Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) was identified as a low abundance protein in bovine uterus that co-purified with estrogen receptor (ER) in a ligand-independent manner and was separated from the ER by its lower retention on estrogen response element (ERE)-Sepharose. In gel mobility shift assays, COUP-TF bound as an apparent dimer to ERE and ERE half-sites. COUP-TF bound to an ERE half-site with high affinity, $K_d = 1.24$ nM. In contrast, ER did not bind a single ERE half-site. None of the class II nuclear receptors analyzed, i.e. retinoic acid receptor, retinoid X receptor, thyroid receptor, peroxisome proliferator-activated receptor, or vitamin D receptor, were constituents of the COUP-TF-ERE binding complex detected in gel mobility shift assays. Direct interaction of COUP-TF with ER was indicated by GST "pull-down" and co-immunoprecipitation assays. The nature of the ER ligand influenced COUP-TF-ERE half-site binding. When ER was liganded by the antiestrogen 4-hydroxytamoxifen (4-OHT), COUP-TF-half-site interaction decreased. Conversely, COUP-TF transcribed and translated in vitro enhanced the ERE binding of purified estradiol (E$_2$)-liganded ER but not 4-OHT-ligated ER. Co-transfection of ER-expressing MCF-7 human breast cancer cells with an expression vector for COUP-TFI resulted in a dose-dependent inhibition of E$_2$-induced expression of a luciferase reporter gene under the control of three tandem copies of ERE. The ability of COUP-TF to bind specifically to EREs and half-sites, to interact with ER, and to inhibit E$_2$-induced gene expression suggests COUP-TF regulates ER action by both direct DNA binding competition and through protein-protein interactions.

The estrogen receptor (ER) is a transactivating enhancer protein that is a member of the ligand-activated steroid/nuclear receptor gene superfamily of proteins that share two conserved regions, the DNA-binding (C) and the ligand binding (E) domains (1). The physiological sequelae of estrogen action involve a passive diffusion of ligands, e.g. estradiol (E$_2$), into cells and the binding of E$_2$ with high affinity and specificity to ER in the nucleus of target cells. Ligand binding initiates a series of steps forming an "activated," homodimeric E$_2$-ER complex that binds with high affinity to specific DNA sequences, estrogen response elements (ERE). Analysis of the 5'-regulatory regions of numerous estrogen-responsive genes revealed a 13-bp palindromic ERE consensus sequence: 5'-GGTCAAnnnTGACC-3' (EREc, where $n = $ any nucleotide in the center spacer region). EREc is the minimal ERE that conferred estrogen responsiveness to reporter genes analyzed by transfection assay (2, 3). However, the crystal structure of the ER DNA binding domain when bound to DNA showed that ER contacts both the 5'-C and A in the more extended palindrome 5'-CAGGTCAAnnnTGACCCTG-3' (4), indicating that a longer inverted repeat (IR) may stabilize ER binding.

Most estrogen-responsive genes identified to date contain one or more imperfect EREs or multiple copies of the ERE half-site rather than EREc (5). The latter genes are also regulated by class II nuclear receptors, e.g. thyroid hormone receptor (TR), retinoic acid receptor (RAR), retinoid X receptor (RXR), and orphan receptors, e.g. estrogen-related receptor (ERR), that bind to direct and inverted repeats (DR and IR) of the ERE half-site 5'-AGGTCA-3' (6, 7). Recent studies demonstrated that ER also binds various spaced DR and IR of the ERE half-site motif, albeit with significantly lower affinity when compared with ER binding to EREc (5, 8).

Once bound to an ERE, the precise mechanism of transcriptional activation, or repression, by the ER is unknown. ER-mediated effects on transcription are thought to involve interaction between the DNA-bound ER and transcription factors, coactivator proteins, e.g. ERAP160; RIP140, SPT6; SRC-1; TIF1, TIF2, or components of the TATA binding complex including TFIIB and TATA-box binding protein (reviewed in Ref. 9). Two distinct ER regions are involved in these interactions.
COUP-TF but Not ER Binds Single ERE Half-sites

as follows: an N-terminal, ligand-independent activation function 1 (AF-1), and a C-terminal, ligand-dependent AF-2 (10, 11). The total and relative activity of each AF varies with the promoter and cell type, indicating that the function of each AF is mediated by interaction with cell-specific proteins (12–14).

In previous work, we quantitated the effect of ERE sequences, spacing, and the role of sequences flanking the ERE on the affinity and stoichiometry of liganded ER-ERE interaction in vitro (15–25). In sum, our results indicate a critical role for sequences flanking the ERE and the relative helix orientation of the EREs on ER binding affinity. We also showed that the ER ligand modulates ERE binding parameters in vitro and that the purity of the ER preparation influences the affinity of ERE binding. Highly purified ER binds to EREs with significantly lower affinity than partially purified ER, i.e., for E$_2$-ER K$_d$ = 1.74 nM versus 0.24 nM (25). The latter result implies that proteins present in a partially purified preparation of bovine uterine ER facilitate high affinity binding of ER to EREs. Here we identified COUP-TF as a constituent of the partially purified bovine uterine ER-ERE binding complex. COUP-TF is an orphan nuclear receptor that binds to DR or of the ERE half-site as a homodimer or as a heterodimer with RXR (26).

We have examined the ability of COUP-TF to bind full and half-site EREs in vitro and to modulate the expression of an estrogen-responsive reporter gene in transiently transfected ER expression MCF-7 human breast cancer cells.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids Containing EREs—The sequences of select synthetic single-stranded oligonucleotides are given in Table I. ERE38 is a 38-bp ER consensus sequence (15). 1/2ERE38 and 1/2ERE33s contain half-site EREs. Double-stranded oligomers were ligated into the Smal restriction site of the vector pGEM-7Zf(+) (Promega) as described (18). The βRare (retinoic acid response element) is a synthetic version of the mouse RAR type β gene (27–29).

Single or multiple, head-to-tail, tandem copies of ERE38 were removed from pGEM-7Zf(+) (18) by double digestion with Kpn1 and Sca1 and cloned directly into the upstream multiple cloning site in pGL3-Promoter vector (Promega). This places the ERE 83 nucleotides upstream of the SV40 promoter. The constructs containing ERE38 are called pGL3–1ERE38, 2ERE38, and 3ERE38 with the number indicating the number of tandem copies of ERE38.

Preparation of ER—ER was partially purified from calf uterine by heparin-agarose (Affi-Gel heparin, Bio-Rad) and ERE-Sepharose affinity chromatography as described previously (30, 31). ER was liganded with $[^3]$H]tamoxifen aziridine (23 Ci/mmol, Amersham Corp.). ER concentration was determined by sedimentation through a G-50 Sephadex spin column in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.3) and electrophoresed at 200 V for 2.25 h at 4 °C. Gels in various amounts of antibodies (see figure legends) in a total reaction volume of 70 μl. Forty-μl aliquots, 70 μl KClfinal, of the preincubated ER-ERE mixture were loaded on 4% non-denaturing polyacrylamide gel electrophoresis using 0.1 M Tris, 83.1 mM boric acid, 1 mM EDTA, pH 8.3) and electrophoresed at 200 V for 2.25 h at 4 °C. Gels were dried under vacuum and autoradiographed on Kodak X-Omat film with an intensifying screen (Lightning Plus from DuPont).

The amount of ERE-ERE complex formed, and that of free ER, was determined by excision of the corresponding regions from the dried gels into scintillation vials containing 3 ml of Ecoscint A (National Diagnostics, Atlanta, GA), and the radioactivity was counted. The fraction of total $[^3]$P-ERE in the ER-ERE complex was calculated as follows: F(t) = (cpm in the ER-ERE complex)/(total cpm in the lane), where the (total cpm in the lane) = (cpm in free ERE) + (cpm in the ER-ERE complex) (35).

Antibodies—H222 monoclonal antibody (Mab) to ER was a gift of Abbott. H222 was diluted 1:10 in TE and 1.0 μl of added to selected samples in each experiment to confirm the identity of ER protein (see figure legends). MAbs 33 and 213, and MAbs NMT-1 and NMT-2 recognize the DNA-binding domain of the human ER and show no reactivity to glucocorticoid receptor, progesterone receptor, or androgen receptor (38). MAbs AER304, AER314, AER308, AER315, AER303, AER311, AER317, and AER320 (36) were generous gifts from Neomarkers (Lab Diagnostics, Atlanta, GA), and the radioactivity was counted. The fraction of total $[^3]$P-ERE in the ER-ERE complex was calculated as follows: F(t) = (cpm in the ER-ERE complex)/(total cpm in the lane), where the (total cpm in the lane) = (cpm in free ERE) + (cpm in the ER-ERE complex) (35).

The following antibodies were gifts of Dr. Abdulmaged M. Traish of Boston University: ER-specific polyclonal antibodies AT2A, AT3A, and AT3B recognize the DNA-binding domain of the human ER and show no reactivity to glucocorticoid receptor, progesterone receptor, or androgen receptor (38). Mabs 33 and 213, and Mabs NMT-1 and NMT-2 react specifically with ER (39–41). Mabs to RARz and RARy were prepared by standard hybridoma technology (42). Polyclonal RAR and RARx antibody RARyIBB was raised against a region just N-terminal to the first zinc finger and recognized all forms of RAR and RXR, but not any of the steroid receptors. Polyclonal antibody R1AB was raised against a polypeptide encompassing amino acids 63–77 (TQSSSSSEEIVPSPPSS) to RARz. Polyclonal antibody γNTB (also called RNTB) was raised against an oligopeptide corresponding to the amino acid sequence encompassing residues between 55 and 68 (STPSPATETQSSS) of RARz. RARx antibody R1AB recognized RARz, but not ER, progesterone receptor, or glucocorticoid receptor (48).

Polyclonal antiserum to RXR was a gift from Dr. Pierre Chambon of the NIDDK, National Institutes of Health. The following antibodies were gifts of Dr. Abdulmaged M. Traish of Boston University: ER-specific polyclonal antibodies AT2A, AT3A, and AT3B recognize the DNA-binding domain of the human ER and show no reactivity to glucocorticoid receptor, progesterone receptor, or androgen receptor (38). Mabs 33 and 213, and Mabs NMT-1 and NMT-2 react specifically with ER (39–41). Mabs to RARz and RARy were prepared by standard hybridoma technology (42). Polyclonal RAR and RARx antibody RARyIBB was raised against a region just N-terminal to the first zinc finger and recognized all forms of RAR and RXR, but not any of the steroid receptors. Polyclonal antibody R1AB was raised against a polypeptide encompassing amino acids 63–77 (TQSSSSSEEIVPSPPSS) to RARz. Polyclonal antibody γNTB (also called RNTB) was raised against an oligopeptide corresponding to the amino acid sequence encompassing residues between 55 and 68 (STPSPATETQSSS) of RARz. RARx antibody R1AB recognized RARz, but not ER, progesterone receptor, or glucocorticoid receptor (48).

Polyclonal antiserum to RXR was a gift from Dr. Pierre Chambon of the Université Louis Pasteur in Strasbourg, France. Mab to pereosixome proliferator-activated receptor was a gift from Dr. Michel Dauca of the
Université de Nancy I in France. MAB to TRα and TRβ were purchased from Affinity BioReagents, Golden, CO. MAB 947 to vitamin D receptor was a gift of Dr. Mark R. Haussler of the University of Arizona. Polyclonal anti sera to COUP-TF and COUP-TFI were purchased from MAB, while anti-serum to TRα and TRβ was purchased from MAB. The concentrations of the glutathione-Sepharose-purified Wisconsin GST fusion proteins and GST expressed from pGEX-2TK was determined by the method of Bradford (45). Protein concentrations were determined by the method of Lowry et al. (46). Protein-Polyacrylamide Gel Electrophoresis, Fluorography, and Western Blotting—Proteins present at various stages of ER purification were analyzed on either 8 or 10% SDS-polyacrylamide gels. Protein concentrations were determined by the method of Bradford (45). Protein molecular weight standards (Mark12 and MultiMark from Novex, San Diego, CA, or, for the prestained protein molecular weight standards, Kaleidoscope (Bio-Rad) were electrophoresed under identical conditions with experimental samples in 25 mM Tris, 192 mM glycine, 0.1% SDS at 135 V for 1.5 h, and the gels were fixed in 10% acetic acid, 50% methanol for several hours. Silver staining was performed using the Bio-Rad Silver Stain Plus kit according to the manufacturer's instructions.

For Western blotting, two identical 8 or 10% SDS-PAGE mini-gels were electrophoresed as above. One gel was silver-stained and proteins in the other SDS-PAGE gel were electrophoblotted onto nitrocellulose (Pharmacia, Dulbin, CA) or polyvinylidene difluoride (NEN Life Science Products) membranes. The transfer was monitored by transfer of the prestained protein markers and staining with 0.5% Ponceau S (Sigma) solution. Following the transfer, the membranes were incubated in a 5% Carnation nonfat dry milk Tris-buffered saline (TBS), pH 7.6, containing 0.1% Tween (Sigma) for 2 h at RT to saturate the nonspecific binding sites. In general, the membrane was incubated with a 1:1000 dilution of primary antibody in the 5% milk/TBS-Tween for 1–2 h at RT and washed three times with a large volume of TBS-Tween for a total of 30 min. The membrane was then incubated with a 1:3000–1:10,000 dilution of secondary antibody in 5% milk/TBS-Tween. After rinsing, the interacting proteins were detected with horseradish peroxidase-conjugated antibodies and Western-Storm (TROPIX, Bedford, MA) for alkaline phosphatase-conjugated antibodies on Reflection (DuPont) or BIOMAX ML (Kodak) film for 10 s to 30 min prior to processing.

GST Protein-Protein Interaction Assays—Plasmids directing the expression of glutathione S-transferase (GST) ER, COUP-TFI, and ERα (Perkin-Elmer) were provided by Dr. Janet E. Mertz of the University of Wisconsin. GST fusion proteins and GST expressed from pGEX-2TK were purified from E. coli BL-21 cells according to protocols supplied by Pharmacia. The concentrations of the glutathione-S-phosphate-purified fusion proteins were determined by DC assay (Bio-Rad), and [H]ERα binding to GST-ER was measured by hydroxyapatite assay (32). Proteins were monitored by separation on 10% polyacrylamide SDS-PAGE followed by Western blot with an anti-GST antibody (Pharmacia). For GST “pull-down” assays, identical amounts of purified GST fusion proteins were preincubated with 30 µl of a 75% slurry of glutathione (GSH)-Sepharose in NENT buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 6 mM MgCl2, 0.5% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 8% glycerol), washed twice with 1 ml of NENT and twice with 1 ml of Transciption Wash buffer (20 mM Tris-HCl, pH 8.0, 60 mM NaCl, 1 mM EDTA, 6 mM MgCl2, 0.05% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 8% glycerol). ER was incubated with the resulting GST fusion protein-bound GSH-Sepharose resin for 2 h at 4 °C on a rotator. The resin was sedimented by centrifugation and the supernatant removed, and the pellet was rinsed three times with 1 ml of NENT. The [3H]-ligand counts were determined immediately in a liquid scintillation counter. The bound protein was eluted from the resin by microwaving the samples in SDS loading buffer and resolved by SDS-PAGE (46). Replica samples were run on duplicate gels. Proteins were transferred to polyvinylidene difluoride membranes and the proteins remaining on the gel were examined by silver staining.

Southwestern Blotting—Proteins on the nitrocellulose membrane were denatured by washing the membrane in 6 M guanidine HCl in 1 × binding buffer (250 mM HEPES, pH 7.9, 30 mM MgCl2, 40 mM KCl, 1 mM DTT) for two 5-min washes at 4 °C (47). Renaturation of the proteins was achieved by two sequential washes in each of the following concentrations of guanidine HCl in 1 × binding buffer: 3 M, 1.5 M, 0.75 M, 0.325 M, and 0.17 M. The membranes were then rinsed in 1 × binding buffer alone, the same buffer containing 5% nonfat milk, and the same buffer containing 0.25% nonfat milk. The membrane was incubated overnight (14 h at 4 °C) in 1 × binding buffer containing 0.25% nonfat milk, 10 µg/ml sonicated denatured salmon sperm DNA, and 108 cpm of [32P]-labeled EREc38. The membrane was rinsed in 1 × binding buffer containing 0.25% nonfat milk, and the same buffer containing 0.1% Tween for 30 min at 4 °C in a total reaction volume of 20 µl for 1 h on ice. Tubes were opened and incubation continued on ice under a UV transilluminator (Spectroline model X-15G, Westbury, NY) at a distance of 6 cm for 0–60 min. DNA polymerase I (Klenow fragment, 68 kDa) and T4 ligase (70 kDa) were cross-linked to [32P]-EREc38 to estimate the distance of the cross-linked probe on the migration of a protein of known size through SDS-PAGE (48). The experiment was performed by addition of 2.5 µl of Laemmli buffer. The samples were boiled for 3 min, loaded onto a 10% SDS-polyacrylamide gel, and electrophoresed as described above. Each gel included pre-stained protein molecular weight standards, Kaleidochrome (Bio-Rad) and/or SeeBlue Standard (Novex). The gel was dried and exposed to X-Omat AR film at −70 °C.

Cell Transfection—MCF-7 human breast cancer cells (2.5 × 106) were plated in each well of a 12-well Corning plate in Iscove's modified Dulbecco's medium (all cell culture reagents were from Life Technologies, Inc.) without phenol red, supplemented with 10% stripped fetal bovine serum, and 1% penicillin-streptomycin. After 24 h, at 50% confluence, the cells were transfected using liposome-mediated transfection (LipoFastAMINE) (49). Cells were co-transfected with 0.5 µg of pCMV-luciferase and 0.25 µg of pGL3-luc reporter vector per well using a DNA/liposome ratio of 1 µg/10 nmol. The DNA was preincubated with LipoFastAMINE in Opti-MEM I without insulin, estradiol, or other growth factors for 45 min. Four hours after transfection, 1 nM 17β-estradiol (Sigma), 100 nM 4-OHT, or an equal volume of ethanol was added to the wells in duplicate. The cells were maintained in Iscove's modified Dulbecco's medium containing 1% stripped fetal bovine serum for 48 h after transfection in 12 well plates. Luciferase and β-galactosidase activities were determined 20 µl of cell extract with the assay performed according to the manufacturer's protocol (Promega). Samples were incubated at 37 °C for 30 min, and the reaction was stopped by addition of 500 µl of 1 M sodium carbonate, and absorbance readings were measured at 420 nm. Activity was calculated from a standard curve generated from a series of dilutions of β-galactosidase (Promega).

RESULTS

ER Binds EREc38 but Not an ERE Half-site in Vitro—We examined the ability of partially purified ERα-ligated ER (Ex5) to bind to EREc38, a consensus ERE derived from three highly estrogen-responsive genes (15), versus a single half-site ERE, 1/2EREc38, which lacks the 3′-ERE half-site but was otherwise identical to EREc38 (Fig. 1, sequences in Table I). Using gel mobility shift assays, we detected the ERE-Ec38 binding complex, of which 85% was supershifted by the ERE-specific antibody H222 (Fig. 1). The specificity of the ERE-Ec38 complex was also demonstrated with displacement by excess unlabeled ERc38 in a concentration-dependent manner (data not shown).

Approximately 15% of the ER-Ec38 binding complex detected...
was not ER. This was indicated by the inability of H222 to shift or inhibit the appearance of this complex (Fig. 1). Similarly, none of the ER-specific antibodies tested altered the amount or appearance of this complex (42). Because the antisera used recognize epitopes spanning the entire ER protein, this result rules out the possibility that the non-supershifted complex was formed by a proteolytic ER product or a naturally occurring truncated ER variant.

In contrast, ER did not bind to 1/2EREc38 (Fig. 1) or 1/2ERE3’c38. Antibodies to ER did not supershift or inhibit the half-site complex, indicating it is not ER (Fig. 1, Ref. 42, and data not shown). However, a complex of similar mobility, but lower intensity, was bound to the ER half-site. Denaturation of the ER preparation (by boiling) or treatment with 0.5 μg/ml trypsin (10 min at RT) destroyed all DNA binding, indicating that a protein(s) is responsible for 1/2EREc38 binding. Because the activity responsible for this binding co-purified with ER by heparin-agarose, phenyl-Sepharose, Yellow 86, Green 19, Affi-Gel Blue, and Blue 4 affinity chromatography (data not shown), we called it estrogen receptor associated factor (ERAF). Since ERAF bound to an oligomer containing a single 5’-AGGTC-3’ sequence and ER did not bind this half-site, measuring ERAF-1/2EREc38 binding provided a sensitive and reproducible assay for its characterization. The relative amount of ERAF activity associated with ER did not change between bovine uterine ER preparations, with ER ligand, i.e. E2, 4-OHT, or TAZ, or in the absence of added ligand.

Cross-linking of ERAF to 1/2EREc38—Photoaffinity UV cross-linking was used to evaluate the molecular weight of ERAF. Heparin-agarose-purified E2-ER was incubated with 5-bromo-deoxyuridine-substituted 32P-EREc38 or 32P-1/2EREc38 and irradiated by UV light, and the protein-DNA adducts were resolved by SDS-PAGE. No cross-linking occurred without exposure to UV light. The amount of protein cross-linked was time-dependent. A band of approximately 146 kDa was cross-linked to 32P-EREc38 at 20 min (Fig. 2A). Additional bands of 234, 78, 50, and 47 kDa appeared at 30 min. UV cross-linking of highly purified TAZ-ER, obtained by two sequential rounds of ERE-Sepharose purification (31), to EREc38 showed a band of 69–77 kDa (data not shown). The specificity of the complex was demonstrated by the complete abrogation of its appearance by the addition of 25-fold molar excess unlabeled EREc38 (Fig. 2A, lane 4). We suggest that the 146- and 69–78-kDa bands correspond to E2-ER dimer and monomer, respectively. The slightly larger size of the ER is consistent with reports showing that UV cross-linking of comparably sized double-stranded oligomers contributes ~10 kDa to the apparent size of the protein (48, 50). We did not detect bands of 55 or 39 kDa that were cross-linked to the Xenopus vitellogenin A2 ERE by rat uterine E2-ER (51). Addition of H222 to E2-ER and 32P-EREc38 increased the intensity of a distinct 146-kDa band (data not shown), corresponding in size to an ER homodimer or an ER monomer plus one light and one heavy chain of the antibody. The size of proteins cross-linked to 32P-1/2EREc38 was time-dependent with a diffuse 78-kDa band appearing by 15 min and bands of 47 and 38 kDa at 45 min (Fig. 2B). Bands of similar sizes were obtained in 34 UV cross-linking experiments performed. Whether the 78-kDa band is identical in composition to that cross-linked to EREc38, i.e. ER monomer, is not known. Unlike EREc38, a 146-kDa complex was not cross-linked to 32P-1/2EREc38 (Fig. 2B). H222 had no effect on the appearance of the bands cross-linked to 32P-1/2EREc38, confirming that ER is not part of the complex. Addition of 25-fold molar excess

**TABLE I**

Sequences of the EREs and βRARE used in gel mobility shift assays

These are sequences of the EREs cloned into the SmaI site of pGEM-7Zf (+) and subsequently used in gel mobility shift assays as described under “Experimental Procedures.” EREc38 is a 38-bp ERE consensus sequence (15). The underlined nucleotides correspond to the minimal core consensus ERE. Nucleotides in italics indicate differences from the EREc38 consensus sequence. The nucleotide in bold indicates an alteration to the ERE inverted repeat (IR). βRARE is a synthetic version of the mouse RAR type β gene (27–29).

| Name         | Sequence                                      |
|--------------|-----------------------------------------------|
| EREc38       | 5’-CCAGGGTCAGAGCTGACCTGAGCTAAAATAACACATTCAG-3’ |
| 1/2EREc38    | 5’-CCAGGGTCAGAGCTGACCTGAGCTAAAATAACACATTCAG-3’ |
| 1/2ERE3’c38  | 5’-CCCTAAGGGTCAGAGCTGACCTGAGCTAAAATAACACATTCAG-3’ |
| 1/2EREc      | 5’-CCAGGGTCAGAGCTGACCTGAGCTAAAATAACACATTCAG-3’ |
| AF-rich region | 5’-CTAAAAATACACATTCAG-3’                       |
| EREc3AΔT     | 5’-CCAGGGTCAGAGCTGACCTGAGCTAAAATAACACATTCAG-3’ |
| βRARE        | 5’-CCCGTGGGTTAGGTCTACCGAAAAGTTCACCTGGA-3’ (DR5) |
tested whether ERAF is a known class II nuclear receptor by using half-site spacing influencing binding specificity (52). We affected neither ER-EREc38 nor ERAF by either EREc38 or 1/2EREc38 by themselves. Antisera to RAR assay conditions. None of the receptor antibodies tested bound epitope recognized by the antibody is not accessible under the DNA binding, inhibition of receptor-DNA binding, a supershift result in four possible outcomes as follows: enhanced receptor-cognate response elements and receptor-specific antibodies can 2EREc38 binding. Incubation of nuclear receptors with their (Fig. 3). Incubation with H222 plus RAR antisera likewise did containing 2

FIG. 2. UV cross-linking of ER and ERAF to EREc38 and 1/2EREc38. Heparin-agarose-purified E2-ER (183 fmol) was preincubated with EREc38 (A) or 1/2EREc38 (B) for 2 h at 4 °C in a reaction containing 5 μg/ml poly(I·C). Reactions were subjected to UV cross-linking for the indicated time (in minutes) as detailed under “Experimental Procedures.” + indicates the addition of 10-fold molar excess of EREc38, and H indicates the addition of H222 antibody. UV cross-linked complexes were separated on by 10% polyacrylamide SDS-PAGE as described under “Experimental Procedures.” Molecular mass marker proteins run in parallel lanes are indicated at the left of each panel (kDa). The Klenow fragment of DNA polymerase I (DNA Pol. I) was incubated with EREc38 and cross-linked for 30 min (B, lane 6).

Effects of Antibodies to Nuclear Receptors on ER-EREc38 or ERAF-1/2EREc38 Binding—Class II nuclear receptors, e.g. RAR, TR, RXR, peroxisome proliferator-activated receptor, and vitamin D receptor, bind to the ERE half-site 5′-AGGTCA-3′ with half-site spacing influencing binding specificity (52). We tested whether ERAF is a known class II nuclear receptor by examining whether selected antibodies altered ERAF-1/2EREc38 binding. Incubation of nuclear receptors with their cognate response elements and receptor-specific antibodies can result in four possible outcomes as follows: enhanced receptor-DNA binding, inhibition of receptor-DNA binding, a supershift of the receptor-DNA complex, or no effect, indicating that the epitope recognized by the antibody is not accessible under the assay conditions. None of the receptor antibodies tested bound either EREc38 or 1/2EREc38 by themselves. Antisera to RAR affected neither ER-EREc38 nor ERAF-1/2EREc38 binding (Fig. 3). Incubation with H222 plus RAR antiserum likewise did not inhibit ERAF-1/2EREc38 binding (Fig. 3).

Interestingly, ERAF activity was separated from ER through an ERE-Sepharose column (Fig. 3, compare lanes 9–14 versus 15–18). Peak ERAF activity eluted at 200 mM, whereas ER eluted at 305 mM KCl. This indicates that ERAF binds EREc38 with lower affinity than ER.

Although MAb to RARα or RARγ and antiserum to RXR did not affect ER-EREc38 binding, these antibodies reduced ERAF-1/2EREc38 binding by >50% (data not shown). This effect was discrete since MAb to TRα, TRγ, or vitamin D receptor did not affect ERAF-1/2EREc38 binding. MAb to peroxisome proliferator-activated receptor increased ERAF-1/2EREc38 binding by 2-fold. The reason for this result is unknown. Although Jun interacts directly with ER (9, 53), addition of a MAb to Jun did not affect ERAF-1/2EREc38 binding, implying that ERAF is not the AP-1 complex.

Effects of Antibodies to Orphan Receptors on ERAF-1/2EREc38 Binding—Because the ERAF activity displayed char-acteristics similar to those described for the related orphan receptors ERR-1 (44), ERRα1 (54), and COUP-TF (43), we tested if antibodies to these receptors affected ERAF-1/2EREc38 binding (Fig. 4). None of the antisera affected the binding of ER to EREc38 (Fig. 4A, lanes 11–14 and 18–20). Antiserum to ERR-1 did not alter ERAF-1/2EREc38 binding.

Preincubation of heparin-agarose-purified ER with an antiserum to COUP-TF produced a dose-dependent supershift of the ERAF-1/2EREc38 complex (Fig. 4A, lanes 5–8). Higher antiserum concentrations completely abrogated ERAF-1/
2EREc38 binding (Fig. 4B, lanes 9 and 10), indicating that COUP-TF is present in the ERAF-1/2EREc38 complex. Whether the COUP-TF detected is COUP-TFI or COUP-TFII is unknown since the antisera recognizes both (43). Because the migration of the COUP-TF-1/2EREc38 complex is similar to that of the homodimeric ER-EREc38 complex, COUP-TF appears to bind 1/2EREc38 as a dimer, e.g. a homodimer or a heterodimer.

Recombinant Human COUP-TFI Binds 1/2EREc38 as an Apparent Homodimer and Migrates at the Same Position as Bovine COUP-TF—To determine whether bovine COUP-TF had DNA binding properties similar to those of human (h) COUP-TF, a plasmid encoding recombinant hCOUP-TFI was transcribed and translated in vitro. As anticipated, [35S]methionine incorporation revealed a prominent band of 47 kDa for hCOUP-TF (Fig. 5A). The hCOUP-TFI bound to both EREC38 and 1/2EREc38 in a dose-dependent manner and migrated at the same position as the bovine COUP-TF-1/2EREc38 complex (Fig. 5B). The specificity of COUP-TFI-DNA binding was tested by displacement, using a 10-fold excess of unlabeled cognate oligomer. These results demonstrate conclusively that COUP-TFI binds EREC38 and 1/2EREc38. The similarity in migration of the COUP-TFI-EREc38 or -1/2EREc38 complexes with that of homodimeric ER-EREc38, indicates that the minimal form of COUP-TFI bound to DNA is a dimer.

Determining the Specificity of COUP-TF-1/2EREc38 Binding—Specificity of the bovine COUP-TF-1/2EREc38 complex was demonstrated by competition with EREC38, a sequence variant ER (Fig. 6, lanes 13–16), and EREC, but not by the AT-rich portion of EREC38 (Fig. 6, lanes 17–20) nor by the region of pGEM-Zf(+) into which EREC38 is cloned, although it contains an imperfect half-site (see “Experimental Procedures”). No component of the partially purified ER preparation bound the AT-rich portion of the 1/2EREc38 construct (Fig. 6, lanes 9–12). Together, these results indicate that COUP-TF interacts specifically with the 5′-AGCTCA-3′ in 1/2EREc38.

Relative Affinity of COUP-TF-1/2EREc38 Versus ER-EREc38 Binding—As one measure of the relative affinity of ER-EREc38 versus COUP-TF-1/2EREc38 binding, the stability of the complexes was measured at different KCl concentrations. We reported that maximum specific E2-ER-EREc38 binding occurred between 100 and 150 mM KCl (16). E2-ER-EREc38 binding was stable up to 500 mM KCl in gel mobility shift assays. Higher KCl concentrations drastically inhibited E2-ER-EREc38 binding. In contrast, COUP-TF-1/2EREc38 binding was considerably less stable with binding reduced to 59% at 200 mM KCl. This observation, with those shown in Figs. 1–6, indicates that the interaction between ER and EREC38 is of higher affinity than that of COUP-TF-1/2EREc38.

By titrating the concentration of 32P-1/2EREc38 or 32P-1/2EREc38 with a fixed concentration of partially purified E2-ER containing bovine COUP-TF, saturation binding experiments were performed (data not shown). Linear regression analysis of the data from Scatchard plots yielded a Kd = 1.24 ± 0.20 nM for COUP-TF-1/2EREc38 and a Kd = 0.94 ± 0.18 nM for COUP-TF-1/2EREc38 complex binding (mean ± S.D. from four separate experiments performed at two different concentrations of partially purified E2-ER). Thus, COUP-TF binds to these half-sites with comparable high affinity and with values similar to
those reported for the binding of in vitro translated COUP-TFI to various retinoid Z receptor (RZR/ROR) elements (55).

Interaction of COUP-TF with ER in Vitro—COUP-TF/1-2EREc38 binding activity was inhibited by approximately 45% by immunoprecipitation of the partially purified bovine ER preparation with ER MAb H222. In contrast, all of the ER-EREc38 binding activity was removed by immunoprecipitation with H222. These results suggest that COUP-TF interacts directly with ER or with a tightly ER-associated protein. Further evidence of a direct COUP-TF-ER interaction was shown by incubating partially purified bovine COUP-TF with ERE-Sepharose-purified E2-ER, which does not contain COUP-TF (Fig. 3) or class II nuclear receptors (25, 31, 42), and demonstrating that COUP-TF/1-2EREc38 binding is enhanced by purified E2-ER in a dose-dependent manner (Fig. 7). In contrast, purified 4-OHT-ER did not enhance COUP-TF/1-2EREc38 binding. Purified ER did not alter the mobility of the COUP-TF/1-2EREc38 complex, indicating that ER did not stably bind COUP-TF-DNA complex.

Direct COUP-TF-ER and ERRα-ER Interaction in Vitro—To address whether COUP-TF interacts directly with ER, GST pull-down assays were performed. Fusion proteins bound to GSH-Sepharose were incubated with \(^{3}H\)TAz-ER or \(^{3}H\)hERE, and specifically retained proteins were eluted by denaturation, resolved by SDS-PAGE, and evaluated by Western blotting using antibodies to ER and GST. ER was retained by the GSH affinity resin in the presence of GST-COUP-TF, GST-hERRα1, and GST-hER, but not by resin with GST or by GSH-Sepharose alone (Fig. 8). To our knowledge, this is the first demonstration of a direct protein-protein interaction between ER and COUP-TF and confirms the recent report of a direct ER-ERRα interaction (54).

COUP-TF Inhibits E2-mediated Transcriptional Activation in Vitro—To address the biological significance of ER-COUP-TF interaction, we co-transfected ER-positive MCF-7 human breast cancer cells with an expression vector for COUP-TFI and examined E2-induced expression of a luciferase reporter gene under the control of three tandem copies of EREc38 (Fig. 9). E2 induced a 24-fold induction of luciferase activity from three tandem copies of EREc38. Addition of a 100-fold excess of 4-OHT inhibited the E2-mediated induction by >90%. Co-expression of COUP-TFI inhibited E2-stimulated luciferase activity in a dose-dependent manner. Co-transfection with identical concentrations of the pCMV5, a control vector, did not inhibit E2-induced luciferase activity, indicating the specificity of the COUP-TF effect (data not shown). Increased expression of COUP-TF in cells co-transfected with the COUP-TF expression vector was confirmed by Western blotting of a slot blot of whole cell extracts from the treated MCF-7 cells (data not shown).

DISCUSSION

An interplay between various nuclear receptors competing for binding to the same target sites in gene promoters modulates hormone-responsive gene expression in mammalian cells. The specificity of transcriptional activation conferred by the cellular levels of cognate ligands, receptors, co-activator proteins, and the chromatin structure of the target gene. We previously showed that partially purified ER binds ERs with higher affinity and stability than highly purified ER (25). In this report, we provide data implicating the COUP-TF orphan receptor as a constituent of heparin-agarose-purified ER. We
show that COUP-TF, like ERR-1 (44, 54), co-purifies with ER on a number of separation matrices, but COUP-TF is separated from the ER on ER-Sepharose because it binds with lower affinity. Using GST fusion proteins in pull-down assays, we provide the first demonstration of a direct protein-protein interaction between ER and COUP-TF and confirm the recent report of direct ER-ERRa1 interaction (54). COUP-TF, unlike ER, binds not only to the fully palindromic ERE, i.e. EREc38, but also to a single ERE half-site.

This is the first report documenting COUP-TF binding as a dimer to an ERE half-site. The specificity of COUP-TF-1/2EREc38 binding was demonstrated by antibody supershift and by competition with unlabeled oligonucleotides. Bovine COUP-TF interacts specifically with a single 5′-AGGTCA-3′ half-site. In contrast to our results, COUP-TF translated in vitro was unable to bind to an ERE half-site (35, 56, 57). However, our results corroborate a recent report that COUP-TF homodimers bind nuclear receptor RZR/ROR response elements consisting of a single 5′-extended half-site (55). We note that the RZR/ROR response element half-sites in COUP-TF but Not ER Binds Single ERE Half-sites
homodimer. Since COUP-TFs range from 43 to 53 kDa (43, 56, 58), we speculate that the COUP-TF-1/2EREc38 complex contains a COUP-TF homo- or heterodimer or possibly a homo- or heterotrimer. COUP-TF is a dimer in solution and binds as a dimer to divergent response elements, e.g. DR0-DR12 and IR, indicating that COUP-TF assumes different conformations to accommodate structural and spatial changes in the DNA (59). The specificity of nuclear receptor response element binding is conferred by the interaction of each receptor monomer with individual half-sites of different spacing and orientation (60). However, a third level of DNA recognition was recently suggested by the cooperative binding of certain nuclear receptors, e.g., RXRs, as higher order oligomers to response elements containing highly reiterated half-sites (61). Recognition of reiterated elements was suggested to extend to other nuclear hormone receptors (59).

Additional evidence that the COUP-TF binds as a dimer comes from two Southwestern experiments. These showed that neither ER nor COUP-TF monomers are capable of binding EREc38 or 1/2EREc38. In contrast, class II nuclear receptors that bind EREs as monomers, e.g., H-2RIIBP (62), are readily detected in Southwestern blots. Our results also demonstrate that COUP-TF differs from orphan nuclear receptors, e.g., ROR, NGFI-B, FTz-F1, ERRα, and Rev-Erbα, that bind as monomers to 5′-AT-rich-extended half-sites (54, 63).

A possible interpretation of our observations is that COUP-TF binds 1/2EREc38 because there is a second half-site in the construct. Inspection of the nucleotide sequences flanking 1/2EREc38 in the EcoRI-BamHI fragment from pGEM-7Zf(+) revealed two imperfect half-sites, 5′-GGTAGC-3′ and 5′-AGCTG-3′ (italicized letters indicate changes from the consensus), located 32 and 8 bp, respectively, 5′ to the complete half-site. Importantly, COUP-TF did not bind an oligomer containing the imperfect 5′-GGTAGC-3′ (data not shown), nor did this oligomer compete for COUP-TF-1/2EREc38 binding (Fig. 6). Indeed, the intensity of COUP-TF bound to an oligomer containing only a consensus half-site plus the AT-rich region was equal to that of the COUP-TF-1/2EREc38 complex. Finally, although the Kd values for COUP-TF-DNA interaction were not reported, COUP-TF bound with progressively lower affinity to a series of constructs as the distance between the half-sites increased (33). In contrast, bovine COUP-TF bound 1/2EREc38 with a Kd = 1.24 nm. These data support our conclusion that COUP-TF binds with high affinity and specificity to a single ERE half-site and preclude the possibility that a "cryptic" half-site(s) is responsible for binding.

Whether bovine COUP-TF bound to 1/2EREc38 is a homodimer or consists of a heterodimer of COUP-TF and another protein(s) is unclear. Our results raise the latter possibility since UV cross-linking of the protein-1/2EREc38 complex generated a diffuse band centering at 78 kDa in addition to a 48-kDa band that we believe is bovine COUP-TF. Although COUP-TF heterodimerizes with RAR, TR, and RXR (59), those receptors were not detected in the COUP-TF-1/2EREc38 complex. COUP-TF also interacts directly with co-repressors N-CoR and SMRT (64). However, neither co-repressor protein appeared to be a constituent of the bovine COUP-TF-1/2EREc38 complex since proteins corresponding to their sizes, i.e. N-CoR = 270 kDa (65) and SMRT = 168 kDa (66), would be expected to slow the migration of the complex more than that detected in gel shift. Finally, bands of neither 270 nor 168 kDa were cross-linked to 1/2EREc38.

We showed that ER interacts directly with COUP-TF1 and ERRα1 in solution. The latter observation agrees with a recent report on ERRα1-ER interaction (54). We emphasize that neither our results (18, 20–22) nor those reported previously (67) indicate that COUP-TF heterodimerizes with ER to bound to EREs nor does ER stably participate in COUP-TF-ERE half-site binding. In an interesting parallel to our findings, two recent reports showed interaction of COUP-TF with orphan receptors nur77 (68) and HNF-4 (69) only in solution but not when bound to DNA.

COUP-TF is highly conserved in evolution, and "knock-out" mutations of COUP-TFI and COUP-TFII are lethal, indicating that COUP-TF performs essential functions in vivo (26, 70). To examine COUP-TF interaction with ER from another estrogen-responsive tissue, we partially purified ER from MCF-7 cells (data not shown). In results virtually identical to those described for bovine ER, ER in the MCF-7 extract showed specific binding to EREc38 but not to 1/2EREc38. The MCF-7 extract showed EREAF-1/2EREc38 binding that appeared identical to the bovine COUP-TF-1/2EREc38. In contrast, a similar pre-preparation of recombinant human ER expressed in yeast (71) showed no 1/2EREc38 binding but did bind specifically to EREc38.3 We conclude that yeast do not express a homologous protein capable of half-site binding.

The potential biological role of ER-COUP-TF interaction was suggested by the ability of COUP-TF to inhibit E2-induced expression of a luciferase reporter gene under the control of three tandem copies of EREc38 in MCF-7 cells. We postulate that in estrogen target tissues such as the uterus, ER is present together with other nuclear and orphan receptors, co-activators, and co-repressors that interact with DNA and perhaps, with each other to regulate gene transcription.

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3 C. M. Klinger, unpublished observation.
