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# Estrogen Receptor Interaction with Specific Histones

## BINDING TO GENOMIC DNA AND AN ESTROGEN RESPONSE ELEMENT

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**ABSTRACT.** Chromosomal proteins that impart high affinity and specificity to the binding of the estrogen receptor (ER) to DNA are termed estrogen receptor binding factors (ERBFs). Certain partially purified chromosomal protein fractions obtained from rabbit uterine chromatin by extraction with various molarities of GdnHCl when reconstituted to double-stranded DNA demonstrated high affinity binding for the ER. We report the purification and characterization of ERBFs in the chromosomal protein fraction extracted with 4 M GdnHCl (CP4) after large scale purification. These protein fractions were further purified by CL-Sepharose 6B column chromatography which resolved fractions from CP4 that recognized the ER bound by estrogen only or antiestrogen only. Thus, these hydrophobic chromosomal proteins enhanced the binding of the ER to reconstituted chromatin. To further investigate the interaction of ERBFs with ER, gel mobility shift assays were performed. The highly purified CP4 fraction with ERBF activity in the binding assay with reconstituted chromatin caused an increase in the formation of the retarded ER-estrogen responsive element (ERE) band. Thus, chromatin contains specific ERBFs for ER bound by estrogen which enhance the binding of ER to genomic DNA and a target ERE sequence. Further purification of the CL-Sepharose fraction with ERBF activity was achieved by preparative SDS–PAGE. ERBF activity was attributed to proteins with approximate molecular weights of 16,000, 13,000, and 12,000 and a pI of >9.0. Peptides were partially sequenced by Edman degradation and were found to have identity with histones H2B and H4. A 17 kDa protein without ERBF activity was identified as H3. Since these histones were not readily extracted from chromatin with 3 M NaCl or 1–3 M GdnHCl, we postulate that some ERBFs may be histone variants or modified histones that display a very high affinity for DNA and ER. BIOCHEM PHARMACOL 52;6:869–878, 1996.

**KEY WORDS.** estrogen receptor; binding factors; chromatin; histones

Although binding of steroid receptors to DNA is generally recognized as necessary for induction of a biological response, it was proposed several years ago that specific chromosomal proteins are also necessary in order for appropriate chromatin binding to occur *in vivo* [1]. That protein–protein interactions in addition to receptor–DNA interactions are necessary for steroid hormone induction of gene transcription is now widely accepted. It has been suggested that the A/B region of the ER† is important for tightness of receptor dimer binding to DNA by interaction of this region with other chromosomal proteins [2]. It has also been proposed recently that steroid receptors are always bound to their respective DNA sequences and that when steroid binds to receptor, chromosomal protein interaction occurs, initiating gene transcription [3]. Steroid hormone receptors have been reported to bind various histones with high affinity [4, 5], and nucleosomes can regulate the interaction of steroid receptors with steroid response elements [6]. In addition, the tight association of steroid receptors with the nuclear matrix, the nuclear scaffolding composed of nonhistone proteins and transcriptionally active DNA, has been reported [7, 8].

Our laboratory has been particularly interested in the role of specific chromosomal proteins bound to dsDNA. The term acceptor site is defined as receptor binding factor (RBF)–DNA complexes that display high affinity and specific binding in their interaction with steroid receptor complexes. We have reported that these acceptor sites have the characteristics of a physiologically significant binding system and serve an important biological role. Experiments utilizing partially deproteinized chromatin fractions from a variety of systems have demonstrated the specific interaction of steroid receptors with acceptor sites. Acceptor sites can distinguish structural/conformational changes induced in the estrogen receptor by estrogens versus triphenylethylene antiestrogens [9–11]. Steroid receptor binding to these acceptor sites was saturable and required intact and transformed receptor complexes. These nuclear acceptor sites were steroid receptor specific, i.e. progesterone receptor and

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† *Abbreviations:* CP, chromosomal (chromatin) protein; dsDNA, double-stranded DNA; ER, estrogen receptor; ERBFs, estrogen receptor binding factors; ERE, estrogen responsive element; HAP, hydroxylapatite; and PMSF, phenylmethylsulfonyl fluoride.

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glucocorticoid receptor did not bind ER acceptor sites [12], and target tissue specific [9, 10]. Our findings with the mammalian ER support the findings of Spelsberg's laboratory on the avian progesterone receptor acceptor sites [1, 13] as well as avian ER acceptor sites [14].

That specific chromosomal proteins are components of chromatin acceptor sites for the mammalian ER was confirmed with initial studies of isolated and partially purified ERBFs [15, 16]. In the present study, we describe the purification and identification of a class of ERBFs after large scale isolation and the interaction of these ERBFs with radiolabeled ER complex after various chromosomal fractions were reconstituted to dsDNA. In addition, we investigated the interaction of these ERBFs directly with the ER using a gel mobility shift assay. We report here the isolation and identification of a class of ERBFs with high affinity for DNA and ER that share sequence homology with certain histones, i.e. H2B and H4. Histone H3, which is also present in the CP4 fraction, was without ERBF activity.

## MATERIALS AND METHODS

### *Cell Lines and Animals*

[6,7-$^3$H]Estradiol (60 Ci/mmol) was obtained from the New England Nuclear Corp. (Boston, MA). The high affinity triphenylethylene antiestrogen [$^3$H]H1285 (22 Ci/mmol) was prepared by our laboratory [17]. Ultrapure guanidine hydrochloride (GdnHCl) was obtained from Amresco (Salon, OH). HAP was Bio-Gel HTP from Bio-Rad (Richmond, CA). Buffer A = 10 mM Tris, 1 mM EDTA, 10 mM monothioglycerol, 5% glycerol, and 5 mM PMSF, pH 7.5. Buffer B = 7 M GdnHCl, 5 mM potassium phosphate, pH 6.0. Buffer C = 2 mM Tris, 0.1 mM EDTA, pH 7.5. Buffer D = 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.6. MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD). Rabbit uteri were purchased from Pel-Freez (Rogers, AK). Immature female Sprague–Dawley rats were obtained from Harlan (Houston, TX).

### *Preparation of Chromatin, DNA, and Steroid Receptors*

The isolation of chromatin from rabbit uterus [9] was as previously described. Rabbit spleen DNA was prepared using a modification of the procedure of Spelsberg *et al.* [18].

ER from rabbit uterus was prepared in buffer A, and transformed ER was 30-fold purified by DEAE-cellulose chromatography as previously described [9] for the chromatin binding studies. For the gel mobility shift assays, rabbit uterine cytosol was incubated with 20 nM estradiol-17β for 2 hr at 20° to transform the ER.

Nuclear extracts containing ER were obtained from MCF-7 cells maintained in DME/F12 medium supplemented with 2.2 g/L sodium bicarbonate, 5% fetal bovine serum, and 10 mL/L antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 150-cm$^2$ culture flasks in an air:carbon dioxide (95%:5%) atmosphere at 37°. Cells were harvested by trypsinization and washed twice in 30 mL of Buffer D. The cells were centrifuged at 10,000 *g* for 5 min, and the pellet was incubated for 10 min in Buffer D without the glycerol. The cells were then homogenized and the homogenate was centrifuged at 40,000 *g* for 10 min. The nuclear pellet was washed twice with Buffer D, resuspended in 1–2 mL of buffer D containing 0.5 M KCl, and incubated for 1 hr at 4° prior to centrifugation at 110,000 *g* to obtain the nuclear extracts containing ER.

Female 21-day-old rats were injected i.p. with 0.5 μg estradiol-17β (Sigma), or vehicle (0.1 mL DMSO) for 3 days. Twenty-four hours after the last injection, the rats were killed with carbon dioxide, and the uteri, stripped clean of mesentery, were removed, weighed, and frozen at −80°. Uteri, 3 per group, were homogenized in Buffer D, and the homogenate was centrifuged at 10,000 *g* for 5 min. The nuclear pellet was washed three times in 10 mL of Buffer D, and the nuclei were extracted as described for MCF-7 cells.

### *Isolation and Purification of Chromosomal Proteins*

In general, the isolation of chromosomal proteins was as previously described [19]. For a large-scale preparation, chromatin (4 g DNA) isolated from 15 kg rabbit uteri was bound to HAP. The HAP was prehydrated in 12 L of 3 M NaCl in 100 mM phosphate buffer, pH 6, for 12 hr and then filtered. The chromatin solution was added (4 g DNA/2.7 kg HAP) and stirred every 15 min with a glass rod at 4° for 1 hr prior to mixing with 12 L of the 3 M NaCl solution for 1 hr. Bulk histones were removed with filtration. A second 2-hr extraction with 12 L of 3 M NaCl was performed followed by a third 3 M NaCl extraction that was filtered immediately after mixing with the HAP-chromatin. Then increasing molarities (1–8 M) of GdnHCl in 100 mM potassium phosphate buffer were used to extract the proteins tightly bound to DNA. Each incubation with the HAP-chromatin slurry for each molarity of GdnHCl (12 L) was for 50 min and was followed by filtration to provide each chromosomal protein (CP) fraction, resulting in eight fractions of protein (CP1–8). Each filtrate was concentrated using an Amicon spiral cartridge system (model S10Y10) allowing 12 L to concentrate to about 1 L. The fractions were then dialyzed against 10 vol. of distilled water with six changes over 48 hr followed by dialysis (mol. wt cutoff-8000) in 5% acetonitrile in water for 1 hr. The samples were freeze-dried and stored desiccated until further use.

Further purification of the chromosomal protein fraction extracted with 4 M GdnHCl (CP4) was achieved by molecular sieve chromatography. Approximately 560 mg of the CP4 fraction was dissolved in Buffer B, centrifuged at 43,000 *g* for 30 min, and the proteins in the supernatant separated by a large (3 L) CL Sepharose 6B column (7 × 80 cm) already equilibrated with Buffer B. Protein concentration was determined in each 70-mL fraction with a modi-

fication of the Bradford assay [20], and ERBF activity was determined with the streptomycin-filter assay (see below). Protein profiles were analyzed by SDS–PAGE [21]. The gels were silver stained (ICN Radiochemicals, Irvine, CA) for visualization.

The fraction from the molecular sieve column that demonstrated specific ERBF activity for the ER bound by estradiol in the streptomycin-filter binding assay was dialyzed and freeze-dried and further purified by isoelectric focusing. The freeze-dried proteins (10 mg) were dissolved in 50 mL of 6 M urea containing 1% pH 3–10, 1% pH 4–7, and 1% pH 5–9 BioLyte ampholytes (Bio-Rad) for 24 hr. The proteins were then separated into 20 fractions by isoelectric focusing in a Rotofor cell (Bio-Rad) for 4 hr at 4° at 12 W. Each fraction was dialyzed in Spectrapor 6 (mol. wt cutoff-8000) bags against 50 vol. of 2 M NaCl with two changes for 16 hr and against 50 vol. of distilled-deionized water for 24 hr with three changes. After an additional 1 hr of dialysis in 5% acetonitrile, each fraction was divided into aliquots and freeze-dried. The protein concentration was measured in an aliquot of each fraction [20], and the fractions were screened for ERBF activity.

To obtain purified ERBFs, the fractions from the isoelectric focusing step that demonstrated ERBF activity were subjected to preparative SDS–PAGE (15% acrylamide for the separating gel, 1.5 mm × 20 cm, 15-well capacity) [21]. The proteins were electroblotted onto two back-to-back sheets of Immobilon P as described by Matsudaira [22]. The second sheet was stained with colloidal gold (Bio-Rad) to visualize the bands. The top sheet was cut into sections corresponding to the protein bands on the template second sheet, and each section was eluted with 70% isopropanol, 5% trifluoroacetic acid, 25% water for 2 hr. Then the samples were dialyzed against distilled-deionized water containing 0.5 mM PMSF for 24 hr and against 5% acetonitrile for 1 hr and freeze-dried. The proteins were solubilized in Buffer B for reconstitution to dsDNA for the streptomycin-filter binding assay or in 2 M urea for the gel mobility shift assay to determine ERBF activity.

### *Cell Free Receptor Binding Assay*

Estrogen receptor binding to reconstituted chromatin was measured using a modification of the method of Spelsberg [19]. Briefly, isolated chromosomal protein fractions were reconstituted to rabbit dsDNA at various ratios of protein:DNA using reverse gradient dialysis in 50 mM sodium acetate, 10 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, pH 6, reducing GdnHCl from 7.5 M to 0 M over a 24-hr time period to produce a reconstituted chromatin (RC) preparation. The RC solutions were centrifuged for 20 hr at 110,000 *g*, and the pellets were dissolved in 1.2 mL of 10 mM KCl in Buffer C. To test for ERBF activity, DEAE partially purified [$^3$H]estradiol–receptor complexes (or in some experiments, [$^3$H]H1285–receptor complexes) were added (0.4 pmol) to approximately 70 μg chromatin-DNA (RC) in a final assay volume of 500 μL (150 mM KCl). After incubation on ice for 1 hr with frequent vortexing, 100 μL of 1.2% streptomycin was added to precipitate the receptor–RC complexes, and incubation was continued for 30 min. The assay tubes were centrifuged at 800 *g* for 10 min, and the supernatant was aspirated. The pellets were washed two times with 1 mL of 0.02% streptomycin in Buffer C, then suspended in buffer, and poured over Millipore (HAWP) filters. The filters were washed four times, air dried for 15 min, and then dried under a Fisher IR lamp for 15 min. The radioactivity on the filters was counted in 10 mL scintillation fluid (0.4% Omnifluor-xylene), the filters were removed and dried, and the DNA content was determined [23]. Aliquots of fractions with ERBF activity were used in the gel mobility shift assay.

### *ERE Gel Mobility Shift Assay*

Complementary strands of a synthetic oligonucleotide containing a wild-type ERE consensus sequence (5′-GTCCA-AAGTCAGGTCACAGTGACCTGATCAAAGTT-3′) that corresponds to −308 to −342 of the promoter/upstream element of the *Xenopus* vitellogenin A2 gene [24] were synthesized, purified by polyacrylamide gel electrophoresis, annealed, and labeled at the 5′ end with [$^{32}$P]ATP using T4-polynucleotide kinase as described [25]. DNA binding was measured using a gel mobility shift assay. Five micrograms of nuclear extract protein from MCF-7 cells containing nuclear ER, or 5 μg of rat uterine nuclear extract, or 20 μg of transformed rabbit uterine cytosol was incubated without or with various concentrations of partially purified chromosomal protein fractions described above for 15 min at room temperature. This mixture was then incubated with 200 ng poly d(I-C) for 15 min at 20° to bind nonspecific DNA binding proteins. After the addition of 1 nM $^{32}$P-labeled ERE, the mixture was incubated for another 15 min at 20°C. Reaction mixtures were loaded onto a 5% polyacrylamide gel (acrylamide:bisacrylamide, 30:0.8) and run at 120 V for 2.5 hr. The gels were dried, and protein–DNA binding was quantitated by a Betascope 603 blot analyzer and visualized by autoradiography.

### *Protein Sequencing Analysis*

An aliquot of CP4 fraction from the CL-Sepharose purification step (CP4-S) was solubilized in SDS-solubilization buffer and separated on tricine SDS–PAGE (1.5 mm × 20 cm × 15 wells, 0.1 M tricine in cathode buffer). The proteins were electro-transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA), stained with Coomassie brilliant blue, destained for 30 sec, and dried. The 17, 16, 13, and 12 kDa bands were subjected to protein sequencing analysis directly from the membrane by the Edman degradation reaction [26] using an Applied Biosystems model 477 protein sequencer. Proteins that were blocked at the N-terminus were subjected to cleavage with cyanogen bromide (CNBr) prior to Edman degradation.

## RESULTS

In the studies reported here, we further characterized and purified ERBFs in CP4. The protein profile after SDS–PAGE and silver stain of CP4 after large scale isolation using DNA affinity chromatography is seen in Fig. 1 (left panel).

### *Fractionation of CP4 using Molecular Sieve Chromatography*

We continued the purification of ERBFs in the CP4 fraction after large scale isolation utilizing separation of the proteins by molecular sieve chromatography. The protein profile of the separation of CP4 through CL-Sepharose 6B is shown in Fig. 1 (center panel). The effectiveness of the purification can be seen by comparing the left and right panels of Fig. 1. ERBF activity of aliquots of each eluted fraction was determined with the streptomycin-filter assay and shown in Fig. 1. ERBF activity for the receptor bound by estrogen was greatest in fraction 18 (approx. mol. wt of 12,000), whereas ERBF activity for the receptor bound by antiestrogen was greatest in fractions 14 and 15 (approx. mol. wt of 30,000), suggesting that CP4 contains ERBFs with independent activities for an estrogen bound receptor or an antiestrogen bound receptor. In the present study, we decided to further investigate the ERBFs that interact with ER bound by estrogen. An aliquot of fraction 18 was separated by SDS–PAGE, and the banding pattern, shown in Fig. 1 (right panel), indicates that the proteins in this fraction are in the approximate molecular weight range of 10,000–23,000. There were no protein bands with molecular weights greater than 30,000 in this fraction.

### *Studies of CP4 Interaction with ER using Gel Mobility Shift Assays*

The above approach to the study of ER interaction with ERBFs measured the binding of a radiolabeled-ER prepara-

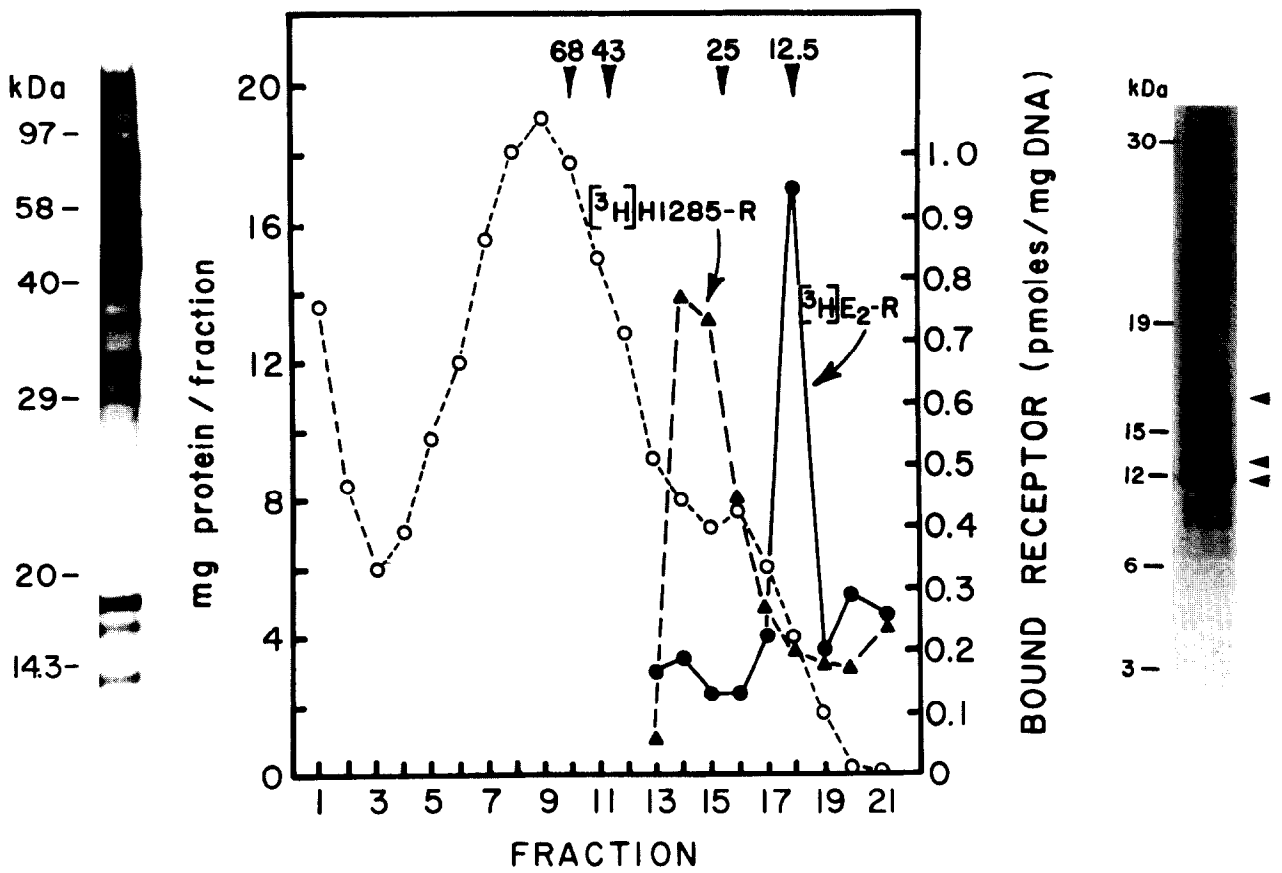


**FIG. 1. Characterization of rabbit uterine CP4 proteins. Left panel: SDS–PAGE profile of proteins, i.e. CP4, eluted from rabbit uterine chromatin with 4 M GdnHCl. The protein fraction was dialyzed, lyophilized, solubilized in sample buffer, and run on a 15% acrylamide gel system. The gel was silver stained for analysis of banding patterns. An aliquot of this protein fraction was dissolved in 7 M GdnHCl and further purified. Center panel: Separation of CP4 ERBFs carried out by CL-Sepharose 6B column chromatography. Protein content (0) and ERBF activity of each fraction were determined and expressed as the mean of 4 experiments. Molecular weight markers were bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,000; cytochrome c, 12,500. Right panel: Protein banding pattern of CP4 fraction 18 containing ERBF activity after separation by molecular sieve chromatography. The protein bands after SDS–PAGE and transfer to Immobilon P were visualized by colloidal gold. Arrows indicate those proteins with ERBF activity.**

tion to ERBFs bound to genomic DNA. To study more directly the interaction of ER with ERBFs, we utilized a gel mobility shift assay in which the direct interaction of ER with ERBFs could be determined by investigating the effect of ERBFs on the interaction of ER with [$^{32}$P]ERE.

Experiments were performed with 0.65, 1.3 and 2.6 μg of fraction 18 from CL-Sepharose-purified CP4 (CP4-S) and nuclear extracts from MCF-7 cells (Fig. 2). CP4-S increased the intensity of ER:ERE, indicating that this chromosomal protein fraction was capable of enhancing ER interaction with a specific ERE in the ER:ERE assay as well as the filter binding assay. CP4-S was also unable to bind ERE directly. To test for nonspecific binding, nuclear extracts of MCF-7 cells were incubated with 5 μg CPNS, a chromatin protein fraction with no ERBF activity in the streptomycin filter assay using reconstituted chromatin. This CPNS fraction was unable to enhance the interaction of ER with ERE (not shown). Similar experiments were conducted to study the effects of CP4-S on rat uterine nuclear extracts. ER from rat uterus routinely resolved into two retarded ERE bands. CP4-S (3 μg) enhanced the ER-ERE interaction (Fig. 3A), primarily with the upper band, although in some experiments the lower band was also enhanced. Incubation of ER with 2.5 μg of CPNS did not cause an enhanced ER:ERE binding (not shown). Experiments were also performed with ER from rabbit uterine cytosol (Fig. 3B). Incubation of cytosol with 5 μg CP4-S (lane 2) caused an enhanced binding of ER to ERE. That both rat uterine ER:ERE retarded bands are ER can be seen in Fig. 3C. The incubation of nuclear extract with anti-ER antibody ER 712 [27] for 2 hr prior to the gel shift assay caused a supershift of both bands (lane 2). The anti-ER antibody was able to supershift rat or rabbit ER:ERE without or with added CP4-S.

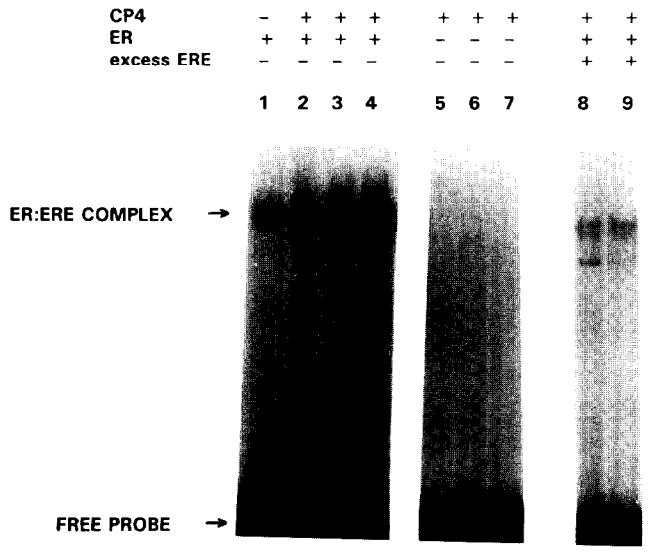


**FIG. 2. Effect of ERBFs on MCF-7 cell ER:ERE binding. Nuclear extracts of MCF-7 cells containing ER were incubated with various concentrations of CL-Sepharose-purified CP4 fraction 18 (CP4-S) prior to the gel mobility shift assay. Lanes 1–4 represent 0, 0.65, 1.3, and 2.6 μg CP4-S. Lanes 5–7 demonstrated no binding of CP4-S to the ERE in the absence of ER. Lanes 8 and 9 included 500-fold excess unlabeled ERE (lane 8 = 2.6 μg and lane 9 = 0.65 μg CP4-S).**

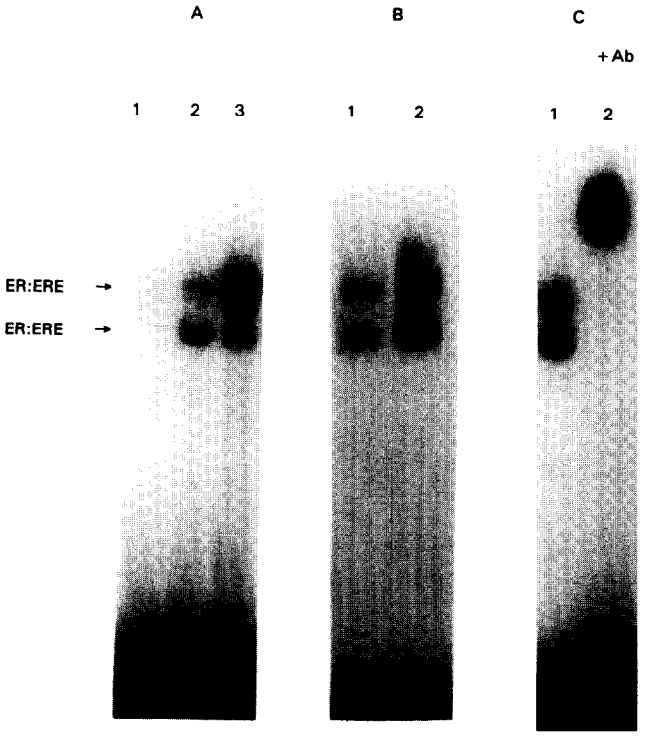


**FIG. 3. Effect of ERBFs on uterine ER:ERE binding. (A) Nuclear extracts (5 μg) from uteri of rats injected with 0.5 μg estradiol for 3 days were incubated without (lane 2) or with (lane 3) 3 μg CP4-S. Lane 1 contained 500-fold excess unlabeled ERE. (B) Rabbit uterine cytosol (20 μg) was incubated without (lane 1) or with (lane 2) 5 μg CP4-S. (C) Effect of anti-ER on ER:ERE binding. Uterine nuclear extract from estrogen-treated rats was incubated with anti-ER antibody for 2 hr at 4° prior to the gel shift assay. Lane 1 = ER in the absence of anti-ER; lane 2 = ER in the presence of 100 ng anti-ER.**

### *Purification of ERBFs*

Continued purification of ERBFs specific for estrogen bound receptors employed preparative SDS–PAGE. An aliquot of proteins in the CL-Sepharose fraction that gave the greatest ERBF activity for ER bound by estradiol (CP4-S) was dialyzed to remove the GdnHCl, freeze-dried, solubilized, separated by SDS–PAGE, and transferred to Immobilon P. The banding pattern obtained with colloidal gold staining from a lane of the transfer top sheet was shown in Fig. 1 (right panel). The ERBF activity (as determined by the streptomycin-filter binding assay of reconstituted chromatin) of each of the protein fractions eluted from cut sections of the transfer sheet of Immobilon P is shown in Fig. 4. Three fractions (10, 11, and 13) containing bands with molecular weights of 16,000, 13,000, and 12,000 yielded ERBF activity. (An intense 17 kDa band in fraction

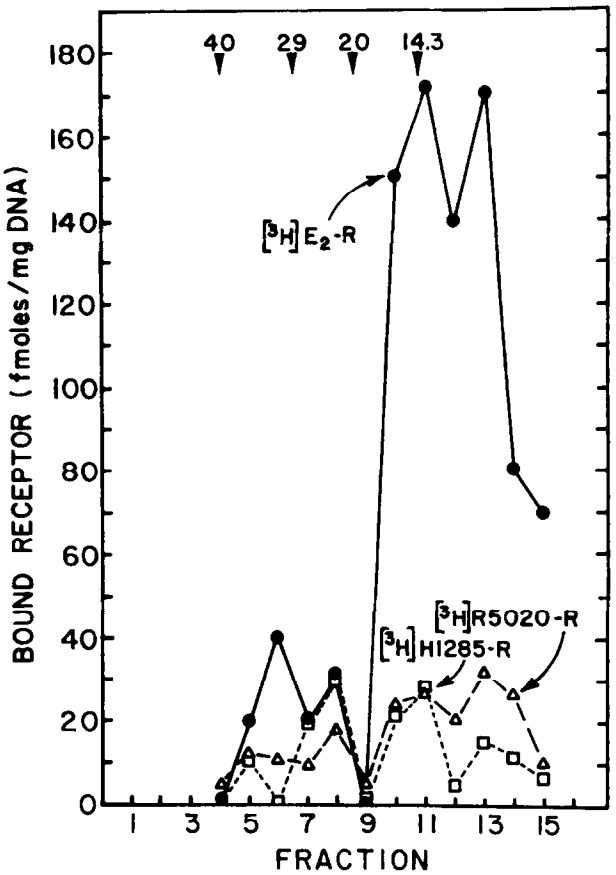


**FIG. 4. Purification of ERBFs with preparative SDS–PAGE. ERBF activity of chromatin proteins eluted from Immobilon P after transfer was determined after reconstitution to dsDNA using a filter binding assay. Data are from binding with ER bound by [$^3$H]estradiol or [$^3$H]H1285 or progesterone receptor bound by [$^3$H]R5020. Fractions 10–13 contained major bands with molecular weights of 16,000, 13,000, and 12,000 (see Fig. 1, right panel). Fraction 9 contained an intense band of 17,000 without ERBF activity in this assay.**

9 did not contain ERBF activity.) The ERBF activity was specific for ER bound by estradiol since antiestrogen–receptor complexes ([$^3$H]H1285-R) did not bind. In addition, this activity was specific for the ER since progesterone receptor complexes ([$^3$H]R5020-R) did not bind. Binding of ER complexes required transformed receptors since molybdate-stabilized receptors did not bind (not shown).

To achieve purification to homogeneity, the fractions from the CL-Sepharose chromatography with ERBF activity were subjected to preparative isoelectric focusing. Aliquots of each of the fractions (pH 3.5 to 10) were analyzed by SDS–PAGE. Although proteins were distributed throughout the pH range, the 16, 13, and 12 kDa proteins with ERBF activity were resolved in two fractions with a pH of about 9–10. Preparative SDS–PAGE of the protein fractions from isoelectric focusing yielded purification of the 16, 13, and 12 kDa ERBFs purified to apparent homogeneity (Fig. 5). These protein bands were used for sequence analysis.

### *Protein Sequencing Analysis*

To determine the amino acid sequence of the proteins with ERBF activity, protein sequence analysis was performed. Proteins were separated by SDS–PAGE and transferred to a ProBlott membrane. Protein bands were detected with Coomassie brilliant blue and excised. Sequencing was performed directly from the ProBlott membrane by Edman degradation [26]. Several attempts to sequence the 12 and 13 kDa proteins revealed that the N-terminus was blocked. These proteins were then subjected to cyanogen bromide treatment, which cleaves peptide bonds on the C-terminal side of methionine, followed by direct sequencing from the membrane. A peptide of 17 amino acids was identified for the 12 kDa proteins and a peptide of 16 amino acids was identified for the 13 kDa protein. These sequences were

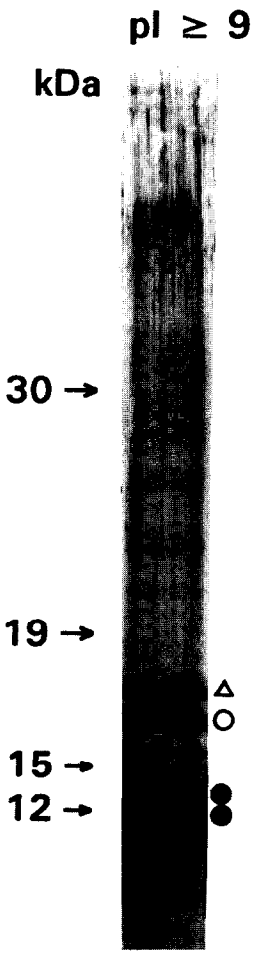


**FIG. 5. Isoelectric focusing (IEF) analysis of ERBFs from rabbit uterine chromatin. An aliquot of CP4-S containing ERBF activity was subjected to isoelectric focusing followed by SDS–PAGE of each focused fraction. Shown is a colloidal gold stain of one lane (pI 9) from an Immobilon P membrane after electro-transfer. The proteins with ERBF activity are indicated by circles. The 16 kDa protein (○) had sequence identity with histone H2B, and the 12–13 kDa proteins (●) had sequence identity with histone H4. The 17 kDa protein (△) had sequence identity with histone H3 and was without ERBF activity.**

analyzed by computer using the FASTA program [28]. A region of 100% identity between these proteins and H4 was obtained. Histone H4 (mol. wt 11,300) contains a single methionine at residue number 84 [29]. The region of identity between the 12 and 13 kDa ERBFs and histone H4 lies in the region just C-terminal to the methionine at position 84. Sequence analysis of the 16 kDa protein band revealed that this protein had 100% identity with histone H2B (mol. wt 13,800) from the N-terminal 25 amino acids. The 17 kDa protein, which was without ERBF activity, was identified as histone H3 (mol. wt 15,200) from the N-terminal 10 amino acids. The apparent molecular weights of the CP4 histones using Gibco-BRL low molecular weight markers were greater than the actual molecular weights determined using total histones as standards. This had been reported previously for histone proteins [30].

### *Interaction of CP4 Histones with ER*

Experiments were also conducted to determine the ability of the extracted individual protein bands to interact with ER in the gel mobility shift assay. Proteins were prepared as described for Fig. 4 except that the proteins were solubilized in 2 M urea. Aliquots were added with increasing concentration to rabbit uterine cytosol. The final concentration of urea was 0.8 M and was constant for all assay tubes. This concentration of urea was shown not to interfere with the gel mobility shift assay. Whereas the CP4 H3 protein (Fig. 6, right panel) was unable to effect the ER:ERE interaction, the CP4 H2B (Fig. 6, both panels) and H4 (Fig. 6, right panel) proteins caused a concentration-dependent (approximate protein concentration = 0.5 μg/μL) and saturable increase in the retarded ER:ERE. Calf thymus H2B and H4 (Boehringer Mannheim) were unable to affect the retarded ER:ERE (not shown) throughout a 10-fold concentration range (0.3 to 3 μg).

## DISCUSSION

Initial experiments with extracted chromosomal proteins from rabbit uterus using reconstitution of the extracted proteins to dsDNA followed by a receptor binding assay complemented our studies using residual chromatin fractions [15, 16]. Reconstitution of CP2, CP4-5, and CP7 to DNA yielded enhanced binding of ER complexes, whereas antiestrogen–receptor complexes bound poorly to sites reconstituted with CP7. The binding was of high affinity (dissociation constants in the nanomolar range) and saturable. Target tissue specificity was also demonstrated since chromosomal proteins extracted from rabbit spleen were unable to form reconstituted acceptor sites. These results also confirmed that specific chromosomal proteins were components of chromatin ER acceptor sites. The protein components of chromatin acceptor sites were designated as ERBFs. We also reported that the binding of ER to CP4-5 reconstituted to dsDNA was saturable with a $K_d$ of 1.25 nM for ER bound by [$^3$H]estradiol and a $K_d$ of 1.16 nM for ER

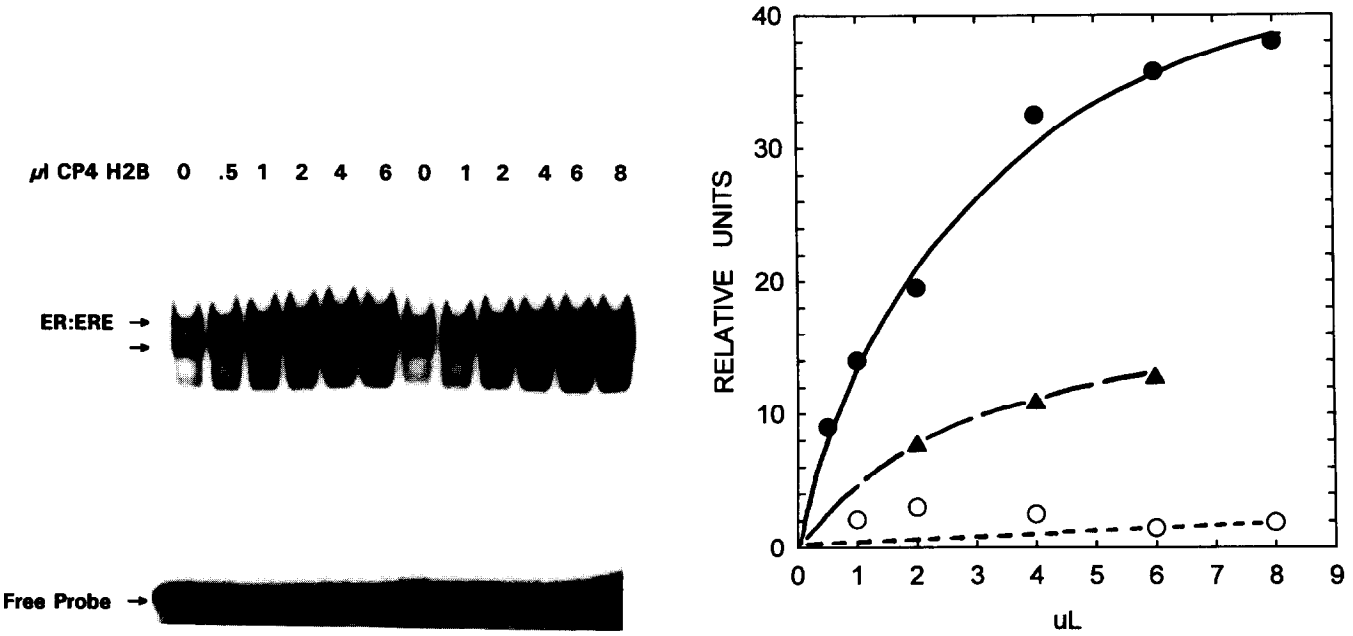


**FIG. 6. Effect of increasing concentrations of the CP4 H2B on ER:ERE binding. The CP4-S fraction containing ERBF activity was purified further by SDS–PAGE (Fig. 4). After transfer to Immobilon P, individual bands were eluted from the membrane, dialyzed, freeze-dried, and solubilized in urea. For the gel shift assay, increasing concentrations (0.5 to 8 μL) of the CP4 H3, H2B, and H4 were incubated with ER prior to addition of poly [d(I-C)]. Left panel: ER:ERE retarded bands with increasing CP4 H2B from two experiments were visualized by autoradiography. Right panel: Intensity of the retarded ER:ERE was measured by phosphor imaging. The value of ER:ERE in the absence of added CP was subtracted from all values. Key: (●) H2B, (▲) H4, and (○) H3.**

bound by [$^3$H]H1285. Binding of ER to dsDNA alone was of low affinity and linear. The number of binding sites for ER bound by either estrogen or antiestrogen from these reconstitution studies using DNA affinity purified CP4 was 8000–9000 sites/cell. In the present study, we further characterized the chromosomal proteins extracted with 4 M GdnHCl (i.e. CP4) from rabbit uterus after large-scale purification and separation by molecular sieve chromatography. We isolated and purified those hydrophobic chromosomal proteins that enhanced the binding of ER to reconstituted chromatin.

The results from studies using gel mobility shift assays to investigate the interaction of ER with CL-Sepharose-purified CP4 fractions with ERBF activity for the estrogen receptor bound by estrogen demonstrated an increased intensity of the retarded band(s). The chromosomal protein fractions with ERBF activity interacted with ER from a variety of species. This is not surprising since ER among mammalian species is highly conserved. The CP4-S did not cause the formation of retarded ERE bands in the absence of ER and thus appeared not to interact directly with DNA sequences present in the oligonucleotide containing the consensus ERE. This is consistent with a model which postulates that ERBFs stabilize ER interaction with ERE and other nuclear transcription factors. This model allows for a high affinity and stable interaction of ER with ERE *in vivo* and may involve alterations in chromatin structure as well as changes in conformation of the various protein components [13, 30, 31].

Interaction of ER from rat uterine nuclear extracts as well as from rabbit uterine cytosol with the oligonucleotide-containing ERE routinely resulted in the formation of two retarded bands. That both retarded bands represent ER:ERE binding was demonstrated by the supershift with the anti-ER antibody. The formation of more than one retarded ER:ERE has also been reported by others [32, 33] using nuclear extracts from estrogen-treated mice. The complexes formed were qualitatively identical with nuclear extracts from mice treated with a variety of estrogenic compounds. The two bands may represent the intact and a slightly degraded ER.

The current study reports the purification to homogeneity of the 16, 13, and 12 kDa ERBFs using isoelectric focusing and SDS–PAGE of the CL-Sepharose fractions with ERBF activity. The general characteristics of ERBFs, i.e. source, hydrophobicity, and small molecular weight, are similar to the characteristics of progesterone RBFs (PRBFs) reported by Spelsberg's group [1, 34, 35]. PRBF-1 is a small basic 10 kDa highly hydrophobic protein that generated high affinity ($K_d \sim 2$ nM) and saturable binding of the progesterone receptor when reconstituted to avian DNA. This protein was reported to have a unique N-terminal sequence that is highly hydrophobic. Consistent with our studies with the rabbit uterine estrogen receptor system, the progesterone receptor binding to the reconstituted avian genomic DNA was saturable and shown to be dependent on an intact transformed progesterone receptor. However, the ERBFs reported here are distinct from PRBFs by sequence analysis; additionally, ERBFs do not react with anti-PRBF antibodies (unpublished observations). Our results also support the existence of multiple ERBFs with different characteristics. However, the ERBFs reported here are distinct from other accessory proteins found in cytosol or nuclear extracts that associate with ER-ERE [36, 37].

The finding that one class of ERBFs shared sequence identity with histones H2B and H4 was somewhat unexpected since we performed not only a series of 3 M NaCl extractions intended to remove histones but several extractions with increasing molarities of GdnHCl. It is well-established that histones are relatively easy to extract in NaCl concentrations of 1 M and less [38], and in our studies the bulk of the histones was extracted with 3 M NaCl. This suggests that the CP4 H2B and H4 differ from histones that are typically extracted under much less stringent conditions. Thus, we believe that we have selected for a subset of histones that were bound tightly to DNA and resistant to salt extraction. Steroid receptor interaction with specific histones has also been reported by others. Estrogen receptors were reported to bind strongly to calf thymus histones H2B and H2A, moderately to histones H3 and H4, and poorly to histone H1 [4]. Conversely, glucocorticoid receptors (GR) were reported to have a higher affinity for H3 and H4 than H2A and H2B [5]. Both groups of investigators postulated that nuclear salt-resistant (0.4 M KCl) steroid receptors represent histone bound receptors. We [39] and others [40] had shown previously that the nuclear salt-resistant form of the estrogen receptor is formed *in vivo* by estrogens but not by antiestrogens.

It is interesting that two independent measures of ER–ERBF interaction used in our studies both indicated that ER had little affinity for H3. In addition, there was negligible H2A present in CP4. The variations or modifications in CP4 H2B and H4 are yet to be determined; however, the isolation of two forms of CP4 H4 already suggests alterations in H4. Histone variants and modified histones (acetylation, methylation, phosphorylation) [41] have been shown to exhibit tissue-specific distribution [42] as well as occurrence within the same nucleus [43, 44]. Histone H4 acetylation has been shown to be involved in the control of transcription, especially in modulating access of transcription factors [45, 46]. As the basic residues in the tails of the core histone $(H3:H4)_2$ tetramer become modified by acetylation, they lose affinity for DNA and permit entry of transacting factors. It is also possible that freeing these tail regions might alter the conformation of the local DNA such that transcription factor binding is favored.

It has been reported that the affinity of the progesterone receptor for its response element is dependent on position of the response element within the nucleosome and that the affinity of DNA–histone interaction influences the function of steroid receptors *in vivo* [6]. Positioning of nucleosomes over steroid-inducible promoters is important

for interaction with steroid receptors as well as affecting accessibility [47–50]. Thus, steroid hormone action *in vivo* requires changes in chromatin structure, and histone proteins appear to be involved. With the multiplicity of variants and modifications possible for the eight histones in a given nucleosome core, the potential for specific regulation of genes through steroid hormone receptor/histone interactions is profound. Our finding of a subset of histones that enhance ER binding to DNA supports the role of specific interaction of steroid receptors with chromatin to allow transcriptional regulation.

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