Estrogen Receptor α Requires No Accessory Factors for High-Affinity Binding to a Consensus Response Element†

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ABSTRACT: Estrogen receptor (ER) α is commonly thought to bind to a consensus estrogen response element (ERE) as a homodimer, but previous experiments have not ruled out the presence of other proteins in the ERα/ERE complex. To characterize this interaction in more detail, we overexpressed mouse (m) ERα in a baculovirus system, using the selective advantage of the apoptosis inhibitor p35. Recombinant mERα possesses the predicted molecular weight and binds 17β-estradiol and an oligonucleotide containing a consensus vitellogenin ERE with high affinity. Over a wide concentration range of mERα protein (0.1−50 nM), only one complex was detected between mERα and vitellogenin ERE in gel shift assays. The ratio of E_2:vitellogenin ERE bound by mERα was close to 2:1, and each complex contained only one ERE. The molecular weight of the complex was determined to be 160 000, very close to that predicted for two mERα proteins and one ERE oligonucleotide, therefore providing strong evidence that no other proteins were present. Recombinant mERα was purified such that it was the only protein observable by silver stain. Purified mERα and mERα in a nuclear extract behaved identically in Ferguson analysis, providing more evidence that only mERα was binding to the ERE. Purified mERα bound vitellogenin ERE with high affinity ($K_d = 0.92 \pm 0.20$ nM), indicating that no other proteins are necessary for high-affinity mERα interaction with a consensus ERE. To determine whether ERα in an estrogen-responsive mammalian tissue behaves the same as the overexpressed mERα, we tested rat uterine cytosol by Ferguson analysis. ERα in rat uterine cytosol behaved identically to overexpressed mERα, suggesting that ERα in the uterine extract also binds to DNA predominantly as a homodimer with no additional proteins.

Estrogens carry out their physiological effects by modifying transcription of specific genes (*1*−*3*), and this function is accomplished by estrogen receptor (ER)[1] α and the recently discovered ERβ (*4*). The ERs possess distinct domains for binding to estrogens and for binding to specific DNA sequences known as estrogen response elements (EREs). The ERE for which ERα has the highest affinity is an inverted repeat of the sequence 5′-AGGTCA-3′ with three nucleotides separating the half-sites, although it is becoming increasingly apparent that EREs can be constituted from other arrangements of half-sites (*5*−*8*).

A number of studies have suggested that ERα binds to EREs as a homodimer. Mixtures of the full-length receptor and truncated forms were shown to form one intermediate gel shift complex on a consensus ERE sequence, as would be expected for a homodimer (*9*). Experiments in which shorter forms of ERα lacking a specific epitope were immunoprecipitated only in the presence of full-length ERα (*10*) showed that ERα could form oligomers, but they did not show how many ERα proteins were interacting. The strongest evidence for ERα binding to a consensus ERE as a homodimer came from the crystal structure of its DNA binding domain (DBD) with a consensus ERE (*11*). Two DBD peptides were found to bind to the ERE, one to each half-site. This is not necessarily the whole picture, however, as the DBD binds to the consensus ERE with much lower affinity than full-length ERα (*12, 13*), and some studies suggest that other domains may influence the structure of the DBD. For instance, full-length ERα bends a consensus ERE to a greater extent than the DBD alone (*14*), and the DBD alone does not distinguish between a consensus ERE and a mutation of this sequence under conditions in which full-length ERα is able to do so (*15*).

All of the studies cited above used ERα and/or fragments of ERα at high concentration or in purified form, and in most cases the protein was produced by transfection of cells that do not normally express ERα. Although ERα may form

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[1] Abbreviations: ER, estrogen receptor; ERE, estrogen response element; DBD, DNA binding domain; RUC, rat uterine cytosol; hsp, heat shock protein; m, mouse; ICPBS, insect cell phosphate-buffered saline; pcv, packed cell volume; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; Hap, hydroxylapatite; STI, soybean trypsin inhibitor; bp, base pairs; SDS, sodium dodecyl sulfate; DRIP, DNA receptor immunoprecipitation; BSA, bovine serum albumin; hsDNA, sonicated herring sperm DNA; E_2, 17β-estradiol; EDTA, ethylenediaminetetraacetic acid; R_f, relative mobility; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; MW, molecular weight; K_{av}, partition coefficient; *R*, Stokes radius; $s_{20,w}$, sedimentation coefficient in water at 20 °C; f/f_0, frictional coefficient; h: human; %T, percentage acrylamide; K_R, retardation coefficient; Y_0, mobility at 0% acrylamide; SfNE, Sf21 cell nuclear extract.

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homodimers under these conditions, that does not necessarily mean that a homodimer is its only active form. In contrast to the homodimer model, Furlow et al. (*16*) estimated the amount of E_2 binding activity versus ERE binding activity in rat uterine cytosol (RUC) and found a ratio of about 1:1. Since these experiments were done at low ERα concentration in an extract of cells that are responsive to estrogens, this raised the possibility that ERα could bind to an ERE as a monomer or heterodimer under these conditions.

The possibility of additional proteins binding to the ERE along with ERα has also been raised. Several proteins have been proposed to enhance the interaction of ERα with an ERE, and these proteins would be expected to associate at least temporarily with an ERα/ERE complex. These include a yeast single-stranded binding protein (*17*), heat shock protein (hsp) 90 (*18*), hsp70, a protein disulfide isomerase, and two uncharacterized proteins (*19*). In support of their interaction with ERα bound to an ERE, several of these proteins were found to coelute with ERα on an ERE affinity column (*19*). Proteins that tightly interact with other nuclear receptors while they are bound to DNA or enhance their binding to response elements have also been reported. For example, HMG1 was found to increase binding of the progesterone receptor to its response element (*20*), and hsp70 was found to interact with the human glucocorticoid receptor bound to a glucocorticoid response element (*21*).

To study ERα interactions with vitellogenin (vit) ERE over a wide ERα concentration range, and to address the involvement of other proteins in this process, we overexpressed mouse (m) ERα in a baculovirus system, and studied its interactions with the consensus ERE. Our results show that purified mERα binds to a consensus ERE with high affinity without the assistance of other proteins. We also provide evidence that purified ERα and ERα in cell and tissue extracts form a complex with a consensus ERE that includes no other proteins.

EXPERIMENTAL PROCEDURES

Plasmids and Viruses. The viruses used were the wild-type *Autographa californica* nuclear polyhedrosis virus (*Ac*MNPV) strain L-1 (*22*), a mutant with the polyhedrin gene removed (Δpol, kindly provided by Dr. L. K. Miller), and vΔ35K/lacZ (*23*). The plasmids used were the *Ac*MNPV transplacement vector pEV/35K/polybsmcr (K. L. Hajek and P. D. Friesen, unpublished results) and pMOR100, which includes the mERα cDNA (kindly provided by Dr. M. G. Parker).

Generation of Recombinant Baculovirus. Standard molecular biology techniques for DNA manipulation were followed (*24*). Sf21 cells (*25*) were propagated as described (*26*). Mouse ERα cDNA was cleaved from the vector pMOR100 with *Eco*RI and ligated into the baculovirus transplacement vector pEV/35K/polybsmcr, which had been digested with *Eco*RI and treated with calf intestinal phosphatase. The resulting plasmid with the mERα cDNA in the correct orientation, pEV/35K/mER, was linearized with *Sca*I. Recombinant viruses were generated by introducing 2 μg of linearized pEV/35K/mER and 0.2 μg of vΔ35K/lacZ viral DNA linearized with *Bsu*36I (*27*) to Sf21 cells in lipofectin as described (*26*). Recombinant viruses were selected from widely spaced plaques to ensure purity. The two viruses with the highest titers were tested for mERα production by Western blot (data not shown), and the one with the higher mERα production was used for all further work.

Sf21 Cell Nuclear Extract. Sf21 cells were infected according to standard procedures (*28*). After 48 h, the medium was aspirated from the dishes. Cells were washed with 5.0 mL of insect cell phosphate-buffered saline (ICPBS; 1.0 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, and 40 mM KCl, pH 6.2) and then scraped up in 2.0 mL of ICPBS and transferred carefully with a Pasteur pipet to a 15 mL centrifuge tube on ice. Cells were spun in a clinical centrifuge for 5 min, and the supernatant was removed with a Pasteur pipet. The packed cell volume (pcv) was estimated and 6 pcv of TDG + PI buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 10% glycerol, 2.0 μg/mL aprotinin, 1.0 μg/mL leupeptin, 0.1 mM phenylmethanesulfonyl fluoride, and 1.0 μg/mL pepstatin A) was added to the cell pellet. The cells were transferred to a Kontes glass/glass homogenizer and broken with 15 strokes of a B pestle. If the cells were not at least 70% broken, as determined by Trypan blue staining, homogenization was continued. The broken cells were spun at 3000*g* for 10 min in an SS-34 rotor to pellet nuclei. Supernatant was removed, and 2.5 pcv of TDG + PI + 0.5 M KCl was added. After a 20 min incubation, the extract was transferred to polycarbonate tubes, spun at 435680*g* for 10 min in a Beckman TLA100 centrifuge and then aliquoted, frozen quickly in liquid nitrogen, and stored at −80 °C.

Purification of mERα. Recombinant mERα was purified from Sf21 cell extract over two columns, hydroxylapatite (Hap) and an ERE affinity column. All steps were carried out at 4 °C. The Hap column (1.0 mL of Hap for each 10 mg of total protein) was prepared by washing with 10 mM K_2HPO_4, pH 7.5. The ERE affinity column was prepared by linking a 29 bp consensus ERE oligonucleotide with an amine tag to CNBr-activated Sepharose beads. The oligonucleotide top strand sequence was 5′NH_2-AGCACGTAG-GTCACTCTGACCTGAAGGCT-3′, and the bottom strand was complementary. The ERE column was prepared by washing with at least four column volumes of T0.1 buffer (50 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 10% glycerol, and 100 mM KCl), where 0.1 in the buffer name refers to the KCl concentration.

Sf21 nuclear extract was thawed and 1.0 M K_2HPO_4, pH 7.5, was added to a concentration of 10 mM. Extract was then added to the Hap column and mixed end-over-end for 30 min. The column was placed in a ring stand, the flowthrough was collected, and then the column was washed with 50 mL of buffer 1 [75 mM K_2HPO_4, pH 7.5, 10% glycerol, 1.0 mM DTT, 20 μg/mL soybean trypsin inhibitor (STI), 1.0 μg/mL each pepstatin A, antipain, leupeptin, and chymostatin, and 2.0 μg/mL aprotinin]. Bound mERα was then eluted with buffer 2 (same as buffer 1 but with 135 mM K_2HPO_4, pH 7.5). Fractions containing mERα were pooled, and a 35 bp oligonucleotide containing no ERE was added as a source of nonspecific DNA. The pooled Hap column eluate was then mixed with 1.0 mL of ERE affinity column beads and mixed end-over-end overnight. The column was then placed in a ring stand and the flowthrough was collected. The column was washed with 4.0 mL of T0.1 + PI (T0.1 as above with 1.0 μg/mL antipain, 1.0 μg/mL

leupeptin, and 2.0 μg/mL aprotinin) and then with 4.2 mL of T0.2 + PI. Finally mERα was eluted with 4.2 mL of T0.4 + PI into tubes coated with 2 mg/mL STI.

Silver Stain and Western Blot of Purified mER. Identical protein samples were separated on two SDS−12% polyacrylamide gels. In one gel, proteins were detected by a quick silver stain protocol (*29*) after the gel was fixed in a solution of 10% ethanol and 5% acetic acid for 30 min. Proteins from the other gel were transferred to a poly(vinylidene difluoride) membrane, and a Western blot was performed with ER 712 antibody, with all steps at room temperature. The membrane was blocked with 5% nonfat dry milk in PBST (20 mM K_2HPO_4/KH_2PO_4, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 2 h at 100 rpm on an orbital shaker. Antibody ER712 (*30*), diluted 1:5000 in PBST + 5% nonfat dry milk, was incubated with the blot for 2 h with shaking, and then the blot was washed extensively with PBST. The secondary antibody, Amersham anti-rabbit IgG linked to horseradish peroxidase, was diluted 1:10 000 in PBST + 5% nonfat dry milk and incubated with the blot for 2 h with shaking, and then the Amersham enhanced chemiluminescence (ECL) kit was used to develop the blot according to the instructions provided.

Oligonucleotide Preparation. Oligonucleotides were synthesized by Research Genetics with the following sequences: vit 31A, 5′-AGCTTCGAGGAGGTCACAGTGACCTGGAGCG-3′; vit 31B,5′-GATCCGCTCCAGGTCACTGTGACCTCCTCGA-3. When annealed, vit 31 A and vit 31 B form a 35 bp double-stranded oligonucleotide with a 4 base overhang on each end. Oligonucleotides were labeled by filling in the overhanging ends using Klenow fragment (*24*). Labeled oligonucleotides were purified on Dupont NENsorb columns and ethanol-precipitated overnight at −20 °C. The oligonucleotides were pelleted at 16000*g* for 30 min, then the supernatant was decanted, and the pellets were desiccated and resuspended in 100 μL of ddH_2O.

DNA/Receptor Immunoprecipitation Assays. The assay described by Furlow et al. (*16*) was modified slightly for use with overexpressed mERα. All assays were carried out in a 100 μL final volume of TDGK buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 10% glycerol, and 0.1 M KCl) with 1.0 mg/mL bovine serum albumin (BSA) from Panvera (Madison, WI). Tubes for measuring total binding contained a 1:20 final dilution of Ab ER712. Tubes for measuring nonspecific binding contained a 1:20 final dilution of nonspecific IgG. Both Ab ER712 and nonspecific IgG contained 50% glycerol and 10 mg/mL BSA. Assays on Sf21 nuclear extract contained 0.1 mg/mL sonicated herring sperm DNA (hsDNA), but assays on purified mERα did not. In all experiments, mERα was occupied either with unlabeled vit ERE oligonucleotide and ^{3}H-labeled 17β-estradiol (E_2) or with unlabeled E_2 and ^{35}S-labeled vit ERE oligonucleotide. After overnight incubation at 4 °C with shaking, 20 μL of a 50% slurry of Pierce ImmunoPure protein A agarose beads equilibrated in TGK buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, and 100 mM KCl) was added, and tubes were shaken at 4 °C for 1 h. Samples were transferred to Bio-Rad PolyPrep columns by adding 3 × 3.0 mL of TT buffer (20 mM Tris-HCl, pH 7.5, and 0.01% Tween-20) to test tubes and pouring into columns. Beads were washed under vacuum with 5 × 3.0 mL of TT buffer, and then the tubes were capped. Labeled DNA was eluted by incubation with 200 μL of TE + 1% SDS for 30 min, followed by uncapping the tube in a scintillation vial and forcing the liquid out with a pipet bulb. Labeled E_2 was eluted similarly with 0.5 mL of ethanol. To each vial, 4.0 mL of ReadySafe (Beckman) scintillation cocktail was added, and each sample was subjected to liquid scintillation counting for 5 min. Binding parameters were calculated by the computer program LIGAND.

ERE Double Labeling Assay. Biotin-labeled vit ERE was obtained from Research Genetics. One 35 base oligonucleotide was labeled on the 5′ end with biotin. The sequence was 5′-biotin-AGCTTCGAGGAGGTCACAGTGACCTGGAGCGGATC-3′. The other 35 base oligonucleotide was complementary. The oligonucleotides were incubated at 95 °C for 10 min, followed by slow cooling to room temperature to promote annealing, and stored at −80 °C. Vit 31A and B were labeled with [α-^{35}S]dATP and purified as described above. Both biotin-vit ERE and ^{35}S-vit ERE were quantified by spectrophotometry at 260 nm, and the specific activity of ^{35}S-vit ERE was calculated. The concentration of vit ERE necessary to saturate 6.7 nM mERα was determined by DRIP assay to be 50 nM (data not shown).

The double label experiment was carried out by mixing various ratios of biotin-vit ERE and ^{35}S-vit ERE at a total ERE concentration of 50.0 nM, and incubating these with Sf21 nuclear extract containing 6.7 nM mERα. Samples (100 μL) were prepared in TDGK buffer with 0.1 mg/mL hsDNA and 0.5 mg/mL BSA. Antibody ER712 was present at 1:20 final dilution. After incubation overnight at 4 °C on an orbital shaker (100 rpm), 20 μL of a 50% slurry of streptavidin−agarose beads (Gibco) was added, and the incubation was continued for 1 h. The samples were then treated exactly as for DRIP assays (above). Nonspecific binding of ^{35}S-vit ERE was measured by running identical assays with ^{35}S-vit ERE and a 35 bp vit ERE with no biotin label. The counts from these tubes were subtracted from the ^{35}S/biotin counts.

Prediction of ^{35}S Binding for Two- and Three-Site Models. If two or more EREs are bound in one mERα/vit ERE complex, then the ERE double labeling assay should measure some specific ^{35}S-vit ERE binding. To predict how much ^{35}S-vit ERE would be detected in this assay, the ER was assumed to have the same affinity for ^{35}S-labeled and biotin-labeled vit EREs. For the two-site model, three species would be present at saturation: those with two biotin-vit EREs (BB), those with two ^{35}S-vit EREs (SS), and those with one of each (SB). The amount of each species is determined by the relative amount of each ERE present. For instance if the reaction contains 10% ^{35}S-vit ERE and 90% biotin-vit ERE, there will be 1% SS (0.10 × 0.10 = 0.01), 81% BB (0.90 × 0.90 = 0.81), and 18%SB (2 × 0.10 × 0.90 = 0.18). The only species detected by the assay is SB, and only one of the two SB binding sites is detected. The concentration of ^{35}S-vit ERE measured by the ERE double labeling assay with any combination of ^{35}S- and biotin-labeled EREs is given by

$$X = 0.5TSB \quad (1)$$

where X is the concentration of ^{35}S-vit ERE bound to mERα and biotin-vit ERE, T is the total concentration of ERE binding sites, and SB is the fraction of mERα bound to one

of each ERE. If we define S as the fraction of total ERE binding sites occupied by ^{35}S-vit ERE (which is the same as the fraction of added ERE labeled with ^{35}S) and substitute for SB in terms of S [$SB = 2\ S\ (1 - S)$], the equation becomes

$$X = T(S - S^2) \quad (2)$$

The analysis for three sites is very similar, the equivalent of eq 1 being

$$X = 0.33T(2S_2B + SB_2) \quad (3)$$

where S_2B is the fraction of mERα bound to two ^{35}S-labeled EREs and one biotin-labeled ERE and SB_2 is the fraction of mERα bound to one ^{35}S-labeled ERE and two biotin-labeled EREs. If we substitute for S_2B and SB_2 in terms of S [$S_2B = 3S^2(1 - S)$ and $SB_2 = 3S(1 - S)^2$], the equation simplifies to

$$X = T(S - S^3) \quad (4)$$

Gel Exclusion Chromatography. A column with an inner diameter of 2.5 cm and length of 50 cm was packed with Sigma S300-HR resin in TDGK buffer at 4 °C. Column flow was controlled with a peristaltic pump. Samples and standards were loaded in 200 μL of TDGK buffer. Before collection of fractions (1.5 mL), a quantity of buffer consisting of most of the void volume of the column (80−90 mL) was gathered. The column was standardized with bovine thyroglobulin, yeast alcohol dehydrogenase, BSA, chicken ovalbumin, and horse heart cytochrome *c* (Sigma), and the void volume was measured with blue dextran. Protein standards and blue dextran were detected by absorbance at 280 nm. The Stokes radii of the standards were calculated from reported diffusion coefficients (*31*−*33*) as described (*32*). Samples containing 0.1 mg/mL hsDNA and 100 nM ^{35}S-labeled vit ERE or mut ERE were fractionated on the column either without protein or with Sf21 nuclear extract containing 35 nM mERα (final concentration). When mERα was included, the samples were incubated on ice for 4 h before being added to the column. Radioactivity was detected by liquid scintillation counting of 200 μL of each fraction.

Glycerol Gradient Sedimentation. Samples for centrifugation contained 100 nM ^{33}P-vit ERE and either Sf21 cell extract containing 35 nM mERα (final concentration) or extract from Sf21 cells infected with Δpol virus. These samples also contained 20 mM Tris, pH 7.5, 0.1 M KCl, 1.0 mM DTT, 100 μg/mL hsDNA, and 50 μg of each protein standard in a 100 μL final volume. The protein standards were horse heart cytochrome *c*, BSA, and sweet potato β-amylase (Sigma). Glycerol gradients (10−30%, with 20 mM Tris, pH 7.5, and 0.1 M KCl) were poured, centrifuged, and fractionated as described by Westwood and Wu (*34*). The positions of the mERα/vit ERE complex and the free vit ERE were determined by liquid scintillation counting of 100 μL of each fraction. The standards were detected by Coomassie staining after trichloroacetic acid precipitation of the remaining 400 μL of each fraction and electrophoresis on an SDS−15% polyacrylamide gel (*34*).

Gel Shift Assays and Ferguson Analysis. Gel shift samples were prepared in TDGK buffer with 0.1 mg/mL hsDNA and 40 000 cpm of ^{32}P-labeled vit ERE. Some tubes also contained Ab ER712 at 1:10 final dilution. Vit and mut oligonucleotides for competition were prepared by annealing complementary 35 base single-stranded synthetic oligonucleotides. The samples were incubated on ice for 4 h before being loaded on a 1.5 mm thick 4% native polyacrylamide gel with an acrylamide:bisacrylamide ratio of 19:1. The high ionic strength gel running buffer contained 50 mM Tris, 390 mM glycine, and 1.0 mM EDTA. The gel contained running buffer and 2.5% glycerol. Orange G dye was loaded in one lane, and the gel was run at 300 V until the dye was close to the bottom of the gel, about 90 min. The gel was then dried onto Whatman 3M paper and exposed to film.

For Ferguson analysis, a series of eight gels from 3.0% to 6.5% acrylamide were run with protein standards on half of the gel and gel shifts on the other half. The protein standards were bovine thyroglobulin, horse spleen apoferritin, sweet potato β-amylase, BSA, and chicken ovalbumin (all from Sigma). After the gels were run, the parts of the gels containing the protein standards were removed and incubated in Coomassie blue staining solution (45% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue dye). After overnight staining the gels were incubated with 2−3 batches of destaining solution (30% ethanol and 5% acetic acid) and then dried on Whatman 3M paper. The parts of the gels containing the gel shifts were transferred directly to Whatman 3M paper, and the tops and bottoms of the gels were marked on the paper. The gels were then dried and exposed to film. After drying, the distances of the protein standards and gel shift bands from the top of each gel were measured and relative mobility (R_f) was calculated by dividing these by the distance of the dye from the top of the gel. The slopes and *y*-intercepts on the graphs were determined by linear regression with the program Graphpad PRISM 2.0.

RESULTS

*Expression of m*ERα *in Baculovirus and Measurement of E_2 and ERE Binding Activities.* The *Autographa californica* nuclear polyhedrosis virus (AcMNPV), the baculovirus commonly used for expression of foreign proteins, encodes a 35 kDa protein that inhibits apoptosis. Viruses unable to produce active p35 protein cause their host cells to initiate apoptosis about 6 h after infection (*35*), and the ability of the virus to replicate is strongly inhibited (*23*). Restoration of p35 allows the virus to replicate normally, thus providing an effective selectable marker for the AcMNPV (*26*). A technique has been developed, based on p35 selection, to produce recombinant baculoviruses more quickly than the visual plaque screening method. The transplacement vector pEV/35K/polybsmcr restores a copy of the p35 gene while at the same time introducing a recombinant protein into the virus under the control of the strong polyhedrin promoter (Figure 1). We cloned the entire mERα cDNA (*36*) into pEV/35K/polybsmcr and introduced this vector with linearized vΔ35K/lacZ baculovirus into Sf21 cells to generate recombinant viruses.

Unlike rat uterine ERα, recombinant mERα could not be extracted from insect cells in low ionic strength buffer; addition of 0.5 M KCl was necessary for extraction. Recombinant mERα had the expected size of about 66 000 based on the amino acid sequence (*36*), although some lower

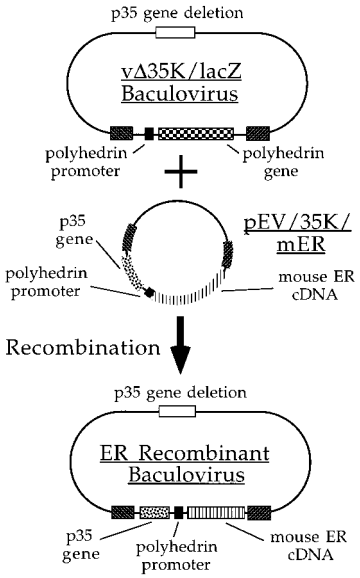

FIGURE 1: Strategy for creation of recombinant baculovirus expressing mERα. vΔ35K/lacZ baculovirus DNA was transfected into Sf21 cells with plasmid pEV/35K/mER to generate mERα-expressing baculoviruses through recombination. vΔ35K/lacZ virus cannot sustain infection of Sf21 cells because it lacks the gene encoding p35. The plasmid pEV/35K/mER contains an active *p35* gene; it also carries the mERα cDNA under control of the polyhedrin promoter. The plasmid sequences flanking the *p35* gene and mERα cDNA (diagonal slashes) are identical to those flanking the polyhedrin gene in the baculovirus, providing substrates for homologous recombination. Viruses that recombine with the plasmid to take up the *p35* gene can sustain an infection and form plaques. Many of these viruses take up the mERα cDNA along with the *p35* gene.

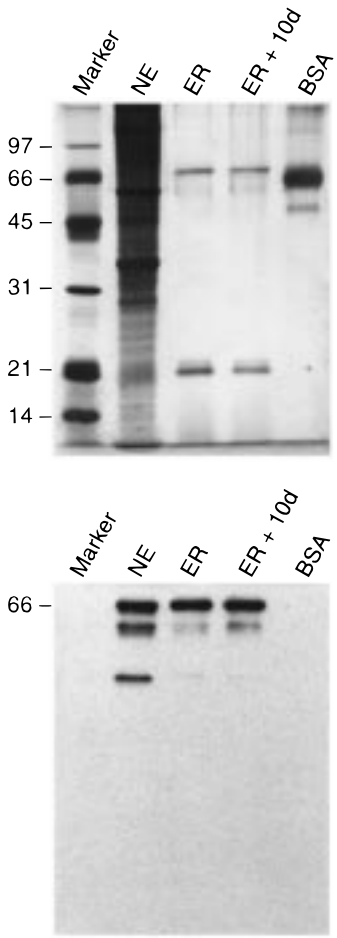

FIGURE 2: Silver stain and Western blot analysis of purified mERα. (Upper panel) Silver stain and (lower panel) Western blot with Ab ER712 of an SDS−12% polyacrylamide gel containing crude and purified mERα samples. Marker, low molecular weight protein standards; NE, nuclear extract from Sf21 cells infected with baculovirus expressing mERα; ER, purified mERα; ER+10d, purified mERα after incubation at 4 °C for 10 days; BSA, bovine serum albumin. The carrier protein during mERα purification is soybean trypsin inhibitor, which has a molecular mass of about 21 kDa.

molecular weight species were present also (Figure 2B). The lower bands are most likely mERα degradation products or incompletely synthesized mERα proteins, because antibody ER712 did not cross-react with any proteins in Sf21 cells infected with Δpol virus (data not shown). On the basis of E_2 binding assays, we estimate that mERα constitutes up to 0.33% of the total protein in infected Sf21 cells, while ERα makes up about 0.01% of protein in rat uterine tissue. The ERα concentration in rat uterine cytosols is generally 1.0−3.0 nM (*16*), while mERα concentration in Sf21 cell nuclear extracts ranged from 350 to 1000 nM.

E_2 binding was measured by standard Hap assay (data not shown), and the measured K_d of 0.5 nM is similar to that measured for ERα from other sources (*37*). The affinity of recombinant mERα for the consensus ERE from the *Xenopus* vitellogenin A2 gene (vit ERE) was measured by the DRIP assay (data not shown). The K_d for this interaction was found to be 5.0 nM, about 10-fold higher than for ERα in rat uterine cytosol measured by the same technique (*16*, *40*, *41*).

Purification of Recombinant mERα and Measurement of Binding Activities. To determine what functions of mERα are carried out by the receptor alone and which may require other proteins, it was necessary to purify the protein from the insect cell extract. ERα has been successfully purified by ERE affinity chromatography previously (*38*, *39*), so this technique was used in combination with a hydroxylapatite (Hap) column. The eluate from the affinity column contained mERα as the major protein band (Figure 2A) except for the carrier protein, soybean trypsin inhibitor (21 kDa). During purification, the lower molecular weight products were removed while full-length mERα protein was retained (Figure 2B). A small amount of degradation was seen after storage of the mER at 4 °C for 10 days, but most of the protein remained intact. In contrast, the E_2 binding activity began to decrease after 3 days storage at 4 °C (data not shown).

Purified mERα was tested for its ability to bind to E_2 and vit ERE by the DRIP assay (Figure 3). The K_d of purified mERα for E_2 was 2.6 ± 0.5 nM, about 5 times the value seen for mERα in nuclear extract. Although there is a decrease in the strength of the interaction, purified mERα retains the ability to bind E_2 with high affinity. The K_d of purified mERα for vit ERE was 0.92 ± 0.20 nM; thus the purified receptor is also able to bind to vit ERE with high affinity. In fact, this K_d value lies within the range of affinities previously reported for ERα in RUC (*16*). Interestingly, the K_d for purified mERα binding to vit ERE is about 5-fold lower than that of mERα in Sf21 nuclear extract, and we attribute this increase in affinity of mERα for vit ERE upon purification to solution conditions. In our procedure, mERα was extracted from insect cells in a high salt buffer, and this would extract other DNA binding

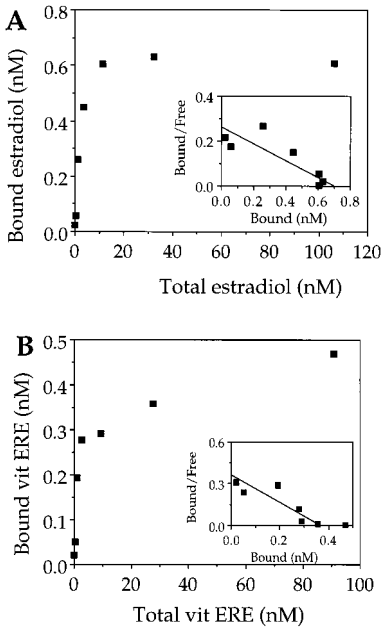

FIGURE 3: E_2 and ERE binding activities of purified mERα. Saturation plots with inset Scatchard plots of (A) E_2 and (B) vit ERE oligonucleotide binding to purified mERα. The K_d for E_2 binding to purified mERα was 2.6 ± 0.5 nM and the K_d for vit ERE binding was 0.92 ± 0.20 nM, calculated by the computer program LIGAND.

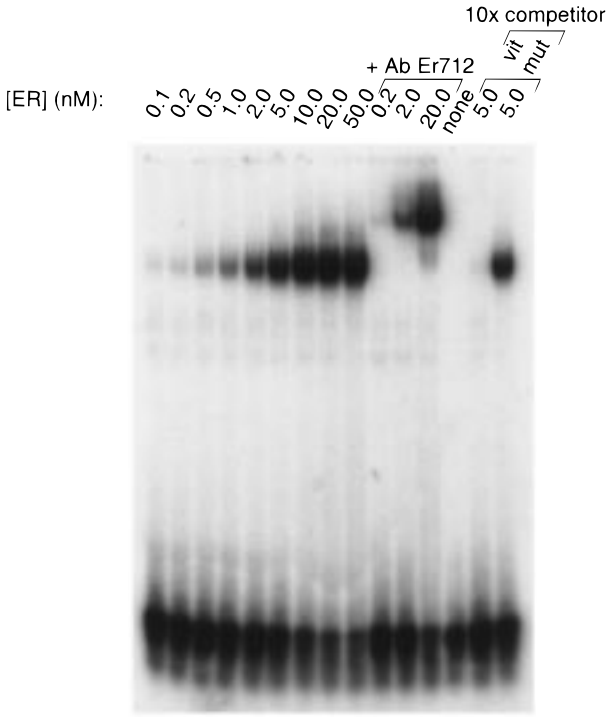

FIGURE 4: Gel shift of vit ERE with Sf21 nuclear extract including increasing mERα protein. Nuclear extract from Sf21 cells expressing mERα was mixed with nuclear extract from Sf21 cells infected with Δpol virus and bound to ^{32}P-labeled vit ERE. The total protein concentration was held constant while the mERα concentration was varied from 0.1 to 50.0 nM. At three concentrations of mERα (0.2, 2.0, and 20.0 nM), Ab ER712 was added to give a supershift. The following lane (labeled none) contains vit ERE with no protein. The last two lanes contain 5.0 nM mERα and a 10-fold excess of unlabeled vit ERE or mut ERE oligonucleotide.

proteins also, while a low ionic strength buffer was used to prepare RUC. Competition from other DNA binding proteins would reduce the apparent affinity of mERα for the ERE in the nuclear extract.

Gel Shift Assay with a Range of mERα Concentration. As a first step toward characterizing the interactions between overexpressed mERα and vit ERE, we investigated how many different complexes were formed in a gel shift assay over a wide range of mERα protein concentrations while the ERE concentration was constant (Figure 4). One major complex was seen over the whole range of concentrations studied, and this complex was shifted further by the addition of antibody ER712, a polyclonal antibody raised against a peptide within the D domain of rat ERα (*30*). Therefore, over almost 3 orders of magnitude of mERα concentration, one predominant complex with vit ERE was detected by gel shift assay.

ERα from RUC has been found to bind to vit ERE about 1000 times more strongly than to a mutated form, mut ERE, which contains a base change in each half site from AGGTCA to AGATCA (*40*, *41*). To test whether overexpressed mERα retains similar specificity, we included 10-fold competing unlabeled vit ERE or mut ERE oligonucleotide along with ^{32}P-labeled vit ERE (Figure 4). Unlabeled vit ERE competes away most of the gel shift signal, while unlabeled mut ERE has no effect. The mERα from baculovirus, then, exhibits similar selectivity for these DNA sequences as ERα from RUC.

ERE Double Labeling Assay. To determine the exact composition of the mERα/vit ERE complex observed in the gel shift assays, we needed to determine how many vit ERE oligonucleotides were present in the complex. To answer this question we used an assay in which mERα was mixed with a combination of biotin-labeled vit ERE and ^{35}S-labeled vit ERE. Streptavidin−agarose beads were used to separate biotin-vit ERE oligonucleotides, and whatever was bound to them, from the other components. Specifically bound ^{35}S-vit ERE could only be associated with the streptavidin−agarose beads through an association with mERα and biotin-vit ERE (Figure 5A). Nonspecific binding was determined by measuring the amount of ^{35}S-vit ERE bound when an unlabeled vit ERE oligonucleotide was substituted for the biotin-labeled vit ERE oligonucleotide. If each mERα/vit ERE complex contained only one vit ERE, then no ^{35}S-vit ERE would be detected. The amount of ^{35}S-vit ERE bound if each complex contained two or three EREs was calculated as described in the Experimental Procedures section and compared with the experimental results (Figure 5B). Although a low level of ^{35}S-vit ERE binding was detected, it was much less than would be found if each mERα/vit ERE complex contained two EREs. The results therefore show that the major mERα/vit ERE complex detected in the gel shift assay contains only one ERE oligonucleotide.

Measurement of the Ratio of Bound E_2 to Bound ERE. To measure the number of mERα proteins bound to a consensus ERE, we used the DRIP assay, which has been adapted for use with ERα by Furlow et al. (*16*). In this assay the ERα-specific antibody ER712 was used to immobilize ERα on beads and then the number of E_2 and ERE binding sites present in a sample of ERα protein were determined by saturation with radioactive ligands. A number of experiments were performed to measure the ratio of bound E_2 to bound ERE at saturating levels of these ligands and at varying mERα protein concentrations. The results of these

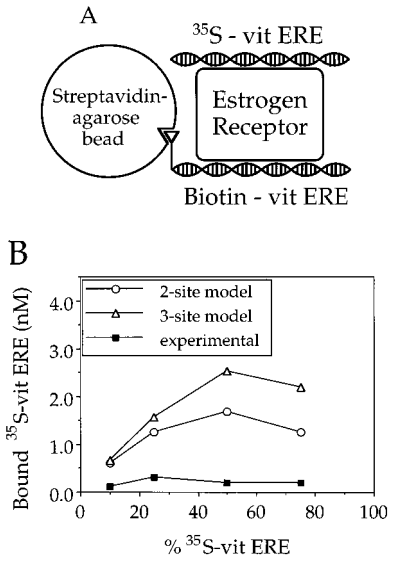

FIGURE 5: ^{35}S/biotin ERE double labeling assay to determine the number of EREs bound in one complex with mERα. (A) In this assay, mERα was incubated with varying ratios of ^{35}S-labeled and biotin-labeled vit ERE oligonucleotides, and then streptavidin−agarose beads were used to capture the biotin-labeled vit ERE. Specifically bound ^{35}S-labeled vit ERE must be bound to mERα that is also bound to biotin-labeled vit ERE. (B) Sf21 nuclear extract containing 6.7 nM mERα was incubated with varying amounts of ^{35}S- and biotin-labeled vit EREs adding up to 50 nM total ERE. The amount of ^{35}S-vit ERE bound was plotted against the percentage of ^{35}S-vit ERE added to the assay (experimental, ■). The amount of ^{35}S-vit ERE predicted to be bound if there were two (○) or three (△) ERE binding sites per mERα/ERE complex were plotted also. These were calculated according to the equations in the Experimental Procedures section.

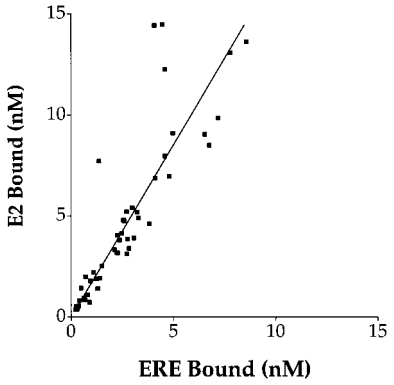

FIGURE 6: Comparison of E_2 and vit ERE bound to mERα in DRIP assays. The amount of ^{3}H-labeled E_2 bound and the amount of ^{35}S-labeled vit ERE oligonucleotide bound were measured in DRIP assays where the ligands were present at saturating concentrations. The line, calculated by the computer program PRISM, was constrained to pass through the origin. The slope is 1.71 ± 0.08 (n = 49).

experiments are combined in Figure 6. A straight line was forced to go through the origin, since the gel shift assay in Figure 4 found one complex at all mERα concentrations. The slope of the line gives the ratio of bound E_2 to bound vit ERE and this slope was found to be 1.7 ± 0.1. In addition, several experiments were performed in which the mERα concentration was held constant while E_2 or ERE concentration was varied (data not shown). From these saturation curves, B_{max} values for E_2 and ERE binding were determined, and the ratio of the E_2 B_{max} to the ERE B_{max} was found to be 1.8 ± 0.2 (n = 4). The recently determined

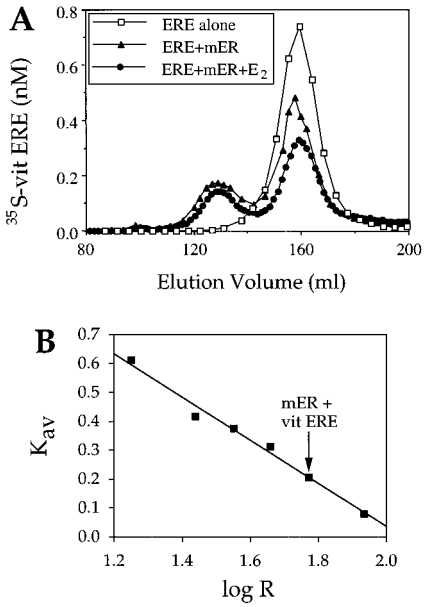

FIGURE 7: Gel-exclusion chromatography of mERα bound to vit ERE. (A) Gel exclusion chromatography was performed with ^{35}S-labeled vit ERE alone or with ERE combined with Sf21 nuclear extract containing mERα, with or without E_2. The amount of ERE in each fraction was determined by liquid scintillation counting and plotted against elution volume. The void volume was 100 mL. (B) Plot of K_{av} vs log R for column standards. The position of the mERα/vit ERE complex is also shown.

structure of the ERα ligand binding domain shows that one estrogen is bound to each domain (*42*). Since we found a ratio of E_2 to vit ERE close to 2.0, it is most likely that two mERα proteins are present for every one ERE; and since we found that only one ERE is present in the complex, it follows that two mERα proteins are present.

Molecular Weight Estimation for the mERα/vit ERE Complex. To determine if additional proteins might be bound to the mERα and/or vit ERE, we estimated the molecular weight (MW) of this complex by a combination of gel exclusion chromatography and glycerol gradient sedimentation as described by Siegel and Monty (*32*). When vit ERE was run over a S-300HR gel-exclusion column with no protein added, it eluted at about 160 mL (Figure 7A). Addition of Sf21 nuclear extract containing mERα resulted in the formation of another species, presumably mERα bound to vit ERE, which eluted at about 130 mL. This species was formed in the presence of E_2 or in the absence of any ligand, as expected for mERα (*16, 40*). To show that this peak was specific for vit ERE, we performed the same experiment with mut ERE. Mut ERE eluted at the same position as vit ERE, about 160 mL, but no peak was observed when Sf21 nuclear extract containing mERα was added (data not shown). The gel-exclusion column results paralleled the gel shift results in that one protein/DNA complex was formed, and it showed much higher affinity for vit ERE than mut ERE. Therefore, this peak most likely contains mERα. A plot of the partition coefficient (K_{av}) versus the log of the Stokes radius (R) gave a straight line for the protein standards (Figure 7B), and R for mERα/vit ERE was found to be 59.2 ± 0.9 Å (n = 2).

For estimation of the sedimentation coefficient ($s_{20,w}$) of the mERα bound to vit ERE, we incubated ^{33}P-labeled vit ERE oligonucleotide with Sf21 nuclear extract from cells

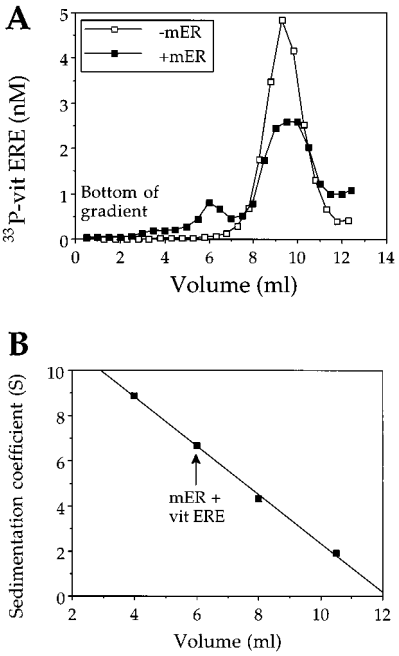

FIGURE 8: Glycerol gradient centrifugation of mERα bound to vit ERE. (A) ^{33}P-Labeled vit ERE oligonucleotide was fractionated on 10−30% glycerol gradients with nuclear extract of Sf21 cells infected either with Δpol virus (−mER) or virus expressing mERα (+mER). Protein standards were cofractionated in each gradient. Radioactivity in each fraction was detected by liquid scintillation counting and plotted against the elution volume. (B) Plot of sedimentation coefficient vs elution volume for protein standards. The position of the mERα/vit ERE complex is also shown.

producing mERα or from cells infected with Δpol virus and then fractionated these mixtures on 10−30% glycerol gradients. Protein standards were included in each gradient along with the oligonucleotide and Sf21 extract. Similar to the gel shift and gel-exclusion column results, we observed one peak when no mERα was present and two peaks when extract containing mERα was included (Figure 8A). We conclude that the peak observed at 6.0 mL from the gradient bottom in Figure 8A contains mERα bound to vit ERE. A plot of the volume at which the proteins were eluted from the gradient versus their previously reported $s_{20,w}$ values (Figure 8B) gave a straight line, and $s_{20,w}$ for mERα/vit ERE was estimated as 6.7 ± 0.0 S (n = 2).

The partial specific volume of mERα was estimated from the amino acid sequence to be 0.721 mL/g (*43*). Since mERα constitutes the majority of the protein/DNA complex, we used the mERα partial specific volume as an approximation for that of the entire complex. With the R and $s_{20,w}$ values determined above, this gives a molecular weight estimate for the mERα/vit ERE complex of 160 000, very close to that predicted for two mERα proteins and one oligonucleotide (2 × 66 000 + 1 × 23 000 kDa = 155 000), with a frictional coefficient (f/f_0) of 1.65. Since we showed above that this complex contains one oligonucleotide and that the ratio of E_2 bound to ERE bound is 2:1, this strongly argues that only two mERα proteins and one oligonucleotide are present.

Ferguson Analysis of ERα from Various Sources Binding to vit ERE. The experiments analyzing the size of the mERα/vit ERE complex were performed with mERα in nuclear extract from Sf21 cells. We wanted to see whether the same complex was formed with purified mERα, purified human (h) ERα, and ERα in RUC. Gel shift assays are convenient for analyzing many protein/DNA interactions simultaneously; however, limited conclusions can be drawn because mobility is affected by the size, charge, and shape of the species being studied (*44*). We therefore compared ERα from these sources binding to vit ERE oligonucleotides by Ferguson analysis. In this procedure, mobility relative to a dye is measured on several gels with varying polyacrylamide concentrations. A plot of the logarithm of relative mobility (log R_f) versus the percentage of acrylamide in the gel (%T) yields a straight line (*45*). The slope is known as the retardation coefficient (K_R), which can be related to molecular weight, and the y-intercept corresponds to the logarithm of the mobility at 0% acrylamide (log Y_0). Figure 9 shows examples of gels used for Ferguson analysis. In Figure 9A, ERα from RUC was compared with mERα in Sf21 cell nuclear extract (SfNE), and both were shifted further with antibody ER712. The Ferguson plots from these data are shown in Figure 10A, and the K_R and log Y_0 values are presented in Table 1. Figure 9B shows a gel on which

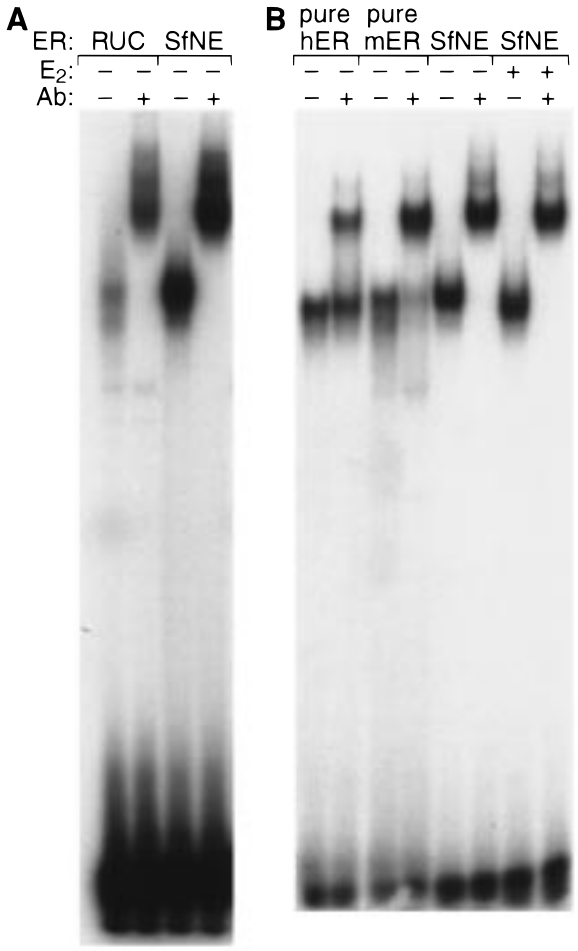

FIGURE 9: Gel shift assay of ERα from several sources binding to vit ERE. Each source of ERα protein was incubated with ^{32}P-labeled vit ERE oligonucleotide for 4 h at 4 °C and then separated on a 4.0% polyacrylamide gel. The ERα concentration in each sample is given below. The second lane of each pair contains antibody ER712 at 1:10 final dilution. (A) RUC, rat uterine cytosol, approximately 1.0 nM ERα; SfNE, Sf21 nuclear extract, 3.5 nM mERα. (B) Pure hER: purified hERα from Panvera, 20 nM; pure mER: mERα purified from Sf21 nuclear extract, 25 nM; SfNE, Sf21 nuclear extract, 6.0 nM mERα. The rightmost two lanes contained 10 nM E_2. In panel B the first lane of each pair contained nonspecific IgG at 1:10 final dilution.

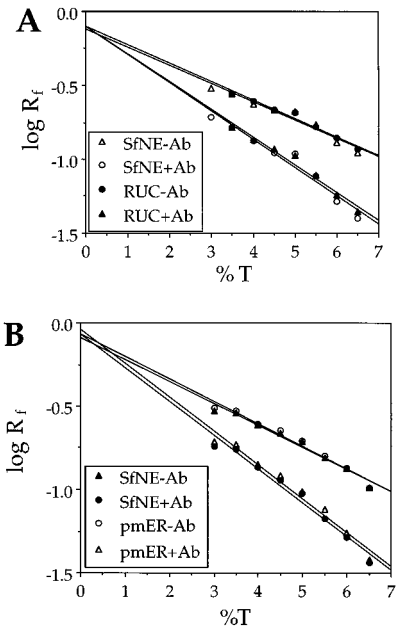

FIGURE 10: Comparison of several sources of ER on Ferguson plots. (A) Ferguson plot of gel shifts and supershifts for Sf21 nuclear extract containing mERα (SfNE) and rat uterine cytosol (RUC). −Ab refers to the absence of ER712 antibody, while +Ab indicates antibody ER712 was present. One of the gels used for this graph is shown in Figure 9A. The slopes and intercepts of the lines are given in Table 1. (B) Ferguson plot of gel shifts and supershifts for Sf21 nuclear extract containing mERα (SfNE) and purified mERα (pmER). One of the gels used for this graph is shown in Figure 9B. The slopes and intercepts of the lines are given in Table 1.

Table 1: Comparison of Ferguson Plot Parameters for Several Sources of ERα[a]

expt	protein	Ab[b]	log Y_0	K_R
A	Sf nuclear extract	−	−0.122 ± 0.052	0.123 ± 0.011
A	rat uterine cytosol	−	−0.103 ± 0.042	0.124 ± 0.008
A	Sf nuclear extract	+	−0.105 ± 0.078	0.191 ± 0.016
A	rat uterine cytosol	+	−0.099 ± 0.073	0.188 ± 0.014
B	Sf nuclear extract	−	−0.090 ± 0.044	0.132 ± 0.009
B	purified mER	−	−0.066 ± 0.047	0.135 ± 0.010
B	purified hER	−	−0.053 ± 0.046	0.133 ± 0.009
B	SfNE + E_2	−	−0.084 ± 0.039	0.128 ± 0.008
B	Sf nuclear extract	+	−0.065 ± 0.068	0.203 ± 0.014
B	purified mER	+	−0.041 ± 0.071	0.202 ± 0.015
B	purified hER	+	+0.009 ± 0.078	0.212 ± 0.016
B	SfNE + E_2	+	−0.077 ± 0.054	0.197 ± 0.011

[a] K_R and log Y_0 values were calculated from Ferguson plots of ERα from several sources binding to vit ERE. These values come from two different experiments: A corresponds to the Ferguson plot in Figure 10A, and B corresponds to the Ferguson plot in Figure 10B. The values were calculated with the computer program PRISM and are shown with standard errors. [b] Minus or plus indicates respective presence or absence of ER712 antibody.

mERα in SfNE was compared with purified mERα and purified hERα. In the same gel, the effect of adding E_2 to SfNE was studied. In Figure 10B, only Ferguson plots for SfNE and purified mERα are shown, for clarity; K_R and log Y_0 values for all of these species are reported in Table 1.

The most intense shifted band seen with RUC runs identically to the mERα band in SfNE (Figure 9A), and the supershifted bands also have similar mobilities. In RUC there is an additional less intense band with higher mobility that is also shifted with the antibody; however, we only studied the major band. The corresponding Ferguson plot (Figure 10A) shows that the mobilities of overexpressed mERα and RUC ERα are very close over the entire range of gel concentrations, both with and without antibody ER712, and the K_R and log Y_0 values are identical within experimental error (Table 1). This indicates that the sizes and charges of the two complexes are virtually identical, and it suggests that ERα in RUC binds to vit ERE as a homodimer. In further support of this conclusion, a series of six experiments performed on RUC using DRIP assays found that the ratio of E_2 bound to ERE bound was 1.85 ± 0.24 (data not shown). This result conflicts with previous data obtained by the same technique (*16*), and the reason for the discrepancy is not known; however, we have confirmed our results using several methods.

We compared purified mERα to unpurified mERα in SfNE to see if the same size complex was formed regardless of the presence of other proteins. The Ferguson plot results clearly show that this is the case (Figure 10B), both in the absence and in the presence of antibody ER712. We also tested commercially available purified hERα in Ferguson analysis, and it behaved similarly to both crude and purified mER (Table 1). Unlike mERα, purified hERα was not completely supershifted by antibody ER712. This is not surprising, as this antibody was produced against a peptide from the rat ERα D domain, which is completely conserved in mERα but not in hERα (*30*). We also tested the effect of adding E_2 to ERα in SfNE. While addition of E_2 caused a noticeable difference in the mobility (Figure 9B), which has been studied in more detail by others (*46*, *47*), it caused no statistically significant difference in the K_R and log Y_0 values determined from Ferguson analysis, suggesting that the difference is caused by a conformation change rather than a change in composition.

Ferguson analysis can be used to estimate MW by relating the K_R of a protein of unknown size to the K_Rs of protein standards of known MW. Three plots have been used for this purpose: MW vs K_R, log MW vs log K_R, and $MW^{1/3}$ vs $K_R^{1/2}$ (*48*, *49*). This technique has recently been used to estimate the sizes of protein/DNA complexes (*50*−*52*), and we applied this technique to the mERα/vit ERE complex, both with and without antibody ER712. For all three of the plots relating K_R with MW, r^2 values were between 0.98 and 1.0 (data not shown). The estimated MW values for mERα/vit ERE with and without antibody and the differences between these values are shown in Table 2. There was some variation in the MW estimates for mERα/vit ERE, depending on which equation was used. All of the mERα/vit ERE estimates are larger than we found by gel-exclusion chromatography and density gradient sedimentation. This is most likely due to the fact that Ferguson analysis cannot separate the contributions of size and shape on gel mobility, whereas the combination of gel-exclusion chromatography and density gradient centrifugation can distinguish between these factors. Therefore the MW estimates from Ferguson analysis are likely to be overestimates. The differences between the MW values with and without antibody were similar for all three equations, between 150 000 and 160 000. Since the MW of one IgG molecule is about 150 000, this result indicates that only one antibody is present in the major supershifted band observed on our gel shift assays, rather than two. This could indicate some asymmetry in the mERα/ERE complex

Table 2: Molecular Weight Estimates of the MERα/Vit ERE Complex from Ferguson Analysis

	molecular weight[a] (MW) estimation (×1000)		
complex	MW vs K_R	logMW vs logK_R	$MW^{1/3}$ vs $K_R^{1/2}$
mER+vitERE	244 ± 6[b]	222 ± 6[b]	214 ± 6[b]
mER+vitERE+Ab	397 ± 12	382 ± 12	373 ± 12
difference (Ab)	153 ± 6	160 ± 7	159 ± 7

[a] The MW of mERα/vit ERE complexes in the presence and absence of antibody ER712 were estimated from seven sets of polyacrylamide gels. For each set of gels, the difference between these values was also calculated, and this corresponds to the increase in MW when the antibody is present. Values are shown as the mean and standard error for seven experiments. MW estimates were determined on the basis of three different plots relating MW with K_R. For all of these plots, $r^2 > 0.980$. The MW estimates were calculated from linear curve fits by the computer program Cricket Graph. [b] For comparison, the MW estimate based on gel-exclusion chromatography and density gradient centrifugation was 160 000.

such that only one epitope is exposed. Alternatively, one antibody could be binding to both epitopes, or binding of one antibody could sterically exclude the binding of another.

DISCUSSION

In this study we have asked two questions about the interaction of the mouse ERα with a consensus ERE oligonucleotide. First, what is the exact composition of the protein/DNA complex? ERα is widely thought to bind to a consensus ERE as a homodimer, but previous experiments analyzing this interaction have not addressed the possibility that proteins other than ERα are present in the ERα/ERE complex. Also, recent results have suggested that ERα in rat uterine cytosol may not always bind to a consensus ERE as a homodimer (*16*). Here we have provided strong evidence that mERα binds to a consensus ERE as a homodimer with no other associated proteins, regardless of whether it is purified or within a cell extract. ERα in rat uterine cytosol and purified human ERα behaved similarly to mERα, suggesting that they also bind to a consensus ERE as homodimers without additional proteins.

Although the ERα complex with a consensus ERE has long been assumed to contain only one ERE oligonucleotide, we felt that this assumption needed to be tested in order for us to determine exactly what is present in the ERα/ERE complex. In this experiment (Figure 5), if ERα was bound to two or more EREs, we would have detected specific binding following a specific pattern depending on how many EREs were bound. Our results showed that only one ERE was bound in the majority of complexes, although a minority contained more than one. Since we measured a ratio of bound E_2 to bound ERE of 2:1 in the DRIP assays, this told us that two ERα proteins were present along with the one ERE. Using the method of Siegel and Monty (*32*), we estimated the MW of the mERα/ERE complex as 160 000. Since two ERα proteins and one ERE would contribute 155 000, this rules out the possibility of another protein interacting with ERα and the ERE. These experiments were carried out with overexpressed mERα in a nuclear extract from Sf21 insect cells. Using Ferguson analysis, we compared mERα in Sf21 nuclear extract with mERα purified from these cells, with rat uterine cytosol, and with commercially available purified human ERα (Figures 9 and 10 and Table 1). ERα from all of these sources formed very similar complexes with a consensus ERE oligonucleotide, providing strong evidence that they are all composed of one ERE oligonucleotide and two ERα proteins.

Second, we asked if purified mouse ERα could bind to a consensus ERE or if other proteins were required for this interaction. There have been conflicting reports on this issue using ERα from other species. Several researchers have found that purified human (*17*, *19*) or calf (*18*) ERα was incapable of binding to an ERE without the assistance of other proteins, and in one of these studies several proteins were observed to copurify with hERα (*19*). On the other hand, Obourn et al. (*38*) observed purified hERα binding to an ERE, although they did not measure the affinity of the interaction, and a 30 kDa protein was purified along with hERα. Recently Ozers et al. (*15*) observed purified hERα binding to a consensus ERE with high affinity (K_d = 1.8 nM) using fluorescence anisotropy, while Cheskis et al. (*53*), using the same protein with a different technique, observed K_d values of 8−62 nM, depending on whether hERα was unoccupied or bound to various ligands. We found that purified mERα still bound to a consensus ERE with high affinity, showing that this interaction can take place without the assistance of other proteins. Purified mERα was the major protein present as detected by silver stain (Figure 2A), and no contaminating proteins were detected. Using the DRIP assay, we found a K_d of 0.92 nM for purified mERα occupied with E_2, showing that purified mERα was able to bind to the consensus ERE with an affinity similar to that of ERα in rat uterus (*16*, *40*, *41*).

Taken together, our results strongly suggest that mERα is capable of binding to a consensus ERE in the absence of other proteins. We found that purified mERα binds to a consensus ERE with high affinity, and no other proteins were present in the mERα/vit ERE complex. Ozers et al. (*15*), using the technique of fluorescence anisotropy, recently found that purified human ERα binds to a consensus ERE oligonucleotide with high affinity. Obourn et al. (*30*) also reported that purified human ERα binds to a consensus ERE. The identification of proteins that assist ERα binding to the consensus ERE has been based on in vitro experiments in which purified ERα alone did not bind to the ERE (*17*−*19*). Therefore our results with mouse ERα and those of others with human ERα cast considerable doubt on the claims that ERα by itself is incapable of binding to an ERE and other proteins are necessary for this interaction. We cannot completely rule out the possibility of other proteins assisting this protein/DNA interaction, however. Even with purified proteins, it is possible that a protein present at a very low level could transiently interact with the receptor to facilitate its interaction with the ERE and then dissociate from the complex.

The discrepancies observed between different laboratories on the question of purified ERα binding to EREs could, however, be due to sample preparation. We believe that others may have seen a drop in ERE binding activity because of ERα protein denaturation or because purified ERα was adsorbed to surfaces. Two groups (*18*, *19*) used harsh conditions for elution of ERα, with denaturants (urea, sodium thiocyanate), organic solvents (dimethylformamide), and/or detergents (CHAPS). These were the same two groups that found heat shock proteins to increase ERα's ERE binding

activity. Possibly the heat shock proteins were refolding ERα disrupted by the harsh solvent conditions, and this increased the ERE binding activity. Another group (*17*) did not report the use of carrier protein in their purification or gel shift assay, and this may have caused the ER to be lost on surfaces. Obourn et al. (*38*) observed purified human ERα binding to an ERE in a gel shift assay, but they noted that if BSA was not included, the binding was lost. Since different groups have found different proteins to help purified ERα bind to an ERE, it is likely that inclusion of a carrier protein is necessary, but many different proteins can suffice.

We used several techniques to analyze the size and shape of the mERα/ERE complex. On the basis of gel exclusion chromatography and gradient sedimentation, the molecular weight of the mERα/ERE complex was 160 000, and the corresponding frictional coefficient was 1.65, indicating a nonspherical structure. Our results agree closely with those of Redeuilh et al. (*55*), who used the same techniques to analyze partially purified bovine ERα without DNA. Their calculated molecular weight was 129 000, consistent with an ERα homodimer, and the frictional coefficient was 1.57. The similarity between frictional coefficients with and without the ERE suggests that ERα may not undergo a large conformation change upon binding to the ERE, although confirmation of this will require determination of the structures. Gel-exclusion chromatography can be used to estimate protein MW by plotting log MW vs elution volume, assuming the protein studied is approximately spherical (*60*). Our frictional coefficient suggests that this is not a good assumption for mERα bound to an oligonucleotide of 35 bp, and a log MW vs elution volume plot with our data results in a MW estimate of 260 000, far from the MW determined when shape is taken into account. In addition, Ferguson analysis, which also does not take shape into account, gave a much higher MW estimate than the combination of gradient sedimentation and gel-exclusion chromatography (Table 2).

While the results reported here and those of others (*9*, *11*) show that ERα binds to a consensus ERE as a homodimer in vitro, and E_2 binding has been reported to show cooperativity in vitro (*38*, *61*, *62*), one might expect estrogen binding and estrogenic responses to display cooperativity in living cells; however, this has not been observed. Neither estrogen binding (*63*) nor E_2-dependent ER downregulation (Y.-J. Lee and J. Gorski, unpublished results) demonstrates cooperativity, suggesting that the apparent cooperativity observed in vitro does not apply to in vivo conditions. One explanation for this incongruity is that ER dimers are not the major species regulating estrogen-induced transcription in vivo. Recently hERRa1 (*64*, *65*) and COUP-TF (*66*) have been found to interact with ERα in vitro, although neither of these proteins has been found to bind to an ERE as a heterodimer with ERα. ERE half-sites are perhaps the most likely sequences on which heterodimerization might occur. Half-sites are capable of promoting estrogen-dependent transcription regulation (*5*−*8*), and we predict that ERα binds to some of these sequences as a heterodimer. Potentially ERα could interact with a wide number of other transcription factors, depending on the sequences adjacent to each half-site.

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