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Rapid Purification of the Estrogen Receptor by Sequence-Specific DNA Affinity Chromatography†

Franklin V. Peale, Jr.,‡ Yuko Ishibe, Carolyn M. Klinge,§ Sayeeda Zain, Russell Hilf, and Robert A. Bambara*

Department of Biochemistry and Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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ABSTRACT: Rapid purification of calf uterine estrogen receptor (ER) to near homogeneity has been accomplished by use of sequence-specific DNA affinity resin. Very high selectivity for the estrogen receptor is achieved through the use of DNA–Sepharose containing eight tandem copies of a consensus estrogen response element (ERE) DNA sequence. The highly purified ER prepared by this new scheme may be labeled economically with ligands of high specific activity. This purification scheme selects for intact receptors retaining function in both estrogen-binding and DNA-binding domains. Purified receptor has an electrophoretic mobility consistent with a molecular weight of 68 000, sediments as a 5S species on sucrose gradients, and reacts with antibody specific to the human estrogen receptor.

Purification schemes for isolating the estrogen receptor protein (ER)[1] have generally depended on the very high selectivity offered by estrogen affinity chromatography. However, resins containing high concentrations of high-affinity estrogens also make difficult the subsequent elution of the purified ER. Various solutions to this problem have been described. Elution of ER from estradiol affinity resins (Sica & Bresciani, 1979; Greene et al., 1980) requires a chaotropic agent such as thiocyanate (Molinari et al., 1977; Kumar et al., 1978), in combination with micromolar concentrations of estradiol. However, such treatment also promotes dissociation of the ER dimer into monomeric subunits (Sica et al., 1976; Weichman & Notides, 1979), necessitating further steps to determine the fraction of purified ER retaining such properties as DNA-binding ability. Alternatively, resins containing low-affinity estrogenic ligands, such as estrone, allow elution of ER with higher affinity 17β-estradiol, without the use of chaotropic agents (Ratajczak & Hähnel, 1980; Atrache et al., 1985). With both of these approaches, the use of high radiospecific activity estradiol at the requisite concentrations is prohibitively expensive, so that the purified ER is labeled to a low specific activity, usually less than 5 Ci/mmol.

We report here a scheme for purifying ER from calf uterus conceptually based on the sequence-specific DNA affinity method of Kadonaga and Tjian (1986). The technique is rapid and yields nearly homogeneous ER labeled with high specific activity ligands at minimal cost. The method should be applicable to other steroid receptors whose hormone response elements have been identified.

Experimental Procedures

Preparation of the DNA Affinity Resin. Double-stranded 38-bp oligonucleotides containing a consensus estrogen-responsive element (ERE)[2] (Peale et al., 1988) were ligated head-to-tail, yielding tandem multimers that were cloned by blunt-end ligation into the *Sma*I site of pGEM-7Zf(+)

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* Address correspondence to this author at the Department of Biochemistry, Box 607, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.

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[1] Abbreviations: bp, base pair; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor; ERE, estrogen response element; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

[2] The ERE sequence is 5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′.

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(Promega). A plasmid containing eight tandem copies of the consensus ERE was grown in *Escherichia coli* JM109 without chloramphenicol to reduce the loss of tandem repeat sequences (Rosenfeld & Kelly, 1986). Plasmid DNA from 2-L cultures was isolated by alkaline–SDS lysis (Birnboim & Doly, 1979). The tandem octamer was excised from 2.2 mg of plasmid DNA by digestion with *Eco*RI and *Hin*dIII. The 337-bp fragment (304 bp of ERE sequences plus 33 bp of flanking DNA) was separated from the plasmid DNA by acrylamide gel electrophoresis and recovered by electroelution. The DNA obtained (20 μg) was resuspended in 100 μL of water and desalted on a 1-mL column of Sephadex G-50 equilibrated in water (Penefsky, 1977), in preparation for coupling to Sepharose resin. An aliquot (1.15 μg) of the isolated DNA fragment was labeled by incorporation of α-[^{35}S]thio-dATP (>400 Ci/mmol) at the recessed 3′-termini, utilizing the Klenow fragment of *E. coli* DNA polymerase I. The labeled DNA (3.8×10^6 dpm total) and unlabeled DNA fragments were coupled to 0.3 g (1-mL settled volume) of cyanogen bromide activated Sepharose 4B-CL (Pharmacia) according to the protocol supplied by the manufacture, except that the dried resin was resuspended in ice-cold water until all crystalline solids had dissolved. The coupling reaction was allowed to proceed overnight at room temperature in a final volume of about 2 mL, and was followed by a blocking step with 1 M ethanolamine, pH 8.0, for 4 h. After the resin had been washed as recommended, the efficiency of DNA coupling was determined by scintillation counting of ^{35}S in aliquots of resin slurry. The 2.9 μg of DNA attached to the resin (14% of input amount) has a theoretical capacity of 104 pmol, or 14 μg of ER dimer, assuming 650 g/mol of DNA bp, eight ER dimers bound per 337-bp octamer fragment, and 134 000 g/mol of ER dimer (Green et al., 1986; Krust et al., 1986; Koike et al., 1986). The resin was washed and stored at 4 °C in 10 mM Tris-HCl, pH 7.6, 300 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, as recommended by Kadonaga and Tjian (1986).

Purification of the Estrogen Receptor. Calf uteri were obtained at a local slaughterhouse, transported in dry ice, frozen in liquid nitrogen, and stored at −70 °C until used. All further steps were performed at 0–4 °C. A 0–30% ammonium sulfate fraction of a high-speed postmicrosomal supernatant solution was prepared as described by Weichman and Notides (1977) and modified by Klinge et al. (1987). The ammonium sulfate precipitated protein obtained from an aliquot of cytosol solution was resuspended in 5–10% of the original cytosol volume of TDP buffer [40 mM Tris·HCl (such that the final pH was 7.5 at room temperature), 1 mM dithiothreitol, 0.5 mM PMSF] containing 175 mM KCl (TDPK-175) and desalted by elution through Sephadex G-25 resin equilibrated in the same buffer. In an adaptation of the Penefsky method, columns were constructed in 60-mL syringe barrels fitted with porous plastic frits (Bolab Inc., Lake Havasu City, AZ) and containing 60 mL of resin. The resin was dehydrated by centrifugation for 2 min at 500*g* at the column tip in an IEC Model PR-2 centrifuge equipped with swinging-bucket rotor Model 253. To each dehydrated column, 10–14 mL of receptor protein solution was applied and eluted by centrifugation as before.

A preliminary measure of the ER content of the spin-column effluent was made to ensure that sufficient estradiol was added to fully saturate the ER in the sample. To determine the total estradiol-binding capacity of the sample, a 20-μL aliquot of spin-column effluent was added to 980 μL of TDP buffer containing 5 nM [^{3}H]-17β-estradiol (Amersham, 158 Ci/mmol). Nonspecific estradiol binding was measured in a parallel sample containing 5 nM [^{3}H]estradiol plus 500 nM unlabeled estradiol. After incubation at 37 °C for 30–60 min, triplicate aliquots (250 μL) from each sample were assayed by the hydroxylapatite method (Pavlik & Coulson, 1976). The specific estradiol-binding capacity of ER in the solution was taken to be the difference in binding between the total and the nonspecific samples.

For heparin affinity chromatography (Molinari et al., 1977), a final volume of 25 mL of centrifuge-column effluent was mixed with 5 mL (packed volume) of heparin–agarose resin (Affi-Gel-Heparin, Bio-Rad) previously equilibrated in TDPK-175. Then, [^{3}H]-17β-estradiol was added to a final concentration (usually 20–25 nM) calculated to be 4–5 nM in excess of receptor estradiol-binding sites, as determined above. The suspension was rotated overnight. The resin was then packed in a 10-mL polypropylene syringe barrel fitted with a plastic frit and rinsed with 10 column volumes of TDPK-175 buffer. The column was developed with a 40-mL linear gradient of KCl from 175 to 500 mM in TDP buffer. Twenty fractions were collected. ER elution was monitored by scintillation counting of tritium in 30-μL aliquots taken from each column fraction.

The peak of estradiol-binding activity eluting from heparin–agarose was pooled, and further buffer components were added to achieve the indicated final concentrations (the stock concentration of each component added is given in parentheses): 20% glycerol (100%), 0.1 mM EDTA (100 mM), 0.1% NP-40 (10%), and 1 μg/mL poly(dI·dC) (1 mg/mL in 10 mM Tris·HCl, 1 mM EDTA, pH 8.0). TDP buffer was added to adjust the sample to a final KCl concentration of 100 mM. One milliliter of the DNA–Sepharose resin prepared above was equilibrated in D buffer (TDP plus 20% glycerol, 0.1 mM EDTA, and 0.1% NP-40) containing 100 mM KCl and gently mixed with the receptor solution in a final volume of 35 mL for 2.5 h. The resin was then packed in a 2-mL polypropylene syringe column and washed with 15–20 column volumes of D buffer containing 100 mM KCl. The column was developed with a 20-mL linear gradient of D buffer containing from 100 to 600 mM KCl. Twenty fractions were collected. ER elution was monitored as for the heparin–agarose column.

Polyacrylamide Gel Electrophoresis. Protein samples were analyzed in 7.5% acrylamide–SDS gels (Laemmli, 1970) with the Bio-Rad low molecular weight protein mixture for standards. Gels were electrophoresed for 14–16 h at 50 V and then silver-stained by the method of Merrill et al. (1981). Stained gels were scanned with an LKB 2222 laser densitometer.

Protein Determinations. Protein concentrations were determined by the microscale method of Bradford (1976) using bovine serum albumin as a standard. For samples of ER eluted from the DNA–Sepharose column, protein concentrations were determined by the following modification of the method of Schaffner and Weissmann (1973). In siliconized microfuge tubes, samples and BSA standards containing 0–1 μg of protein in 270 μL were mixed with 30 μL of 1 M Tris·HCl, pH 7.5–1% SDS and then precipitated with 60 μL of 60% TCA for 10 min at 4 °C. The precipitated protein was filtered, with three 300-μL rinses of 6% TCA, onto a nitrocellulose membrane (BA-85, 0.45-μm pore size, Schleicher & Schuell) with a slot-blot apparatus that produced uniform sample spot sizes of 0.5 mm by 4.5 mm. After the filter had been stained with amido black and destained as described by Schaffner and Weissmann (1973), the absorbance of the sample spots was determined by scanning the wet nitrocellulose filter with an

Table I: Purification of Calf Uterine Estrogen Receptor

step	volume (mL)	protein (mg)	ER dimer (pmol)[a]	sp act. (pmol of ER dimer/mg)	purity (%)[b]	yield (%)
postmicrosomal fraction	325	870	654	0.75	0.01	100
0–30% $(NH_4)_2SO_4$ fraction	15	64	146	2.3	0.03	22
heparin–agarose pooled fractions	11	0.31	52	170	2.3	8.0
DNA–Sepharose pooled fractions	5.8	0.0017	12	6700	90	1.8

[a] The amount of ER dimer was calculated from tritium dpm in column fractions, assuming two estradiol-binding domains per dimeric ER. [b] Calculated purity assumes that the dimeric bovine ER is of M_r 134 000.

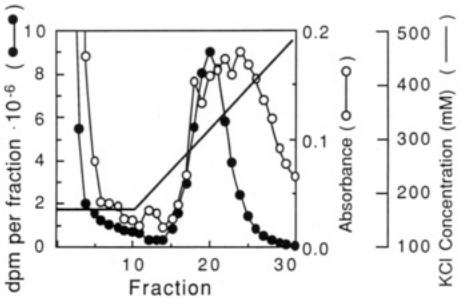

FIGURE 1: Elution profile of ER from heparin–agarose. The position of ER is indicated by the presence of [^{3}H]estradiol (closed circles). Fractions 17–23 were retained for further purification. Total protein is indicated by the absorbance at 280 nm (open circles). KCl concentration in the collected samples was determined by conductivity measurements (solid line).

LKB 2222 laser densitometer. The filter itself gave a uniform background absorbance of 0.95–1.0 OD unit. Areas of the nitrocellulose through which only buffer and TCA had been filtered ("blank") gave absorbance values of 0.02–0.03 OD unit above background. The BSA standards gave absorbance values of approximately 0.12–0.15 OD unit above background per 100 ng of protein.

Sucrose Gradient Analysis of ER. The sedimentation rate of purified ER eluted from the DNA–Sepharose resin was determined in 3.8-mL sucrose gradients of 5–30% prepared with TDP buffer containing 400 mM KCl and 100 μg/mL reduced and carboxymethylated BSA as a carrier protein. The method of preparing reduced and carboxymethylated BSA was adapted from King and Spencer (1970). Prior to application of the ER samples to the sucrose gradients, 50-μL aliquots of ER [(2–3) × 10^4 dpm, 30–45 fmol of ER dimer] in D buffer were diluted with 3 volumes of TDP buffer in order to reduce the glycerol concentration to 5%. For each gradient, 10 μg of calf intestinal alkaline phosphatase (6.2 S) and 250 μg of BSA (4.6 S) were included as internal standards. The gradients were spun for 14 h at 50 000 rpm (337000g_{max}) in a Beckman SW-60Ti rotor at 4 °C. Five-drop fractions (32–34 per gradient) were collected by bottom puncture of the centrifuge tubes. Alkaline phosphatase was detected by the colorimetric method of Garen and Levinthal (1960). BSA was detected by the Bradford protein assay.

Western Blotting. DNA affinity purified ER samples and protein molecular weight markers (2 μg of protein per band) were resolved by SDS–PAGE and then transferred to nitrocellulose according to Burnette (1981). ER was detected by use of monoclonal antibody H222 (Abbott Laboratories; Greene et al., 1984). Marker lanes were stained separately with 0.2% amido black in 7% acetic acid and then destained in water.

Results

ER was eluted from the heparin–agarose column as a single peak of tritium-binding activity centered between 260 and 325 mM added KCl (Figure 1). The ER eluted from the DNA affinity resin as a single peak between 275 and 290 mM added KCl (Figure 2A). SDS–PAGE analysis reveals a single major band migrating with an apparent molecular weight of 68 000, which eluted from the DNA affinity resin in the same fractions as the [^{3}H]estradiol binding activity (Figures 2B and 3).

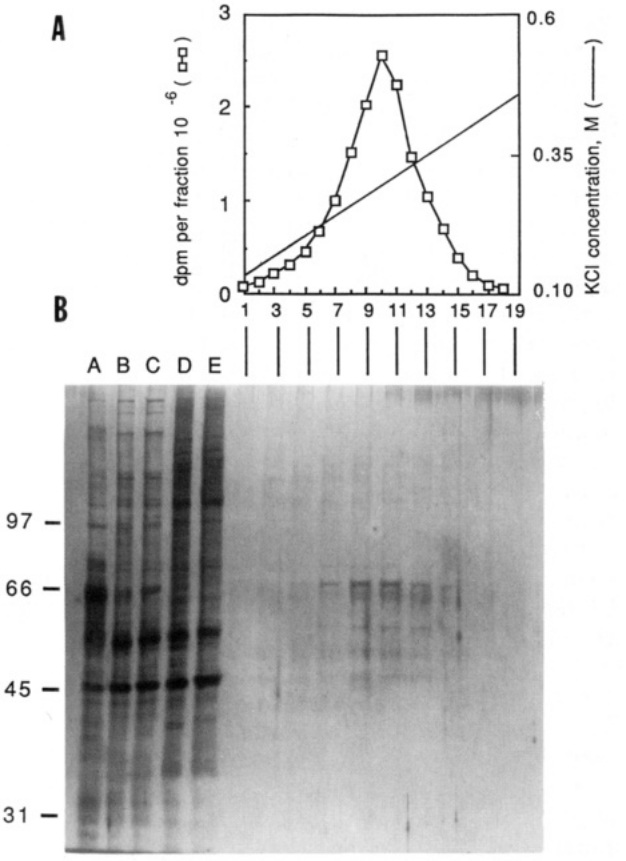

FIGURE 2: Elution profile of ER from DNA–Sepharose. (Panel A) The position of ER is indicated by the presence of [^{3}H]estradiol (open squares). KCl concentration in the collected samples was determined by conductivity measurements (solid line). (Panel B) SDS–PAGE analysis of purification fractions is shown. Numbered wells correspond to the numbered fractions shown in panel A. Proteins with apparent molecular weights of 45 000–66 000 seen here are occasional contaminants of the ER preparation. Other fractions include (A) postmicrosomal supernatant fluid (0.5 μg of protein), (B) ammonium sulfate precipitate (0.5 μg of protein), (C) material not bound by heparin–agarose (0.5 μg of protein), (D) pooled heparin–agarose fractions 17–23 (1 μg of protein), and (E) material not bound by DNA–Sepharose (1 μg of protein). The apparent molecular weight (×10^{-3}) of standard proteins is indicated.

Laser densitometer scanning of a subsequent preparation of ER demonstrated that the band of M_r 68 000 represented nearly all of the protein in the sample (Figure 3), consistent with the 90% purity calculated on the basis of protein mass and estradiol-binding capacity present in the purified material (Table I). In three steps, the estradiol-binding specific activity of the ER was enriched 8900-fold over its value in the starting postmicrosomal high-speed supernatant solution, to a final purity of 90%, with a yield of 1.8% (Table I). A second round

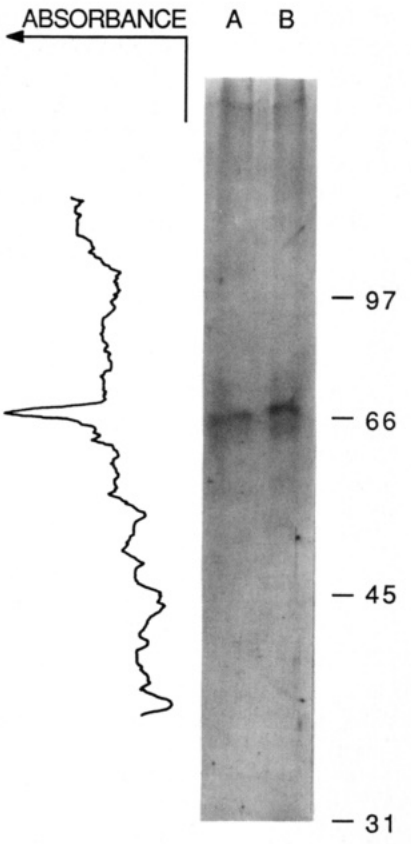

FIGURE 3: SDS–PAGE analysis of DNA–Sepharose-purified ER. (Lane A) ER after one cycle of DNA–Sepharose purification. (Lane B) ER after two cycles of DNA–Sepharose purification. An absorbance profile of lane A is shown at the left. The migration positions of standard proteins, with their respective apparent molecular weights ($\times 10^{-3}$), are indicated by the dashes.

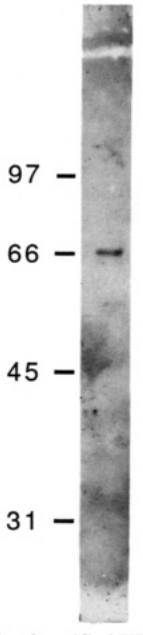

FIGURE 4: Western analysis of purified ER. Details are given in the text. The migration positions of standard proteins, with their respective apparent molecular weights ($\times 10^{-3}$), are indicated by the dashes.

of DNA affinity chromatography (not shown) yielded slightly further enrichment of ER (96% pure), but the overall yield was reduced to 0.2%. Western analysis demonstrated that the band of M_r 68 000 is recognized by the monoclonal antibody H222, generated against the human estrogen receptor (Greene et al., 1984) (Figure 4). The light bands of contaminating

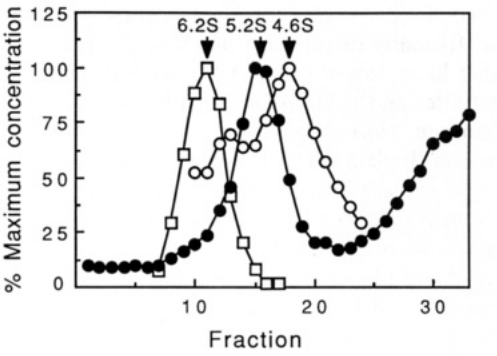

FIGURE 5: Sedimentation analysis of purified ER. The positions of ER (closed circles) and the protein standards alkaline phosphatase (open squares) and BSA (open circles) are indicated. The amount of a particular protein in each fraction is given as the "% maximum concentration" of the fraction containing the greatest amount of that protein. Sedimentation values for the protein standards and the interpolated sedimentation value for the ER are shown. Protein concentrations were determined as indicated in the text. The fraction with maximum ER concentration contained 1057 dpm of tritium per 60 μL.

protein seen in Figure 3 were not seen in the Western analysis, suggesting that they do not contain epitopes recognized by the antibody. To determine whether a significant portion of the protein of M_r 68 000 in the purified ER sample might be BSA, a major contaminant in the starting postmicrosomal supernatant solution (Figure 2, lane A), a mock purification was conducted. A solution containing 64 mg of BSA, equal to the entire mass of protein in a typical ammonium sulfate fraction of uterine cytosol, was applied sequentially to the heparin–agarose and DNA–Sepharose columns. While significant protein (19 mg) was retained and eluted from the heparin affinity resin, no detectable protein (less than 200 ng) was retained and eluted from the DNA affinity resin. From this and the data in Table I, we predict that the purified ER contains no more than 0.2% by weight of contaminating BSA.

Sucrose gradient analysis of the purified receptor demonstrated a single symmetrical peak sedimenting as a 5.1S–5.2S particle (Figure 5), consistent with the value expected for the dimeric ER protein complex (Notides et al., 1975).

Discussion

The sequence-specific DNA affinity method reported here yields a 90% homogeneous ER sample after a single cycle of DNA affinity chromatography. This ER necessarily retains estradiol-binding activity and sequence-specific DNA-binding activity in order to be selected by the purification scheme. SDS–PAGE reproducibly demonstrates a band with an apparent molecular weight of approximately 68 000, consistent with the molecular weight of cloned human, chicken, and rat ER (Green et al., 1986; Krust et al., 1986; Koike et al., 1986). The physical integrity of the purified ER is further demonstrated by the absence of a 4S species, representing proteolytic fragments or dissociated subunits, in sucrose gradient profiles. The purification therefore efficiently selects for dimeric ER molecules, consistent with the report that ER binds to DNA as a dimer (Kumar & Chambon, 1988). Theoretically, the method could select ER fragments containing DNA-binding domains but lacking steroid-binding domains (regions E and F) (Krust et al., 1986) or transcription-activating domains (regions A and B). The absence in most preparations of contaminating low molecular weight species in SDS–PAGE analyses argues against this possibility. In addition, receptor dimers that have truncated steroid-binding domains bind to DNA less stably than do intact ER dimers (Kumar &

Chambon, 1988). Such proteolytic fragments would therefore be less efficiently retained by the DNA–Sepharose resin. On the other hand, loss of the A or B region of the ER can occur without altering the DNA-binding ability of the ER (Kumar & Chambon, 1988).

The overall yield of approximately 2% obtained from the starting postmicrosomal supernatant solution does not reflect the practical yield from the ammonium sulfate fraction of ER routinely used in our laboratory. Only about half of the ammonium sulfate fraction of ER is functional in DNA binding (F. Peale and C. Klinge, unpublished observations). Therefore, relative to the amount of ER in the ammonium sulfate fraction that can bind DNA, the ER purified by the subsequent steps represents approximately a 15% yield, with a 2900-fold increase in purity. While the 20–25% yield of ER in the DNA affinity step is less than the 40–90% yield reported for estradiol affinity methods (Greene et al., 1980), it is still adequate for the preparation of useful quantities of functional ER.

This purification method has three advantages over methods based on estradiol affinity chromatography (Greene, 1984; Maurer & Notides, 1987). First, it selects for ER with functional consensus sequence binding capacity. Second, it allows options for the ER to be purified while bound to scarce or expensive ligands, such as nonsteroidal anti-estrogens, or ligands with a modest affinity for the ER. Third, it allows economical labeling with very high specific activity ligands. The second and third options are precluded in procedures requiring exchange of ER from a steroid affinity column using high concentration of a high-affinity ligand. The procedure described here substantially improves the convenience with which highly purified functional ER can be obtained. These advantages are possible while still achieving a purity (90%) and specific activity (13 400 pmol of ER monomer/mg of protein) equivalent to those obtained with the estradiol affinity methods cited above. It should therefore be immediately useful in laboratories where receptor–DNA equilibrium binding experiments and enzymatic and biochemical kinetic studies are being conducted.

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