Identification and Characterization of an Estrogen-Responsive Element Binding Protein Repressed by Estradiol†

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ABSTRACT: Cytosolic proteins from uteri of 19-day-old rats were analyzed by an electrophoresis mobility shift assay (EMSA) using a 31 base pair DNA probe containing an estrogen-responsive element (ERE) from the vitellogenin A2 gene. EMSA identified three distinct cytosolic protein−DNA complexes that are separable by Q-Sepharose anion exchange chromatography into an estrogen receptor (ER)-containing fraction (150 mM NaCl eluate) and a non-ER-containing fraction (250 mM NaCl eluate). We thus refer to the non-ER fraction as the ERE binding protein (ERE-BP). The ERE-BP-containing fraction was repressed to 40−50% of its normal levels following a single injection of estradiol. In addition, ERE-BP levels were repressed to the same extent (greater than 50%) by day 20 of the rat's gestational period. Examination of the expression pattern of ERE-BP shows that this activity is differentially expressed in both estrogen-responsive and nonresponsive tissues, with the highest levels of expression occurring in the pituitary. We next examined the specificity of ERE-BP binding by competition analysis using DNA sequences corresponding to binding sites of several known transcription factors. ERE-BP was found to be specific for both the ER binding site (ERE) and TATA binding protein binding sites. Furthermore, saturation analysis demonstrated that ERE-BP binds to the ERE and TATA binding protein sequences with an apparent K_d of 1.2 and 0.12 nM, respectively. Partial purification of ERE-BP using three chromatography steps (Q-Sepharose, hydroxyapatite, and Sephacryl S300) followed by sodium dodecyl sulfate analysis indicated the presence of three major protein bands (p102, p81, and p48) as judged by Coomassie staining. UV cross-linking of the ERE-BP/DNA complex followed by sodium dodecyl sulfate analysis−polyacrylamide gel electrophoresis analysis indicates that the 48 kDa band seen in the final, partially purified fraction correlates with the ERE-BP activity. Thus, this study has identified a unique uterine cytosolic protein that binds to the ER binding site and may influence ER binding.

In addition to hormone and DNA binding, interactions with other accessory proteins appear to be a critical event in transcriptional regulation by the estrogen receptor (ER).[1] There have been several reports regarding the interaction of ER with general transcription factors (TF) as well as other proteins (Baniahmad et al., 1995; Brou et al., 1993; Cavailles et al., 1994; Halacchmi et al., 1994; Ing et al., 1992; Jacq et al., 1994; Landel et al., 1994). Ing et al. (1992) demonstrated the association of the ER with TF-IIB using an in vitro protein−protein interaction assay. Although the functional relevance of this interaction is unknown, the ER TF-IIB association introduces the possibility that TF-IIB may mediate ER actions in vivo. Further evidence that an estrogen-dependent increase in transcription is modulated through the association of ER with other proteins was demonstrated by the change in DNase hypersensitivity of the prolactin gene after estradiol treatment (Seyfred & Gorski, 1990). This increase in hypersensitivity is interpreted as the ER or another protein factor (such as pituitary factor 1) associating and dissociating from the DNA.

An interplay of different ER−protein complexes during estrogen-dependent transcription has been reported by several laboratories. Cavailles et al. (1994) reported an estradiol-dependent association of three proteins (p160, p140, and p80) with the transcription activating function 2 (TAF-2) region of ER. These proteins appear to interact with the active form of ER and function differently depending upon the cell type. Experiments by the Jacq group (Jacq et al., 1994) demonstrated an ER−hTAF$_{II}$30 association in a distinct subpopulation of a transcriptionally active TF-IID fraction. They were able to show that an antibody to TAF$_{II}$30 was able to inhibit estradiol-activated but not basal transcription in vitro. These data suggest a functional role for the ER−TAF$_{II}$30 interaction.

Recent data concerning the architecture of ER−protein associations (Cavailles et al., 1994; Jacq et al., 1994; Landel et al., 1994) suggest an intricate interplay of ER/protein/DNA complexes in ER activation of transcription. The different ER accessory proteins may interact with ER as well as the ER binding site (estrogen responsive element, ERE) as evidenced by the different strategies (direct protein−protein interaction, ERE-affinity column, immunoprecipitation) used to identify these accessory proteins. In order to better understand ERE−proteins and ER−protein interac-

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[1] Abbreviations: ER, estrogen receptor; TF, transcription factor; TAF-2, transcription activating function 2; ERE, estrogen response element; kDa, kilodalton(s); PBS, phosphate-buffered saline; TBP, TATA binding protein; EMSA, electrophoresis mobility shift assay; vit-ERE, vitellogenin estrogen response element; DTT, dithiothreitrol; T-250, Q-Sepharose T-250; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ERE-BP, estrogen response element binding protein.

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tions, we have partially purified and characterized a 48 kDa (kDa) protein from rat uterine cytosol that (i) binds to both the ERE and the TATA box enhancer sequences with nanomolar affinities, (ii) is repressed by estradiol and the onset of gestation, and (iii) is expressed in both estrogen- and non-estrogen-responsive tissues.

MATERIALS AND METHODS

Materials. Nineteen-day-old immature and timed pregnant rats were purchased from Sprague-Dawley (Madison, WI). The immature rats were injected with 3 μg of estradiol for 1, 3, and 6 h. The animals were sacrificed, and their uteri were removed and frozen at −80 °C until used. Pregnant rats were sacrificed, and their uteri were removed and washed 3 times with cold phosphate-buffered saline (PBS) before being frozen at −80 °C. All radioisotopes used in this study were purchased from Du Pont Research Products (Boston, MA). Recombinant TATA binding protein (TBP) and anti-TBP antibody were purchased from Promega Corp. (Madison, WI).

Fractionation of ERE Binding Activity. The cytosolic fractions were prepared from normal, pregnant, or estradiol-treated rat uteri in T-0 buffer (T-0:50 mM Tris-HCl, pH 7.4, 10% glycerol, and 1.5 mM EDTA) and loaded batchwise on a 1 mL Q-Sepharose column. The column was washed with 5 column volumes of T-10 (T-0 plus 10 mM NaCl) and eluated stepwise with 2.5 mL of T-150 followed by 2.5 mL of T-250. The eluated fractions were pooled and concentrated with Centric 30 (Millipore, Bedford, MA). The protein concentration was determined by the Bradford method (Bradford, 1976), after which the proteins were frozen at −80 °C in 25 mg/mL aliquots until used. The samples are stable up to 6 months when stored at −80 °C.

DNA Binding Studies. Electrophoresis mobility shift assay (EMSA) was carried out as previously described (Murdoch et al., 1990). A standard binding reaction was performed at room temperature for 15 min. The reaction mixture (25 μL) contained 0−25 μg of sample, 100 pg of ^{32}P-labeled, double-stranded DNA containing the vitellogenin ER binding site (vit-ERE) (5′-GATCCAGGTCACTGTGACCTGGATC-3′) consensus sequence, 1.0 μg of poly(dI-dC), and 1.0 μg of sheared salmon sperm DNA in 10 mM Hepes (pH 7.9), 0.02% Ficoll 400, 10% glycerol, 100 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT), and 1.0 μg/mL each of leupeptin, pepstatin, antipain, aprotinin, and chymostatin. The protein−DNA complexes were separated on a preelectrophoresis 4% polyacrylamide gel [40% 19:1 acrylamide/bis(acrylamide)] containing 1.0 M glycine, 0.125 M Tris-HCl, and 4 mM EDTA (2× Tris/glycine plus EDTA, pH 8.0). The gel was run in 2X Tris/glycine plus EDTA at 30 mA, dried, and exposed to film for 16−20 h.

For saturation binding analysis, a subsaturating concentration (7−10 μg) of Q-Sepharose T-250 eluate (T-250) was incubated with varying concentrations (0.2−4.6 nM) of [^{32}P]-vit-ERE or [^{32}P]-TF-IID oligonucleotide (5′-GCAGAGCATATAAGGTGAGGTAGGA-3′) at room temperature for 20 min. The bound DNA was separated on a 5% polyacrylamide gel in 2× Tris−glycine buffer. The total and bound fractions were detected quantitatively by a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and analyzed using a Molecular Scanner (Molecular Dynamics). The binding constant was determined from the data obtained from the scanner according to the Scatchard method (Scatchard, 1949).

Competition analysis was performed using 50 pg of ^{32}P-labeled vit-ERE and 25 μg of T-250 eluate in the presence and absence of 23-fold molar excess cold competitor DNA under standard conditions. The oligonucleotides used in the competition studies corresponded to the binding sites of known transcriptional factors and were purchased from Promega Corp. or synthesized by Research Genetics (Huntsville, AL). The random DNAs were of two sources. Random polylinker DNA was excised from the pBluescript IISK plasmid with *Bss*HII and a 173 base pair DNA fragment (multiple cloning site) and gel-purified before use. Random herring sperm DNA (2.0 μg/μL) was sonicated and passed 2 times through a 18 guage needle before use in a competition assay.

UV-cross-linking experiments were carried out using 62 μg of T-250 fraction, 10 ng of ^{32}P-labeled vit-ERE, or TF-IID oligonucleotide in the presence or absence of 600 ng of radioinert TF-IID DNA under standard conditions. The reaction was cross-linked on ice for 15 min using a 312 nm UV light positioned 6 cm above the sample. The UV cross-linked complexes were resolved by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), dried, exposed to a Phosphorimager screen, and analyzed using a Molecular Scanner.

Purification of ERE-BP Activity from Rat Uterus. Typically, 50−100 mg of normal rat uteri was used to prepared cytosol as described above. The cytosol was loaded on a 5 mL Q-Sepharose column connected to a fast-flow performance liquid chromatograph (Pharmacia Biotech, Piscataway, NJ) at 1.0 mL/min. The column was washed with 20 column volumes of T-100, and then eluated with a 50 mL linear gradient of T-100 to T-250. The active fractions were located by EMSA, pooled, made to 10 mM phosphate, and loaded on a 2.0 mL hydroxyapatite column. After being washed with a 10 column volume of 10 mM phosphate buffer, the column was eluated with a 20 mL linear phosphate gradient from 10 to 250 mM phosphate. The active fractions were located by EMSA, pooled, and concentrated with a Centricon 30 spin column to 0.2−0.5 mL. The Centricon 30 retentate was applied to a 3.5 × 100 cm Sephacryl S300 column equilibrated with T-100, followed by elution with 300 mL of T-100, and the eluated fractions were assayed by EMSA.

RESULTS

We previously reported the presence of a protein factor in rat uterine cytosolic extract that interferes with ER binding to ERE found in the upstream flanking region of the prolactin gene (prolactin ERE), but fails to affect ER binding to ERE derived from the vitellogenin A2 gene (Murdoch, 1995). Furthermore, when rat uterine cytosol is assayed by EMSA using ERE derived from the vitellogenin A2 gene, three different protein−DNA complexes are formed (Gray, 1994). In order to further characterize these activities, we used Q-Sepharose anion exchange chromatography to fractionate cytosol from 19-day-old rat uteri into ER-containing (T-150) and non-ER-containing fractions (T-250) (Figure 1A). Mobility shift analysis of these fractions identified two ERE binding proteins (Figure 1B). The two slowest migrating protein−DNA complexes formed from the T-150 eluate

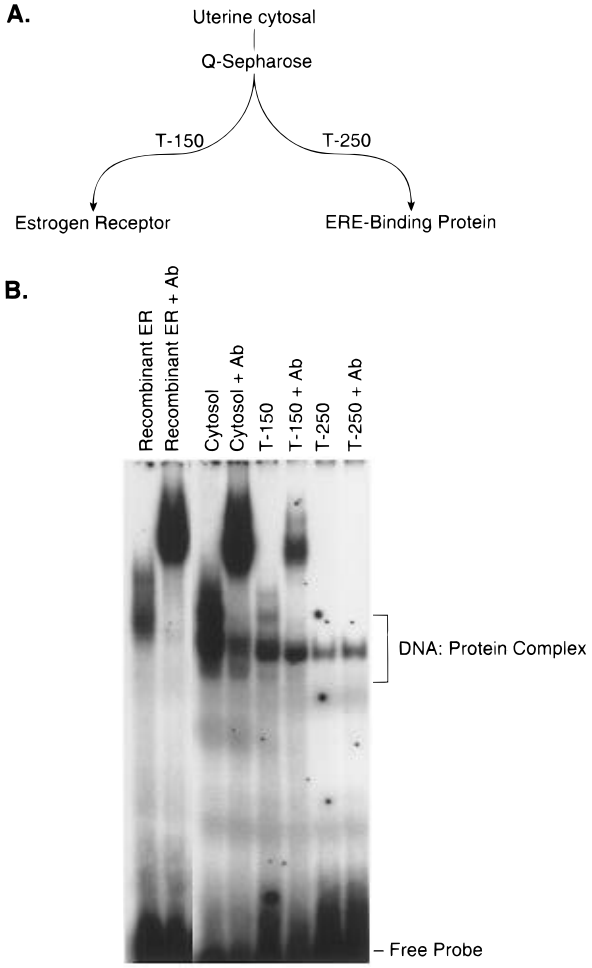

FIGURE 1: Anion exchange chromatography demonstrates the presence of ERE binding protein (ERE-BP) activity in the rat uterus. Cytosols were prepared from the uteri of 19-day-old, female, Sprague-Dawley rats and fractionated by Q-Sepharose ionic exchange as described under Materials and Methods. An electrophoresis mobility shift assay (EMSA) was performed using equal amounts of protein (0.25 μg of recombinant ER, and 25.0 μg of either cytosol, Q-150, on Q-250 eluates) and 100 pg of ^{32}P-labeled, doubled-stranded vit-ERE in the presence and absence of ER antibody. The bound protein complexes were separated on a 5% nondenaturing polyacrylamide gel in 2× Tris/glycine buffer. (A) Schematic diagram for ERE-BP fractionation. (B) EMSA of ERE-BP fractions. Cytosol; T-150 and T-250, 150 and 250 mM NaCl eluates of the Q-Sepharose, respectively; ER, estrogen receptor.

contained the ER as judged by the decreased mobility in the presence of anti-ER antibody (Figure 1B, lanes 4 and 5) (Murdoch et al., 1990). Addition of ER antibody to the T-250 fraction fails to retard the ERE binding activity (Figure 1B, lanes 7 and 8). We refer to the ERE binding activity present in the T-250 Q-Sepharose fraction as the ERE binding protein (ERE-BP).

Characterization of the ERE-BP Binding Site. The presence of an ERE-BP, in addition to the ER, may result in inhibition of the ER binding by competing for binding to the ERE (squelching of ER). Therefore, it was imperative that we determine the specificity and affinity of DNA for ERE-BP. DNAs corresponding to the binding sites of known transcriptional factors and polylinker DNA (Figure 2) were used as competitors of ^{32}P-labeled vit-ERE in competition analysis. When ERE-BP was assayed in the presence of 23-fold excess of unlabeled vit-ERE, 55% of the binding was lost (Figure 2). Examination of the ability of several

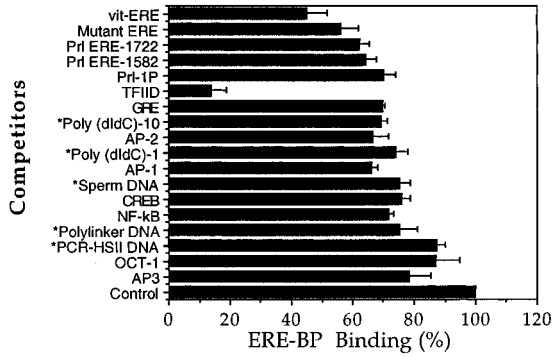

FIGURE 2: Specificity of ERE-BP binding. Competition analyses were performed using 50 pg of ^{32}P-labeled vit-ERE and 25 μg of T-250 eluate in the presence and absence of 23-fold molar excess cold competitor DNA under standard conditions. The level of competition was calculated from densitometric analysis of the EMSA gel and is expressed as a percentage of control. The random DNA [*poly(dI-dC), sperm DNA, PCR-HSII DNA, and polylinker DNA] was added at 23-fold excess by weight. The graphs represent the average of two experiments performed in duplicate ($n = 2$). Control, no added DNA.

unrelated DNA sequences to compete with vit-ERE for binding to ERE-BP reveals a 50−60% inhibition of binding. It appears that DNA sequences with ERE-like (having one half-site) motifs are able to challenge 50% of vit-ERE binding. In addition, assaying ERE-BP in the presence of 23-fold molar excess of the TF-IID sequence eliminated over 80−90% of the binding (Figure 2, compare control to TF-IID), indicating that ERE-BP binds to the TF-IID sequence with greater specificity. To corroborate the specificity of ERE-BP binding, several unrelated DNA sequences were used in the competition assay. Figure 2 shows that none of the random DNA or binding sites of several well-characterized transcriptional factors show any significant binding to ERE-BP. Thus, it appears that ERE-BP is specific for both TF-IID and vit-ERE DNA sequences, although ERE-BP binds to the latter with a lower affinity.

A comparison between the DNA sequences that bind the ER and TATA binding subunit (TBP) reveals no similarity. Furthermore, when the competing oligonucleotides were labeled to the same specific activity and used in EMSA, similar results were obtained (data not shown). The absence of sequence similarity between vit-ERE and TF-IID and the high affinity of ERE-BP for the TF-IID sequence suggest that this protein may be the TBP of the TF-IID transcriptional factor (Kim et al., 1993). To address this issue, Western analysis (Figure 3A) was performed with 0.10 μg of recombinant TBP and 6 μg of ERE-BP. When the samples were probed with anti-TBP, an immunoreactive protein corresponding to the predicted size of TBP was observed with the recombinant TBP (Figure 3A, lane 2). However, no immunoreactive protein was detected in the ERE-BP fraction (Figure 3A; compare lanes 2 and 3).

We were concerned that the anti-TBP could not detect ERE-BP in our Western analysis because the protein was denatured. To examine this possibility, EMSA was performed using ERE-BP and recombinant TBP in the presence and absence of TBP antibody. Figure 3B indicates that ERE-BP and TBP form distinct protein−DNA complexes when TF-IID oligonucleotide is used as a probe. Addition of anti-TBP after the complex is formed fails to retard the migration of either ERE-BP or TBP (Figure 3B). The inability of this antibody to retard the migration of the TBP−DNA complex

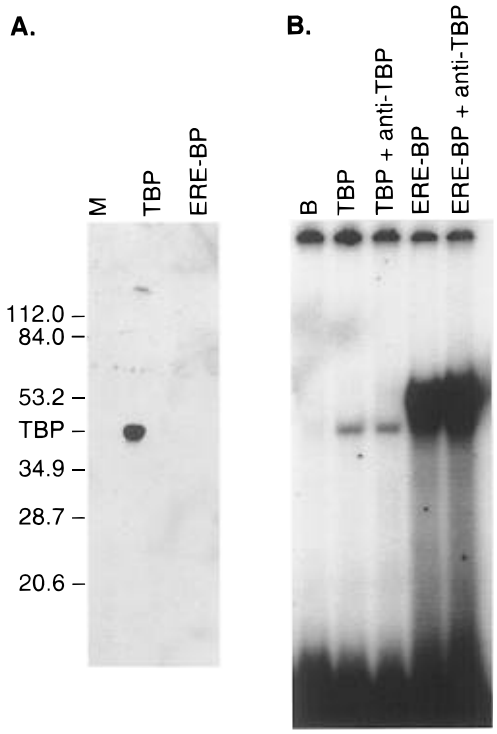

FIGURE 3: Immunological analysis of ERE-BP. (A) Western blot of TBP and ERE-BP. 0.10 mg of TBP and 6.0 mg of ERE-BP were fractionated on a 12% SDS−PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a 1:1000 dilution of anti-TBP, and the immunoreactive proteins were visualized by the ECL Western blotting system. Lane 1, marker; lane 2, TBP; lane 3, ERE-BP (T-250). (B) EMSA of TATA binding protein (TBP) and ERE-BP. 20 ng of TBP and 20 μg of ERE-BP were assayed in the presence and absence of TBP antibody under standard conditions.

indicates that the antibody binding site is masked when TBP is bound to DNA. These data confirm that ERE-BP is a distinct protein that is able to bind to both the ERE and the TATA binding sites.

In order to determine the apparent K_d of ERE-BP, we took advantage of the high sensitivity and wide linear dynamic range of the Molecular Dynamics Phosphorimager (Johnston, 1990). The advantage of using this instrument to measure binding parameters is that it allows direct quantification of both the bound DNA−protein complex and free DNA directly from mobility shift assay gels (Johnston, 1990; Williams, 1995).

Saturation analysis was performed with a subsaturating concentration of ERE-BP over a wide concentration range (0.2−4.6 nM) of either vit-ERE or TF-IID consensus oligonucleotide sequence. Figure 4 shows that ERE-BP binding to vit-ERE DNA resulted in a saturable response, consistent with the existence of a single binding site (Figure 4A). Examination of the saturation binding data by the method of Scatchard indicates that the ERE-BP binds to the vit-ERE with an apparent 1.0 nM K_d (Figure 4B, insert). This affinity is comparable to the affinity of the ER binding to this same sequence (Murdoch, 1995). Conversely, when saturation analysis was performed using the TF-IID oligonucleotide, a nonsaturable response was observed (Figure 4C). Transformation of the saturation data reveals the presence of two sites, K_d 0.12 and 1.18 nM, as judged by the nonlinear appearance of the Scatchard plot (Figure 4C, insert). However, if the data are fitted to a one-site model, linear regression analysis argues for the presence of a single, high-affinity site (Figure 4C, insert, K_d no. 1, $R^2 = 0.91$). Visual representation of the saturation binding (Figure 4D) demonstrates that only one DNA−protein complex was formed. This observation is consistent with the linear regression analysis of the data, but does not rule out the presence of a low-affinity site that is undetectable by EMSA.

To delineate further ERE-BP involvement in this estrogen-regulated pathway, immature rats were injected with a single 1.0 μg dose of estradiol for 1, 3, or 6 h. Uteri were homogenized at equal tissue weight per unit volume, and the resulting cytosol was fractionated as described above and assayed by EMSA. Figure 5A demonstrates that ERE-BP is present in high amounts (0.59 pmol of ERE-BP binding activity/mg of uteri) in the untreated animal but decreases to less than 50% (0.38 pmol of ERE-BP binding activity/mg of uteri) at 1−3 h of estradiol treatment. In most experiments, this repression was sustained for up to 3 h and returned to 80% of its normal levels after 6 h. However, in some experiments, the repression persisted up to 6 h without returning to normal levels. The repression of ERE-BP by estradiol may be occurring through the ER. It should be noted that estradiol also represses ER levels within 3 h following estradiol injection (Ogle, 1995a). Therefore, the apparent stimulation of ERE-BP observed in some experiments after 6 h may represent loss of the ER. Furthermore, the repression of ERE-BP by estradiol is not a dilution effect caused by an increase in uterine weight due to estradiol, because the experiment produced the same result when equal weight per unit volume or equal volume per unit weight was examined (data not shown). Thus, the 2-fold repression in the levels of ERE-BP suggests that this protein is involved in estradiol-dependent pathways.

We next determined if the decrease in ERE-BP by estradiol is physiologically relevant. This issue was addressed by comparing the levels of ERE-BP during gestation. EMSA analysis of timed pregnant rats indicates that ERE-BP decreases to 50% of its normal levels (0.59 pmol of ERE-BP binding activity/mg of uteri to 0.237 pmol/mg of uteri) during gestation (Figure 5B). The highest level of repression occurred on or by day 20 of gestation (Figure 5B, compare days 0 and 20). This maximal repression of ERE-BP corresponds to a period during gestation when the rat's serum estradiol concentration reaches a maximum of 150−160 pmol/mL (Bridges, 1984), concentrations analogous to our in vitro experiments. These results show that the rat uterus contains a protein that is able to bind to the ER binding site and is regulated by estradiol and the onset of gestation.

The presence of an additional ERE binding protein that may influence ER action prompted us to examine the expression pattern of this unique activity. Since no antibody or DNA probes are available for ERE-BP, two criteria were developed to assess the expression levels of ERE-BP in different tissues. The first criterium takes advantage of the fact that ERE-BP is unresponsive to the ER antibody (Figure 1A), and the second makes use of its high specificity for the TF-IID consensus oligonucleotide (Figure 2). Using these two criteria to monitor ERE-BP, cytosolic fractions from various tissues were fractionated and assayed by EMSA in the presence or absence of ER antibody, anti-TBP, or 25-fold excess of TF-IID DNA. Figure 6 shows that ERE-BP is a ubiquitous protein because it is expressed to some degree in all tissues examined. ERE-BP is expressed in estrogen-

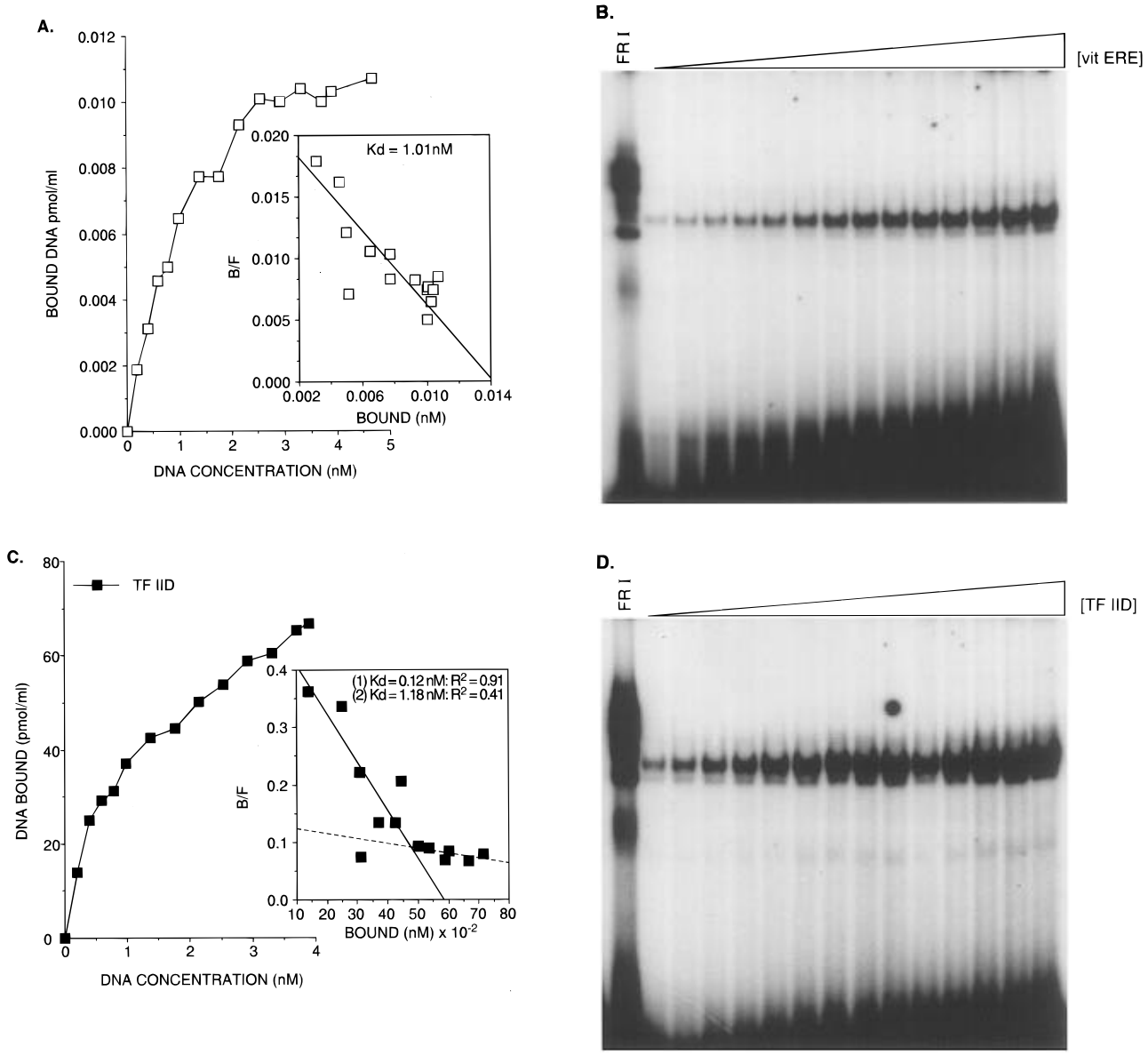

FIGURE 4: Saturation analysis of ERE-BP binding. Subsaturating concentrations of ERE-BP were incubated with varying concentrations (0.2−4.6 nM) of ^{32}P-labeled vit-ERE or TF-IID consensus oligonucleotide and 10 μg of T-250 eluate under standard assay conditions. The total and bound oligonucleotides were detected quantitatively by exposure to a Phosphorimager screen (Molecular Dynamics) and analyzed using a Molecular Scanner (Molecular Dynamics). Binding constants were determined from the data obtained from the Molecular Scanner according to the method of Scatchard (1949). (A) Saturation binding analysis with the vit-ERE as a probe. The insert is a Scatchard plot of saturation binding data. The graph fits a one-site model. (B) EMSA of saturation data in (A). Lane 1, FR I (cytosol); lanes 2−15, 0.2−4.6 nM vit-ERE probe. (C) Saturation binding analysis using TF-IID oligonucleotide as a probe. The insert is a Scatchard plot of saturation binding data in (C). This graph fits a two-site model. The first K_d was obtained by performing linear regression on the points connected by the solid line and the second K_d from the points connected by the dashed line. (D) EMSA of saturation data shown in (C). Lane 1, FR I (cytosol,vit-ERE probe); lanes 2−15, 0.2−4.0 nM TF-IID oligonucleotide as probe.

responsive (uterus and pituitary) as well as non-estrogen-responsive (spleen and hypothalamus) tissue. A comparison of the relative expression levels of ERE-BP in estrogen-responsive tissues indicates that pituitary shows the highest expression levels (Figure 6, compare pituitary with uterus and liver). However, heart, a non-estrogen-responsive tissue, shows the lowest expression levels (Figure 6; compare pituitary with heart). The wide distribution of ERE-BP suggests that this protein may function in estrogenic and nonestrogenic processes.

To rule out the possibility that the binding observed in Figure 6A was not a result of contaminating TBP, we performed Western blot analysis on the samples using anti-TBP. Analysis of 20 μg of total protein from each tissue (ERE-BP fractions) failed to detect any TBP-specific protein (Figure 6B, lanes 3−13). However, analysis of 0.02 μg of recombinant TBP detected a specific 38 kDa protein band that corresponds to the predicted size of TBP (Figure 6B, lanes 2 and 4). The anti-TBP used in this study also detected a specific 60 kDa protein band that was present in all tissues examined (Figure 6B, band A). Although band A in Figure 6B shows strong cross-reactivity to anti-TBP and is present in all tissues examined, it is not responsible for the ERE-BP activity (compare band A in Figure 6B with Figure 6A). Quantification of band A in Figure 6B reveals that its distribution pattern differs from that of ERE-BP. A comparison of the relative levels of band A indicates that it is not expressed in the pituitary (0.57% of levels in the uterus)

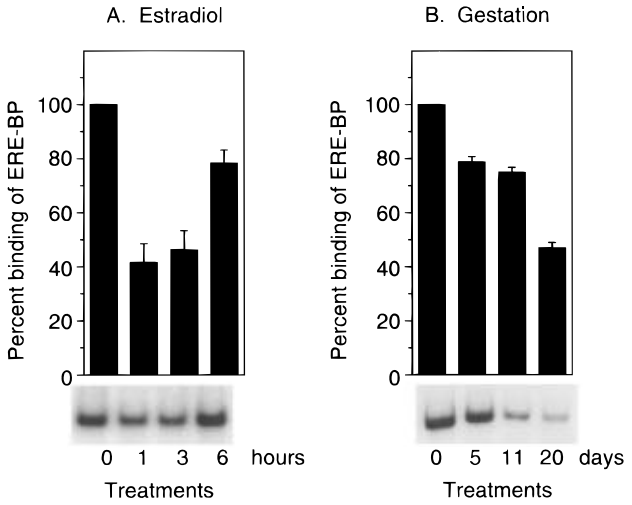

FIGURE 5: Modulation of ERE-BP by estradiol injection or the onset of gestation. Cytosols were prepared from the uterus of estradiol-treated or pregnant rats and fractionated as described under Materials and Methods. EMSA was performed on the T-250 eluate using 25 μg of protein and 100 pg of ^{32}P-labeled vit-ERE DNA as a probe. The binding was quantified by densitometric analysis and expressed as a percentage of control. The graphs represent the average of four experiments ($n = 4$), and the figure is representative of a typical experiment.

whereas ERE-BP is present in high amounts in this tissue (291% of levels in the uterus). Furthermore, in tissues where band A is present in high amounts (heart, 313% of levels in the uterus), ERE-BP is not expressed (heart, 3.4% of that in the uterus). These data demonstrate that the expression observed in Figure 6A is not a result of TBP binding to the TF-IID consensus DNA.

Partial Purification of ERE-BP. To further characterize ERE-BP activity, the T-250 fraction was partially purified by three chromatography steps (Q-Sepharose, hydroxyapatite, and Sephacryl S300). Analysis of the individual column fractions and the pooled column fractions by EMSA indicates that ERE-BP migrates as a single complex on each column (data not shown, Figure 1). SDS−PAGE of the final ERE-BP fraction indicates the presence of three major protein bands (p102, p81, and p48) as judged by Coomassie staining (Figure 7A, lane 3). To corroborate which proteins were responsible for the ERE-BP activity, the DNA−protein complex was allowed to form, UV cross-linked, and separated by SDS−PAGE (Figure 7B). Autoradiography of the gel indicates that the ERE-BP/DNA complex migrates as a 60 kDa band (Figure 7B). The 60 kDa complex is specific for ERE-BP as evidenced by the fact that the same complex is formed with either labeled vit-ERE or labeled TF-IID. Furthermore, an excess of unlabeled TF-IID oligonucleotide obliterates the cross-linked product formed by both vit-ERE and TF-IID (Figure 7B; compare lanes 2 and 3 with lanes 4 and 5). The 60 kDa complex that was formed represents both the bound protein and the labeled DNA (see Materials and Methods). In order to obtain the apparent molecular mass of ERE-BP, the relative mobility of the DNA in the absence of protein was subtracted from the relative mobility of the ERE-BP/DNA complex. After correcting for the mass contributed by the DNA, the ERE-BP should migrate as a 48 kDa band (Figure 7). This molecular mass corresponds with the p48 protein seen in the final purified fraction (Figure 7A, lane 3, asterisk).

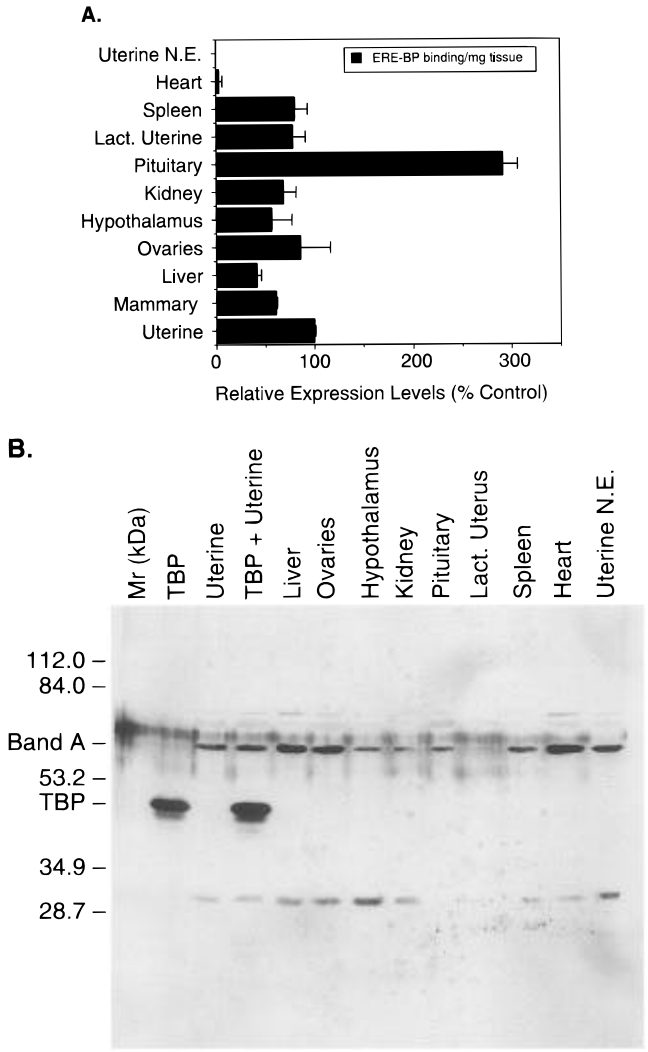

FIGURE 6: Expression pattern of ERE-BP. (A) EMSA analysis of different rat uterine tissues for the presence of ERE-BP. The graph represents two independent experiments performed in duplicate. (B) Western analysis of the samples in (A) for TBP-specific protein using anti-TBP antibody. Cytosol was prepared from equal weights of each tissue (as indicated on the figure) and fractionated as described under Materials and Methods. 25 μg of total protein was assayed via EMSA for ERE-BP binding in the presence or absence of either anti-ER, anti-TBP, or 25-fold excess of TF-IID consensus oligonucleotide or by Western blot using anti-TBP antibody. The relative amount of ERE-BP binding was determined using a Molecular Dynamics Phosphorimager and reported as a percentage of the binding obtained in the uterus.

DISCUSSION

Binding of the ER to its ERE is one of the critical events in transcriptional modulation by ER. Therefore, an understanding of the different types of interactions that occur at the ERE is central to understanding ER action. Accordingly, we have made the following observations: (i) The rat uterus contains a unique protein that binds with high affinity to the ER-responsive element. (ii) In addition to binding to ERE, this protein binds to the TATA binding sequence with high affinity. (iii) The levels of this protein are modulated by estradiol and during gestation of the rat. (iv) This protein is widely distributed among estrogen-responsive and estrogen-unresponsive tissues.

Recently, a number of reports identified several ER accessory proteins that may be involved in ER regulation of transcription (Cavailles et al., 1994; Halacchmi et al., 1994;

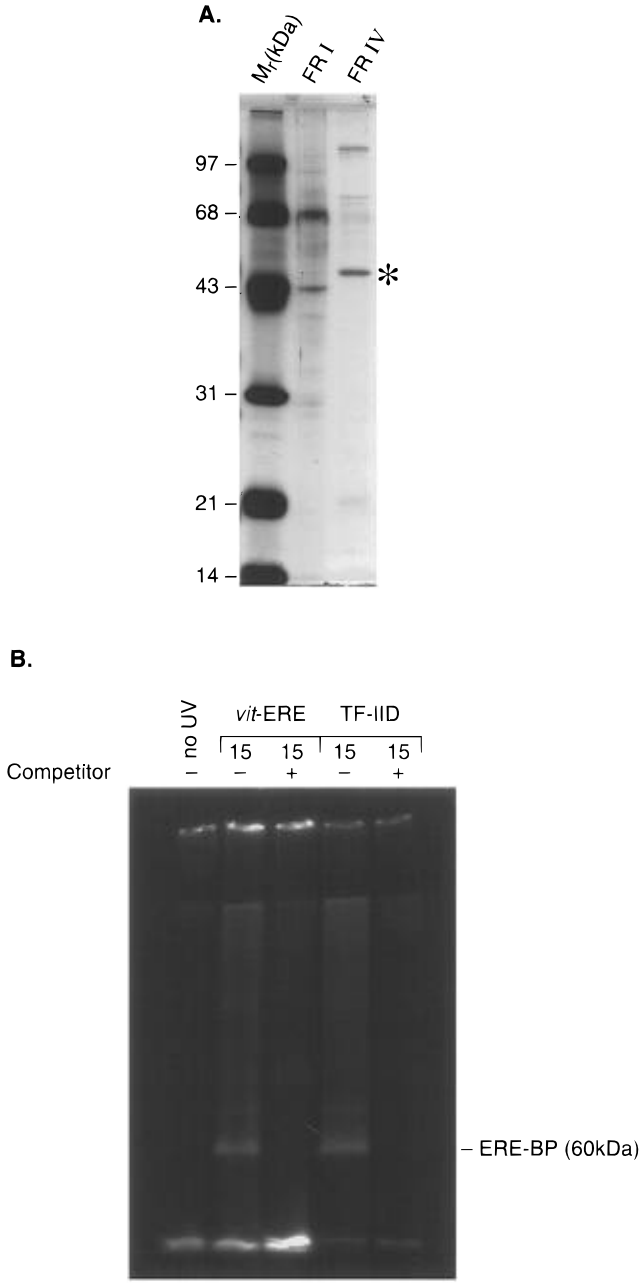

FIGURE 7: Estimation of ERE-BP molecular mass by UV cross-linking. (A) SDS−PAGE of partially purified ERE-BP. Normal uterine cytosol was partially purified by three successive chromatography steps (Q-Sepharose, hydroxyapatite, and Sephacryl S300), and the ERE-BP was localized by EMSA. 25 μg of cytosol (FR I) and 2.0 μg of Sephacryl S300 (FR IV) were separated on a 10% SDS−PAGE, and the fractionated proteins were visualized by Coomassie staining. (B) Autoradiography of a UV cross-linked gel. 25 μg of FR II was incubated with 10 ng of ^{32}P-labeled vit-ERE or TF-IID oligonucleotide in the presence or absence of 600 ng of radioinert TF-IID DNA under standard conditions. The reaction was UV cross-linked, and the complexes were resolved on a 10% SDS−PAGE, dried, exposed to a Phosphorimager screen, and analyzed using a Molecular Scanner.

Jacq et al., 1994). These accessory proteins are thought to be coactivators that function in conjunction with the ER in regulating transcription. However, none of these proteins have been shown to be ER-specific coactivators or specific for a particular estrogen-responsive gene. Although the precise role of these accessory proteins in estrogen action is unknown, it is postulated that they play a central role in ER action. The protein described in this study has the potential to function as a regulator of estrogen action. First, it possesses the properties of a positive repressor protein, and, second, it may function as an ER accessory protein.

A comparison of the DNA binding affinity of ERE-BP and ER (Figure 4; Murdoch, 1995) suggests that both proteins should be able to recognize and distinguish between ERE and non-ERE sequences to the same degree. Thus, ERE-BP would be able to repress ER action by competing with or blocking ER access to DNA. Our competition data indicate that ERE-BP is able to bind to all the DNA containing an ERE sequence. The slight competition produced by some sequences may be due to the partial EREs (one half-site) present in these DNA sequences. We speculate that the ability of ERE-BP to interact with such a diverse set of EREs may lie in its ability to recognize ERE half-sites. It should be pointed out that most of the ERE present in the mammalian gene does not possess the consensus ERE found in the vitellogenin gene. Rather, they contain one full half-site and one or two base mutations in the other half-site. It is possible that in tissues where ERE-BP is present in high levels, it could function in conjunction with the ER in regulating certain genes in much the same way as the chicken ovalbumin upstream promoter transcription factor (COUP-TF) functions with the ER in induction of the lactoferrin gene (Youhua, 1993).

Although the exact identity and function of the ERE-BP are unknown, our data suggest that it is a ubiquitous protein. The wide distribution pattern of ERE-BP is consistent with that of a repressor protein. For example, the ERE-BP is expressed in extremely high levels in the pituitary and uterus as compared with the liver, suggesting that it may be specific for estrogen-responsive tissues (Figure 6). In these tissues, ERE-BP may function as a repressor of ER action. However, ERE-BP expression in non-ER-responsive tissue such as the spleen indicates that the ERE-BP may be involved in other processes that do not involve the ER, presumably by its ability to bind TATA-containing sequences. Since the method that was used to assess the distribution of ERE-BP is subject to interference from other proteins that may bind to the ERE half-sites with low affinity (Rishi, 1995), we may not be obtaining an accurate measure of ERE-BP distribution.

Estrogen, progesterone, and other ovarian hormones are responsible for maintenance of the uterus. These hormones lead to stage-specific gene expression and a conversion of the uterus from a pre-implantation to an implantation stage. Of the genes expressed in response to estrogen or to the onset of pregnancy, only a few have been identified (Chiappetta, 1992; Huet-Hundson, 1990; L'Horest, 1990; Zhu, 1995). The genes responsible for the estrogen-induced decidualization of the uterus in response to estradiol or pregnancy remain unknown. We have shown that in addition to binding to the ERE and the TATA binding sequence, ERE-BP is down-regulated by the injection of estradiol and the onset of gestation. Although the mechanism of down-regulation was not examined, our data imply that the ER is involved. The 2-fold repression of ERE-BP parallels the loss of ER (Figure 5; Ogle, 1995b). In addition, ERE-BP was found to be repressed during the period of the rat's gestation when the serum estradiol concentration was comparable to the in vitro-injected estradiol concentration (Bridges, 1984). The fact that ERE-BP repression appears to parallel ER repression (Ogle, 1995b) suggests that both ERE-BP repression and loss of ER may be related. However, a more detailed analysis

of the degree of ERE-BP repression and the disappearance of ER is needed.

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