that has a different size and charge from the complex between mERα and vit ERE.

Ferguson analysis was also performed with purified human ERα (Figure 5). The supershifts in Figure 2 had shown that ERα was present in the protein/DNA complex, but they did not address whether additional proteins were present. We found that purified human ERα behaved very similarly to mERα in Ferguson analysis, providing strong evidence that no other proteins were present in these complexes. The K_R values for mERα/vit ERE and hERα/vit ERE complexes were identical, while the log Y_0 value of the hERα/vit ERE complex was slightly higher than that of the mERα/vit ERE complex. These results are similar to those observed previously with the same technique (*26*). The K_R values for mERα and hERα bound to the PRL2 half-site were also very similar, and here also the log Y_0 value for the hERα complex was higher than that of the mERα complex.

Ferguson analysis of mERα interaction with both EREs was repeated 3 times with similar results, and the numerical values determined from the plots are presented in the top part of Table 1 with standard errors. The differences observed between K_R and log Y_0 values for the two complexes are statistically significant (paired *t* test, $P < 0.05$), reflecting differences in size and charge. Normally, one would predict

Table 1: Ferguson Plot Parameters and Molecular Weight Estimates for Complexes of mERα with Consensus ERE and Half-Site Oligonucleotides[a]

		mERα/vit	mERα/PRL2	difference
	K_R	0.122 ± 0.004	0.146 ± 0.008	0.024 ± 0.003
	log Y_0	−0.125 ± 0.022	0.042 ± 0.044	0.167 ± 0.022
MW ($\times 10^{-3}$)	K_R vs MW	250 ± 6	301 ± 16	51 ± 11
	log K_R vs log MW	227 ± 12	282 ± 20	55 ± 8
	$\sqrt{K_R}$ vs $\sqrt[3]{MW}$	218 ± 10	273 ± 18	55 ± 7

[a] Values of K_R and log Y_0 were determined from three experiments. The differences between these values for mERα/vit and mERα/PRL2 are statistically significant (paired t test, $P < 0.05$). The MW estimates were determined from three different plots relating K_R and MW.

that a faster migrating complex on a gel shift had a lower molecular weight. Surprisingly, we found that the mERα/half-site complex, which migrated faster on the 4% polyacrylamide gels, had a higher K_R value than the mERα/vit ERE complex, reflecting a larger molecular size. The log Y_0 value for the mERα/half-site complex was also larger, indicating a higher negative charge on this complex than on the mERα/vit ERE complex. Therefore, at low polyacrylamide concentrations, the higher negative charge offsets the larger molecular size and allows the mERα/half-site complex to move more quickly.

Protein standards were co-electrophoresed with the gel shift assays, but in separate lanes. From these standards, we were able to estimate the molecular weights of the complexes and the difference between them. Three equations have been used to correlate K_R with molecular weight (see *34* and *35*), and we have applied these to the mERα/vit and mERα/PRL complexes (Table 1, lower part). Although the three equations give varying values for the molecular weights of the complexes, the molecular weight difference between the complexes is quite constant, between 50 000 and 55 000. This value is smaller than expected for an additional mERα protein (66 000) and larger than expected for an additional oligonucleotide (23 000). The increase in negative charge of the mERα/half-site complex, however, strongly suggests that another oligonucleotide is present in this complex. Thus, the higher charge and higher molecular weight of the mERα/half-site complex can be accounted for by a model in which one mERα dimer is bound to two half-site oligonucleotides.

DISCUSSION

It has been known for some time that estrogen regulation can occur through half-sites as well as through inverted repeats, but the way in which the ER interacts with half-sites has not been determined. From X-ray crystallography results (*36*), we know that two ERα DNA binding domains (DBDs) interact with one consensus ERE oligonucleotide, with each DBD binding to one half-site. Also ERα is known to dimerize, with the major interactions occurring through the ligand binding domain (*37*, *38*). There are several theoretically possible ways in which ERα could interact with half-sites: (1) one ERα monomer could interact with one half-site; (2) one ERα dimer could interact with one half-site; or (3) one ERα dimer could interact with two half-sites. In the second possibility, one of the ERα DBDs would interact with the half-site while the other would interact with the adjacent DNA. This type of interaction was observed in the crystal structure of the glucocorticoid receptor DBD binding to an oligonucleotide with two half-sites separated by four base pairs instead of the usual three (*39*). The results presented here argue strongly for the third model. Ferguson analysis was used to demonstrate that the complexes between ERα and half-site oligonucleotides are larger and have higher negative charge than the complex between ERα and a consensus ERE, which contains two ER proteins and one oligonucleotide (*26*). If model 1 was correct, the mass of the ERα/half-site complex would be 40% less than that of the ERα/consensus ERE complex; if model 2 was correct, the size and charge of the ERα complexes with half-site and consensus oligonucleotides would be identical, and there would be little or no difference between their gel shift mobilites. From the results presented here, it is clear that model 3 applies to this interaction. This result also agrees with previous data from Murdoch et al. (*25*) in which the ratio of ER protein to PRL2 half-site oligonucleotide was 1:1.

The best explanation for the observed results, then, is a dimer of ERα binding to two half-site oligonucleotides. In this model, the ligand binding domain of ERα forms a strong dimerization interface that holds the two ERα proteins together (*37*, *38*), and each DNA binding domain binds to a half-site. Since ERα binds to each of the four half-site oligonucleotides tested here in the same manner, this appears to be a general property of ERα which could apply to any half-site sequence. It is likely that the interaction we have observed in vitro also occurs within the cell. Although we had to increase the ERα protein concentration in order to observe interaction with half-sites, the concentration of ERα protein was lower than the estimated physiological concentration of ER within nuclei (*31*).

While the PRL1 imperfect ERE appears to be required for estrogen regulation of prolactin (*21*, *22*), there is evidence that it is not the only sequence within HSII capable of binding to ER and that it is not the only sequence required for E_2 regulation. Maurer and Notides (*20*) identified the PRL1 sequence as an ER binding site by nitrocellulose filter binding with purified ER, but noted that additional sequences also appeared to be involved in ER binding. Another ER binding site within HSII was identified in a region with unusual secondary structure (*24*). Murdoch et al. (*25*) studied the interaction of ER in vitro with the PRL1 imperfect ERE and the PRL2 half-site, and found that ER bound with higher affinity to the PRL2 half-site. The importance of the PRL1 sequence in E_2 regulation is shown by the fact that mutation of PRL1 eliminates E_2 induction in cells (*21*, *22*); however, mutation of the half-site in the PRL5 oligonucleotide has the same effect (*23*). Therefore, at least one of the half-sites studied here is essential for estrogen regulation of the rat prolactin gene. The effects of mutations in the other half-sites have not been studied.

How could these half-site sequences be influencing estrogen regulation of the rat prolactin gene? It is interesting to note that the four half-sites tested here fall into two pairs arranged in direct repeats: PRL4 and PRL2 are separated by 40 base pairs, while PRL5 and PRL3 are separated by 24 base pairs. Direct repeats with similar spacing have already been shown to confer estrogen regulation on a reporter gene in transient transfection assays (*11*). The same

kind of interaction could occur in the nucleosome-free HSII region of the rat prolactin upstream region. Alternatively, ER could interact with the half-sites so that a large loop is formed, with the two ER dimers acting as a clamp to hold the loop in place. In this case, one dimer would interact with PRL2 and PRL5 half-sites, while the other would interact with PRL3 and PRL4 half-sites.

Since other nuclear receptors can interact with half-sites and widely spaced direct repeats, these elements may allow fine-tuning of the response to estrogen. For instance, retinoic acid receptors and vitamin D receptors can also activate from direct repeats (*11*), and PRL2 contains the sequence TAAAG-GTCA to which the orphan receptor NGFI-B binds as a monomer (*40*). Different sets of nuclear receptor family proteins may bind to these sites depending on which hormones and nuclear receptor proteins are present at a particular time. Thus, the use of half-sites in addition to consensus and imperfect EREs may allow for more flexibility in estrogen responsiveness.

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