PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 71 (1999) 1–19

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Role of estrogen receptor ligand and estrogen response element sequence on interaction with chicken ovalbumin upstream promoter transcription factor (COUP-TF)

Carolyn M. Klinge*

Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 319 Abraham Flexner Way, Louisville, KY 40292, USA

Received 3 November 1998; accepted 17 August 1999

Abstract

Estrogen-responsive genes are regulated by altering the balance of estrogen receptor (ER) interaction with transcription activators and inhibitors. Here we examined the role of ER ligand on ER interaction with the Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) orphan nuclear receptor. COUP-TF binding to half-site estrogen response elements (EREs) was increased by the addition of estradiol (E_2) -liganded ER (E_2-ER), but not by ER liganded with the antiestrogens 4-hydroxytamoxifen (4-OHT-ER) or tamoxifen aziridine (TAz-ER). ER did not bind to single half-sites. Conversely, COUP-TF enhanced the ERE binding of purified E_2-ER, but did not affect TAz-ER-ERE binding. In contrast, only antiestrogens enhanced direct interaction between ER and COUP-TF as assessed by GST pull-down assays. Identical results were obtained using either purified bovine or recombinant human ERα. Co-immunoprecipitation assays showed that ER and COUP-TF interact in extracts from MCF-7 and ERα-transfected MDA-MB-231 cells. Here we document that ER ligand impacts COUP-TF-ER interaction. COUP-TF interaction is mediated by the DNA binding and ligand-binding domains of ER. We suggest that changes in ER conformation induced by DNA binding reduce ER-COUP-TF interaction. Transient transfection of human MCF-7 breast cancer cells with a COUP-TFI expression vector repressed E_2-induced luciferase reporter gene expression from single or multiple tandem copies of a consensus ERE. COUP-TFI stimulated 4-OHT-induced luciferase activity from a minimal ERE. Alone, COUP-TFI increased transcription from ERE half-sites or a single ERE in a sequence-dependent manner. These data provide evidence that the ERE sequence and its immediate flanking regions influence whether COUP-TF enhances, inhibits, or has no effect on ER ligand-induced ERE reporter gene expression and that COUP-TFI activates gene transcription from ERE half-sites. We suggest that COUP-TFI plays a role in mitigating estrogen-responsive gene expression.

1. Introduction

Estrogen receptors (ERs) α and β mediate the actions of estrogens in target tissues thus regulating estrogenic effects on reproduction, bone homeostasis and mammary gland structure and function [1]. Estrogens play a pivotal role in the development of breast cancer. ER is a member of the steroid/thyroid superfamily of related proteins [2]. After binding its cognate ligand, e.g. estradiol (E_2), a series of activation events occur that increase ER binding to estrogen response elements (EREs), located in or adjacent to the coding regions of estrogen-regulated genes. The detailed mechanism accounting for estrogen-dependent transactivation remains to be fully elucidated, but recent reports indicate that ERE-bound ERα interacts with nuclear proteins including co-activator proteins, e.g., SRC-1 and components of the TFIID complex, e.g. $TAF_{II}30$, that enhance gene expression [3].

Antiestrogens are used clinically to prevent the recurrence of breast cancer [4]. 4-hydroxytamoxifen (4-OHT), a metabolite of tamoxifen (TAM), the most widely used antiestrogen, binds ER and activates ER-ERE binding [5]. However, the specific details of its

* Tel.: +1-502-852-3668; fax: +1-502-852-6222.
E-mail address: carolyn.klinge@louisville.edu (C.M. Klinge)

0960-0760/99/$ - see front matter
PII: S0960-0760(99)00124-7

Table 1
Sequences of the EREs used in experiments. The single-strand nucleotide sequence of the double-stranded EREs used in EMSA, described in Materials and Methods, are presented below. The underlined nucleotides correspond to the *minimal* core consensus ERE. Nucleotides in italics differ in sequence from the consensus EREc38

Name	Sequence
EREc38	5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3′
1/2EREc38	5′-CCAGGTCAGAG*CATTTC*GAGCTAAAATAACACATTCAG-3′
1/2ERE3′c38	5’-CC*CCTAAG*GAGTGACCTGAGCTAAAATAACACATTCAG-3′
EREm	5′-C*T*GGTCAGAGTGACC*G*-3′
AT	5′-AGCTAAAATAACACATTCA-3′
pS2	5′-CTTCCCCCTGCAAGGTCAGCGTG*GCCA*CCCCGTGAGCCACT-3′

mechanism of action remain unknown. Tamoxifen aziridine (TAz) is an analog of TAM that binds covalently to ER [6]. TAM, 4-OHT and TAz are type I antagonists that induce high affinity ER-DNA binding but have mixed agonist/antagonist properties depending on the cell type and promoter [5]. The current model of estrogen action postulates that interaction of agonist-liganded ER with co-activators, e.g., SRC-1, recruits CBP/p300 which has histone acetyltransferase activity [7]. Acetylation of histones in the nucleosome creates a more ‘open’ chromatin structure that facilitates transcriptional activation [3,8]. Conformational differences in AF-2 within the ligand binding domain (LBD) between antiestrogen versus E_2-liganded ER are thought to preclude ER interaction with certain co-activators, e.g., SRC-1 [9,10].

Nuclear receptors also interact with corepressors, two of which have been identified to date: Nuclear Co-Repressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) [8,11–16]. NCoR and SMRT interact with mSin3A in association with histone deacetylase 1 (HDAc1) [12]. Histone deacetylation represses gene transcription. Recent evidence suggests that interaction of NCoR with 4-OHT-ER may contribute to antiestrogen action. Treatment of ER-expressing cells with 4-OHT relieved transcriptional repression mediated by RU486-liganded progesterone receptor (PR)-B, presumably by 4-OHT-ER sequestering NCoR [17]. However, in vitro assays showed no effect of ER ligand on interaction with NCoR [17] or SMRT [18]. These latter observations indicate that factors other than ligand may influence ER interaction with the corepressors NCoR and SMRT.

Accumulated evidence indicates that a complex interplay between nuclear receptors and other transcription factors at the promoter regions of responsive genes facilitates the assembly of the RNA polymerase II pre-initiation complex [3]. Estrogen-responsive gene expression is modulated not only by ER-ERE interaction, but by ERα interaction with transcription factors including AP-1 [19–21], Sp1 [22,23], class II nuclear receptors, i.e., RAR, retinoid X receptor (RXR) and TR [24] and orphan receptors: ERRα1 [25], COUP-TF [26] and SHP [27]. COUP-TF, ERRα1 and SHP are ‘orphan receptors’ whose ligands, if necessary, are unknown [28]. The specificity of transcriptional activation is conferred by the cellular levels of cognate ligands, receptors, co-activator and co-repressor proteins, the phosphorylation state of the various proteins, facilitated by ‘cross talk’ with cell-membrane-mediated phosphorylation cascades [29] and by the chromatin structure of the target gene [8].

We recently reported that COUP-TF co-purifies with bovine ER and blocks E_2-stimulated reporter gene induction in transiently-transfected ER-positive MCF-7 human breast cancer cells [26]. COUP-TF is a highly conserved and is involved in diverse biological functions, most prominently in the repression of gene expression [30], perhaps through its interaction with co-repressors N-CoR and SMRT [31]. Here we examined the role of ER ligand on COUP-TF interaction with ER or with DNA in vitro. We also examined how overexpression of COUP-TFI affected reporter gene activation from various ERE sequences in transiently transfected MCF-7 human breast cancer cells treated with ER ligands. We demonstrate that ER liganded by E_2, but not by 4-OHT or TAz, enhanced COUP-TF-DNA binding. Likewise, COUP-TF enhanced ER-ERE binding when ER was bound by E_2, but not antiestrogens. Conversely, antiestrogens increase direct interaction between ER and COUP-TF. These results suggest that DNA binding induces a change in the conformation of ER that decreases its interaction with COUP-TF. We present evidence that COUP-TF stimulates transcription from certain EREs and half-site EREs, but inhibits E_2-stimulated transcription from EREs in transiently transfected cells.

2. Materials and methods

2.1. Preparation of plasmids containing EREs

The sequences of select synthetic single-stranded oligonucleotides are given in Table 1. EREc38 is a 38bp

ERE consensus sequence [32]. EREc38 was cloned as a single, two, three, or four tandem (head-to-tail) direct repeats into pGEM-7Zf(+) (Promega, Madison, WI) as described [33]. 1/2EREc38, 1/2ERE3′c38 and EREm were ligated into pGEM-7Zf(+) [33].

Single or multiple tandem copies of various EREs were removed from pGEM-7Zf(+) and cloned into the upstream multiple cloning site in pGL3-Promoter (Promega) vector [26]. The constructs containing EREc38 are called pGL3-1EREc38, -2EREc38, -3EREc38 and 4-EREc38 with the number indicating the number of tandem copies of EREc38. The pS2 oligomer was synthesized by Genosys and cloned into *Kpn* I/*Sac* I digested pGL3-pro-luciferase. Sequences were confirmed by DNA sequencing using the Sequenase Ver. 2.0 kit (Amersham).

2.2. Preparation of estrogen receptor (ER) from calf uterus

ER was prepared from calf uterus as described [5,34]. Partially purified and purified ER refer to the receptor following heparin agarose and ERE-Sepharose affinity chromatography, respectively. ER was liganded with 17β-[2,3,6,7-^{3}H(N)]estradiol ([^{3}H]E_2, 84.1 Ci/mmol, NEN, Boston, MA), (Z)-4-hydroxytamoxifen (4-OHT) (Research Biochemicals International, Natick, MA), or [ring-^{3}H]tamoxifen aziridine ([^{3}H]TAz, 23 Ci/mmol, Amersham, Arlington Heights, IL). ER concentration was determined by adsorption to hydroxyapatite (HAP) [35]. All receptor concentrations refer to dimeric ER (i.e. with 2 molecules of bound ligand).

2.3. Preparation of baculovirus-expressed recombinant human ERα

A recombinant AcMNPV containing the coding sequence for wild-type recombinant human ERα was generously provided by Dr. Nicholas J. Koszewski of the University of Kentucky [36]. Recombinant human ERα (rh ERα) was prepared from the nuclear extract (NE) from baculovirus infected IPLB-SF-21AE insect cells in 50 mM Tris-HCl pH 7.5, 0.6 M KCl, 10% glycerol, 10 mM DTT, 1 mM EDTA, 1 mM PMSF, 2 mg/l each in aprotinin, leupeptin and pepstatin and 10 mg/l E64 as described [37]. The ER concentration in the NE was determined by HAP assay [35].

2.4. Preparation of recombinant COUP-TFI

The pRSV-COUP-TFI plasmid, encoding recombinant human COUP-TFI, was a gift of Dr. Sophia Y. Tsai [38]. COUP-TFI was transcribed and translated in vitro using the TNT rabbit reticulocyte lysate system from Promega (Madison, WI) according to the manufacturer's instructions. As a positive control, a plasmid encoding luciferase was transcribed and translated in parallel with COUP-TFI. The relative amounts of the translated proteins were determined by [^{35}S]methionine (1175 Ci/mmol from NEN) incorporation [26].

2.5. Electrophoretic mobility shift assay

ERE-containing oligomers were labeled with [^{32}P]α-dATP (800 Ci/mmol from NEN) [26]. The size of the ERE oligomers used was 77 nucleotides for EREc38, 1/2EREc38 and 1/2ERE3′c38, 49 for EREm and 43 for AT. ER-ERE binding reactions included [^{32}P]labeled oligomer (50,000 cpm), liganded-ER (concentrations given in Figure legend) and/or the indicated amount of COUP-TF. Reaction and electrophoresis conditions have been described [26]. The amount of protein-DNA complex was determined as described [26].

2.6. Antibodies

H222 monoclonal antibody (MAb) was a gift of Abbott Laboratories (Abbott Park, IL). MAb AER304, AER320 and AB10 to ER [39] were purchased from Neomarkers (Lab Vision Corp., Fremont, CA). Antiserum to COUP-TF was kindly provided by Dr. Janet E. Mertz [25]. Monoclonal ER antibody EVGF-9 was a gift from Dr. Abdulmaged M. Traish [40].

2.7. GST protein preparation and GST-protein:protein interaction assays

Plasmids directing the expression of GST fusion proteins COUP-TFI or ERRα1 were kindly provided by Dr. Janet E. Mertz. The plasmid for GST-C-SMRT was generously provided by Dr. Ronald Evans [41]. 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 (GSH)-Sepharose-purified fusion proteins was determined by DC assay (BioRad). GST 'pull-down' assays were performed using identical amounts of purified GST-fusion proteins as described [26].

2.8. Co-immunoprecipitation in cell extracts

MCF-7 or MDA-MB-231 cells were plated at a density of 2×10^5 cells/well in 12-well plates in IMDM (all cell culture reagents were from Life Technologies) medium without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum (FBS) and 1% Pen-Strep. The MDA-MB-231 cells were co-transfected

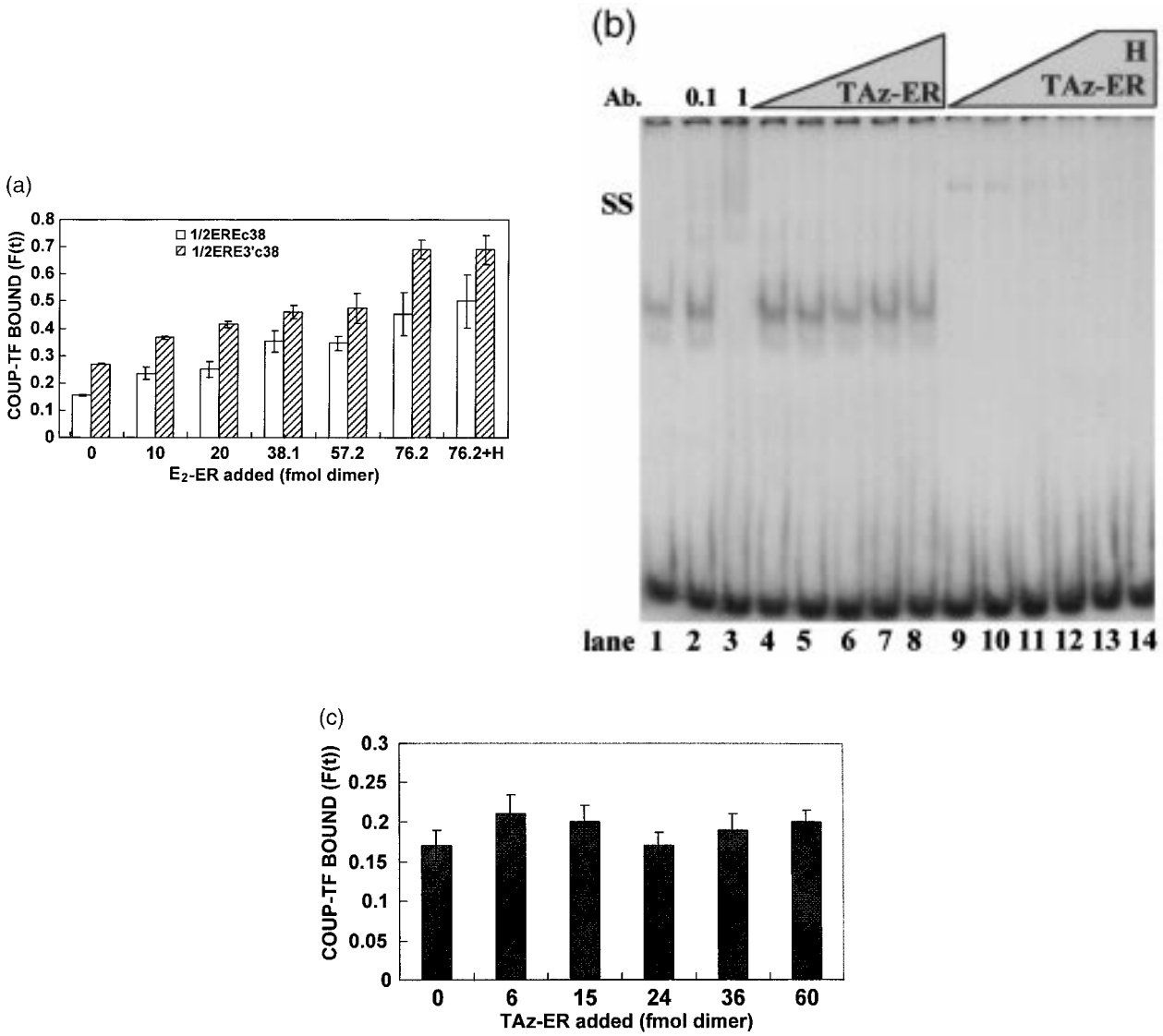

Fig. 1. Purified E_2-ER but not TAz-ER enhances GST-COUP-TFI binding to ERE half-sites. (A) EMSA was performed to quantitate how addition of ERE-affinity purified calf uterine E_2-ER affected the binding of a fixed amount (0.5 μl) of purified GST-COUP-TFI to ERE half sites. Quantitation of [^{32}P] counts in the COUP-TF-1/2EREc38 (open bars) and COUP-TF-1/2ERE3′c38 complexes (hatched bars) with added E_2-ER was performed as described in Materials and Methods. The values are mean ± S.E.M. from 3 and 2 EMSAs, respectively. (B) Increasing amounts (indicated by the shaded triangle) of ERE-affinity purified calf uterine TAz-ER (3, 6.1, 9.1, 12.2 and 15.3 fmol/reaction in lanes 4–8 and lanes 9–14, respectively) was incubated with [^{32}P]1/2EREc38 alone (lanes 9–14) or with added purified GST-COUP-TFI (0.5 μl in the reactions for lanes 1–8). Polyclonal antiserum to COUP-TF (Ab., volume in μl) was added to lanes 2 and 3. H222 (H) was added to the reaction for lane 14. Assay conditions are described in Materials and Methods. (C) Quantitation of [^{32}P] counts in the COUP-TF-1/2EREc38 (closed bars) with added TAz-ER was performed as described in Materials and Methods. The values are mean ± S.E.M. of two separate experiments.

with pCMV-ERα, generously provided by Dr. Benita Katzenellenbogen [42]. Co-transfection was performed as described [26]. After 24 h., the cells were treated with ethanol, 1 nM E_2, 100 nM 4-OHT, or 1nM E_2 plus 100 nM 4-OHT. Six h after treatment, the cells were washed with PBS and scraped off the plates in 500 μl of Lysis buffer (50 mM Tris-HCl, pH 7.4; 600 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM PMSF and 2 μg/ml each of aprotinin, leupeptin and pepstatin. That volume of cell extract corresponding to 400 μg of protein was diluted in TDPEK (40 mM Tris-HCl, pH 7.5; 1 mM DTT; 0.5 mM PMSF; 1 mM EDTA, 100 mM KCl) buffer containing 10% glycerol and antibody was added. After 1 h incubation at room temperature (RT), 50 μl of a 50% slurry of Protein A-Sepharose (Pharmacia) was added and incubation continued for an additional h at RT. The Sepharose resin was pelleted by sedimentation, rinsed twice as

described for GST-pull-down assays [26]. The bound proteins were eluted from the resin [43] and resolved on 10% SDS-PAGE gels.

For Western blotting, proteins in the SDS PAGE gel were electroblotted onto PVDF (NEN) membranes, blocked and incubated with the primary and secondary antibodies as described [26]. The interacting proteins were detected by chemiluminescence (ECL, Amersham or Renaissance, NEN) on BIOMAX ML (Kodak) film [26].

2.9. Cell transfection

MCF-7 human breast cancer cells (2.5×10^5) were plated in each well of a 12-well Corning plate in IMDM medium without phenol red, supplemented with 10% charcoal-stripped FBS and 1% Pen-Strep. After 24 h, the cells were transfected using liposome-mediated transfection (LipofectAMINE, Gibco-BRL). Cells were co-transfected with 0.4 μg of pCMV-β-gal (Clonetech) and 0.6 μg of pGL3-luc reporter vector per well using a DNA:liposome ratio of 1μg:10 nmol. For specified experiments, cells were co-transfected with the indicated amount pRSV-COUP-TF, a gift of Dr. Sophia Tsai of Baylor University. As a negative control, cells were co-transfected with identical concentrations of pCMV5 [26]. Four hours after transfection, 1 nM 17β-estradiol, 100 nM 4-OHT, or an equal volume of ethanol was added to the wells in triplicate. The cells were maintained in IMDM medium containing 1% charcoal-stripped FBS. The cells were lysed 24 h after treatment in 150 μl of 1X reporter lysis buffer (Promega) and the cleared extract was assayed for luciferase and β-gal activities as described [26].

3. Results

3.1. COUP-TF-half-site binding is enhanced by E_2-liganded, but not antiestrogen-liganded ER

We recently reported that COUP-TF interacts directly with ER and that ER liganded by E_2, but *not* 4-OHT, increased the binding of partially purified bovine COUP-TF to an ERE half-site [26]. To examine the specificity of this response, purified GST-COUP-TFI was incubated with two different ERE half-site constructs in the presence of increasing amounts of ERE-affinity purified E_2-ER. The amount of COUP-TF-DNA binding was determined by EMSA (data not shown). As shown previously [26], E_2-ER alone did not bind to either half-site. GST-COUP-TFI bound specifically to 1/2EREc38 and 1/2ERE3′c38 with more GST-COUP-TFI bound to 1/2ERE3′c38 than to 1/2EREc38. 1/2EREc38 contains a 6 bp half-site and a consensus AT-rich located 10 bp from the

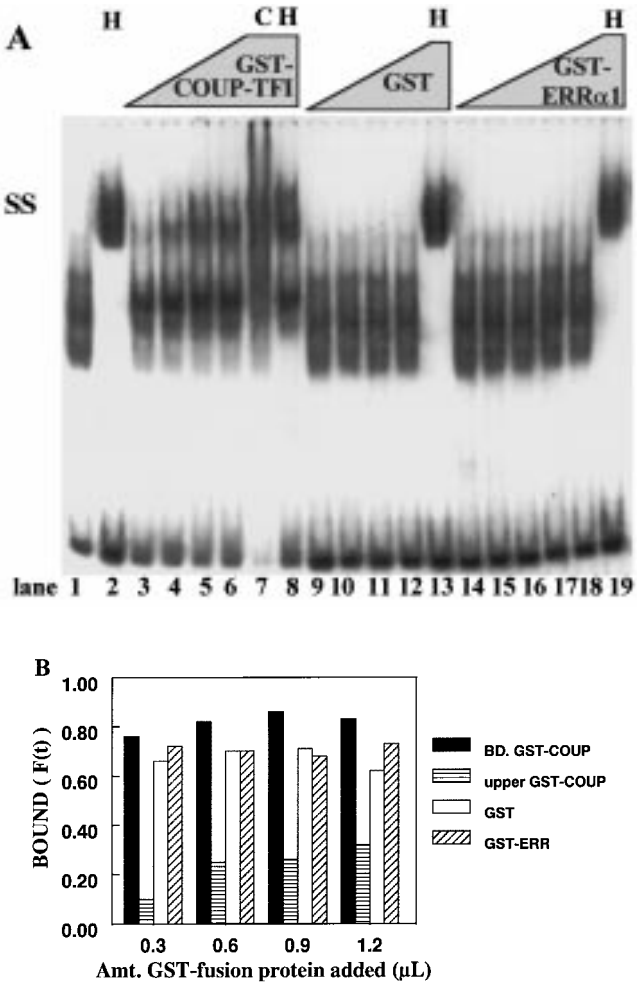

Fig. 2. GST-COUP-TF, but not GST or GST-ERRα1, enhances E_2-ER binding to EREc38. (A) ERE affinity purified E_2-ER (2.29 nM in each reaction, 46 fmol/ lane) was incubated with increasing amounts (indicated by the shaded triangle) of purified GST-COUP-TFI, GST, or GST-ERRα1 (0.3, 0.6, 0.9 and 1.2 μl/reaction) and [^{32}P]1/2EREc38 (50,000 cpm per reaction, 10 fmol/reaction) in a final reaction volume of 30 μl for 2 h at 4°C. Polyclonal antiserum to COUP-TF (C) was added to the reaction for lane 7 and H222 anti-ER antibody (H) was added to the reactions for lanes 8, 13 and 19. Twenty-five μl aliquots of the reaction mixture were loaded onto 4% polyacrylamide gels and EMSA was performed as described in Fig. 1. (B) Quantitation of [^{32}P] counts from the gel shown in A was performed as described in Materials and Methods. The $F(t)$ of bound counts in the lower and upper complexes with the addition of GST-COUP-TFI is shown in solid and vertical striped bars, respectively. The $F(t)$ of bound counts with added GST and GST-ERRα1 are shown as open and diagonally-hatched bars, respectively.

half-site [33]. 1/2ERE3′c38 also contains the 6bp half-site, but it is located immediately adjacent to the consensus AT-rich region. We speculate that the proximity to the AT-rich region enhances COUP-TF binding stability. Addition of purified E_2-ER increased the amount of complex formed. Addition of E_2-ER also generated the appearance of a less defined, but more retarded complex. Quantitation of the DNA-protein complexes from repeated experiments shows the con-

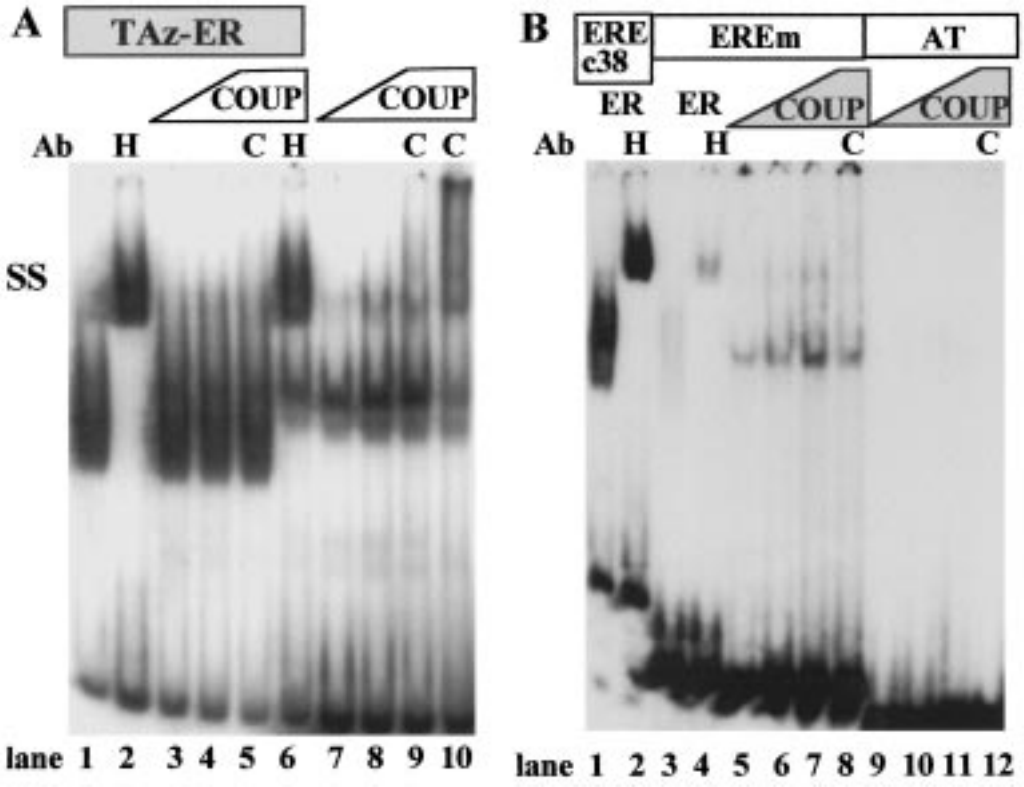

Fig. 3. GST-COUP-TFI does not enhance TAz-ER-EREc38 binding. (A) Purified TAz-ER (1.2 nM in the reaction, 40.6 fmol/lane) was preincubated with [^{32}P]EREc38 alone (lanes 1 and 2) or with the addition of increasing amounts of purified GST-COUP-TFI (0.5, 1, 1 and 1 μl/reaction for lanes 3–6, respectively) for 2 h at 4 C. (B) Purified E_2-ER (2.29 nM in the reaction, 57.4 fmol/lane) was incubated with [^{32}P]EREc38 (lanes 1 and 2) or [^{32}P]EREm (lanes 3 and 4). Increasing amounts of purified GST-COUP-TFI (0.5, 1, 1.5 and 1.5 μl/reaction for lanes 5–8 and 9–12, respectively) were incubated with [^{32}P]EREm or [^{32}P]AT (lanes 5–8 and 9–12, respectively) for 2 h at 4 C. H222 anti-ER antibody (H) was added to the reactions for lanes 2 and 6 in A and 2 and 4 in B. Polyclonal antiserum to COUP-TF (1, 0.1 and 1 μl to lanes 5, 9 and 10 in A and 0.1 μl to the reaction in lanes 8 and 12 in B, respectively) was added to the indicated reactions (C). EMSA was performed as described in Fig. 1.

centration-dependent increase in GST-COUP-TFI-half-site binding with added E_2-ER (Fig. 1A).

The inability of 4-OHT-ER to enhance the binding of partially purified bovine COUP-TF to 1/2EREc38 [26] might be attributed to an antiestrogen-induced ER conformation that impeded COUP-TF-ER interaction or an apparent change in ER conformation upon 4-OHT ligand dissociation [44]. To distinguish between these possibilities, another antiestrogen was tested. Purified ER was liganded covalently by TAz, hence obviating the effect that 4-OHT ligand dissociation might have on the interaction between ER and COUP-TF. Although purified GST-COUP-TFI bound 1/2EREc38, neither the amount nor the migration of GST-COUP-TFI-1/2EREc38 complex was altered by the addition of TAz-ER (Fig. 1C, lanes 4–8). TAz-ER did not bind 1/2EREc38 (Fig. 1C, lanes 9–14). The specificity of GST-COUP-TFI -1/2EREc38 binding was confirmed by the inability of purified GST to bind 1/2EREc38 or EREc38 (data not shown) and by the concentration-dependent supershift and ablation of the complex with COUP-TF antiserum (Fig. 1C, lanes 2 and 3). Identical experiments using higher concentrations of purified TAz-ER showed similar results (Fig. 1C). Although ER liganded with E_2 increased COUP-TF-1/2EREc38 binding, ER liganded by the antiestrogens 4-OHT and TAz did not alter the amount of COUP-TF-1/2EREc38 complex formed. Since TAz covalently binds ER, 4-OHT dissociation is not the reason for the lack of COUP-TF interaction with ERE-bound 4-OHT-ER.

3.2. E_2-ER-ERE binding is enhanced by added COUP-TFI

Purified ER binds EREs with reduced affinity compared to partially purified ER [45]. Adding GST-COUP-TFI increased the total amount of E_2-ER-EREc38 complex formed in a dose-dependent manner (Fig. 2A). In contrast, neither GST nor GST-ERRα1 altered the amount or migration of the E_2-ER-EREc38 complex. Addition of GST-COUP-TFI generated the concentration-dependent appearance of a defined, more slowly migrating complex (Fig. 2A, lanes 3–8). Since these binding reactions contained highly purified proteins and addition of GST or GST-ERRα1 did not

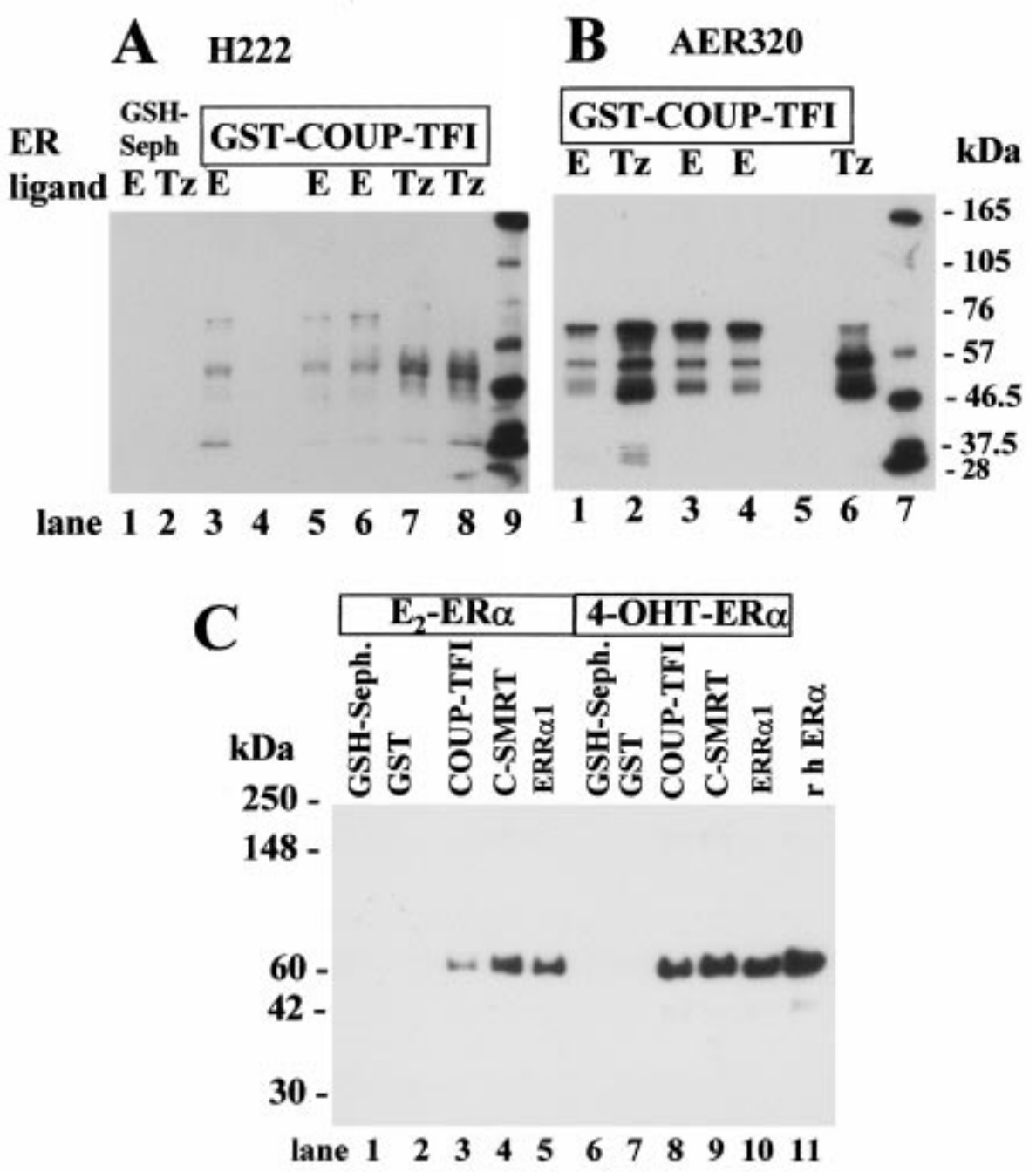

Fig. 4. TAz-ER interacts more strongly with COUP-TF than E_2-ER. (A and B) Heparin agarose purified calf uterine ER liganded by E_2 (lanes 1, 3 in A and 1 in B) or by TAz (lanes 2 in both A and B) was incubated with the GSH affinity resin alone or bound by purified GST-COUP-TFI, lanes 3–8 in a and lanes 1–6 in b. Purified E_2-ER was incubated with GST-COUP-TFI in lanes 5 and 6 in a and lanes 3 and 4 in b. Purified TAz-ER was incubated with GST-COUP-TFI in lanes 7 and 8 in A and lane 6 in B. Reactions for lanes 4 in A and 5 in B contained the ER buffer. Reactions for lanes 3, 5 and 7 in A and 1 and 3 in B include GST-COUP-TFI from one preparation of the fusion protein while lanes 6 and 8 in panel A and lanes 4 and 6 in panel B contain GST-COUP-TFI from a different preparation. Lanes 9 in A and 7 in B contain biotinylated markers whose molecular weight is given at the far right of panel B in kDa. (C) A nuclear extract of rh ERα (144 fmol ER/reaction) was incubated with the indicated ER ligand followed by GST pull-down assays using identical amounts of the indicated GST-fusion protein, the control GSH-Sepharose resin (lane 1), or GST (lane 2). After washing the GSH-Sepharose resin, the retained proteins were eluted by denaturation and separated by SDS-PAGE. Proteins were transferred to PVDF membranes. Lane 11 contains the input nuclear extract of rh ERα (144 fmol ER). The membrane in A was probed with H222 ER antibody, membrane B was probed with AER320 ER antibody and membrane C was probed with ER Ab10 antibody. The interacting proteins were visualized by chemiluminescence [26]. MW markers are indicated in kDa at the left of the gel in C.

generate this complex; this complex is unique for GST-COUP-TF and may represent a ternary complex of an ER homodimer plus either a COUP-TFI monomer or dimer. Support for this suggestion comes from the supershift in the bound complex with COUP-TF antiserum (Fig. 2A, lane 7). As indicated by both the amount of shifted complex and the decrease in the amount of free [^{32}P]EREc38, the COUP-TF antiserum increased the total amount of binding (Fig. 2A, lane 7). The COUP-TF antiserum did not bind EREc38 alone (data not shown). The ability of nuclear receptor antibodies to enhance the specific DNA binding of their cognate receptors is well established (reviewed in [46]). ER antibody H222 did not generate a further supershift in the E_2-ER-GST-COUP-TFI-EREc38 complex (Fig. 2A, lane 8). Additionally, the total amount of H222-supershifted complex was reduced compared to that detected in the presence of E_2-ER alone plus H222 (Fig. 2A, compare lanes 2 and 8). One possible explanation for the reduced intensity of the binding complex is that ER interaction with H222 interferes with COUP-TF-ER interaction.

Quantitation of the data shows a concentration-dependent increase in the upper shifted band in lanes 3–8 with added GST-COUP-TFI (Fig. 2B). Little increase in the lower ER-EREc38 band was detected with added GST-COUP-TFI. These observations lead us to postulate that ER and COUP-TF form a ternary complex on EREc38.

In a similar experiment, recombinant human COUP-TFI synthesized in vitro also enhanced the binding of ERE-affinity purified E_2-ER to EREc38 in a concentration-dependent manner (data not shown). The specificity of this response was demonstrated by the lack of effect of in vitro synthesized Luciferase, prepared using the same rabbit reticulocyte lysate preparation, to alter E_2-ER-EREc38 binding. Thus, two different preparations of COUP-TF increased the binding of purified E_2-ER to a consensus ERE sequence.

3.3. TAz-ER-EREc38 binding is not altered by COUP-TFI

Since neither 4-OHT-ER nor TAz-ER enhanced COUP-TF-1/2EREc38 binding, we tested whether GST-COUP-TFI would alter the binding of purified TAz-ER to EREc38 (Fig. 3A). GST-COUP-TFI did not affect the amount or mobility of the TAz-ER-EREc38 complex (Fig. 3A, lanes 3–6). In contrast to the formation of a second, more slowly migrating complex when GST-COUP-TFI and E_2-ER were incubated with EREc38 (Fig. 2A), no secondary complex was formed with TAz-ER. We noted that purified GST did not affect the ERE binding of either TAz-ER or 4-OHT-ER (data not shown). These observations indicate that when ER is bound to EREc38 and is liganded by either TAz-or 4-OHT, the ER interacts less avidly with COUP-TF compared to E_2-ER.

3.4. COUP-TFI binds as multimers to EREs

GST-COUP-TFI bound to EREc38 in a dose-dependent manner and generated two complexes (Fig. 3A, lanes 7–10). Purified GST did not bind EREc38 (data not shown), indicating that co-purifying bacterial proteins are not responsible for the slower migrating band. Addition of COUP-TF antiserum reduced the intensity of the faster migrating band in a concentration-dependent manner and generated a supershifted complex (Fig. 3A, lanes 9 and 10). Larger volumes of COUP-TF antiserum completely blocked COUP-TFI-EREc38 binding (data not shown). Given the similar migration of COUP-TFI and ER when bound to EREc38, we conclude that COUP-TFI binds EREc38 minimally as a dimer.

GST-COUP-TF also formed two distinct complexes, i.e., of faster and slower migration, with EREm, but did not bind to the AT-rich region that immediately flanks the ERE palindrome in EREc38 (Fig. 3B). This is a minimal ERE that contains only the 13 bp consensus ERE. These data exclude the possibility of COUP-TF interaction with a site other than the ERE in EREc38. The specificity of the GST-COUP-TFI-EREc38 binding was also demonstrated by complete inhibition of EREc38 binding by GST antibody, apparently by occluding the DNA binding domain of COUP-TFI or preventing COUP-TFI dimerization (data not shown).

3.5. ER ligand alters direct COUP-TF-ER interaction

To address whether ER ligands affect the direct interaction between COUP-TF and ER, GST 'pull-down' assays were performed. Purified GST fusion proteins bound to glutathione-Sepharose were incubated with TAz-ER or E_2-ER. The affinity resin was washed and specifically retained proteins were eluted, resolved by SDS-PAGE and evaluated by Western blotting using antibodies to ER and GST. The latter served as a positive control (data not shown). ER was retained by the GSH-Sepharose in the presence of GST-COUP-TF, but not by GST or resin alone (Fig. 4). Interestingly, when identical concentrations of E_2-ER and TAz-ER were added, more TAz-ER was retained in the presence of GST-COUP-TF than E_2-ER (Fig. 4B, compare lanes 3, 5 and 6 with 7 and 8; Fig. 4B, compare lanes 1 and 2; and lanes 3 and 4 with 6). Regardless of ligand, both partially and highly purified ER interacted with GST-COUP-TFI. This indicates that ER itself and not an intermediary protein is responsible for ER-COUP-TF interaction. To assure the reproducibility of these findings, two different

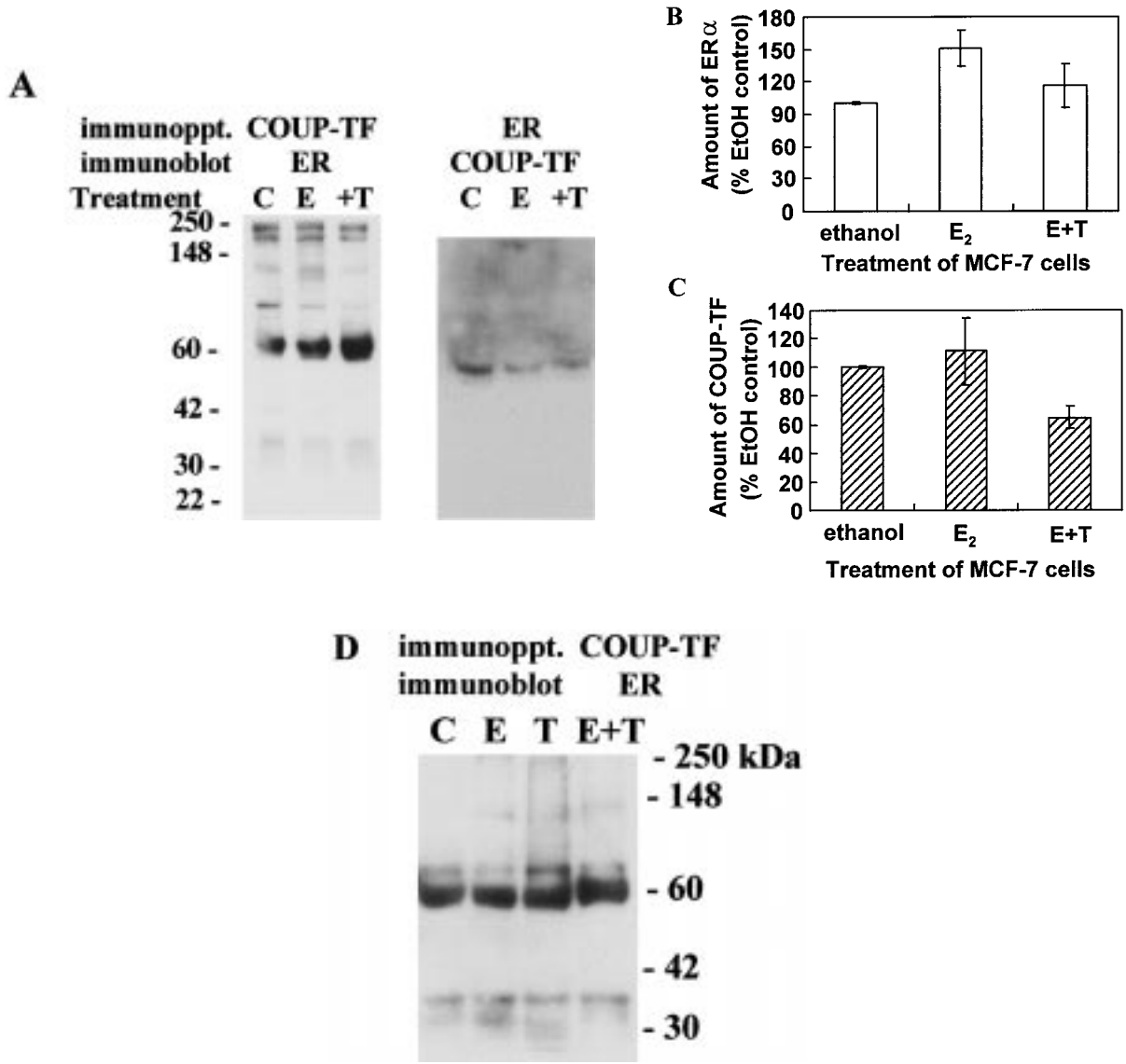

Fig. 5. ER co-immunoprecipitates with COUP-TF in cell extracts. (A) Identical protein concentrations (250 μg) of whole cell extracts from MCF-7 cells treated for 24 h. with ethanol (indicated as C, control, at top), 1 nM E_2 (E), or 1 nM E_2 + 0.1 μM 4-OHT (+T) were incubated with AER320 monoclonal antibody to ER (left panel) or COUP-TF antisera (right panel) as described in Materials and Methods. The proteins interacting with the antibodies were separated on Protein A-Sepharose, resolved by 10% SDS PAGE and immunoblotted as described in Materials and Methods. The membranes were probed with monoclonal antibody AER320 or COUP-TF antiserum, as indicated. The sizes of protein MW markers are indicated in kDa. (B and C). Data from the quantitation of the amount of ERα (B) or COUP-TF co-precipitated with COUP-TF antiserum (B) or ERα antibody (C). Densitometric scans of three different immunoblots were normalized by setting the number of pixels of ERα (B, open bars) or COUP-TF (C, hatched bars) in the control (EtOH)-treated cells to 100%. Bars are the mean ± S.E.M. of three separate experiments using different cell extracts. (D) Co-precipitation of ERα with COUP-TF antiserum from identical protein concentrations (141 μg) of whole cell extracts from MDA-MB-231 cells that were transiently transfected with pCMV-ERα expression vector. Cell treatments are identical to those in 5B, except that T = 0.1 μM 4-OHT alone and $E+T$ = 1 nM E_2 + 0.1 μM 4-OHT.

preparations of GST-COUP-TFI were used. Additionally, two separate blots probed with different ER-specific antibodies showed stronger interaction of TAz-ER with GST-COUP-TF compared to E_2-ER. Thus, neither the particular GST-COUP-TF preparation nor the ER antibody used as probe alters the conclusion: antiestrogen-liganded ER increases ER interaction with GST-COUP-TFI in vitro.

3.6. Recombinant human ERα interacts directly with COUP-TF, ERRα1 and SMRT in vitro

To assess whether human ER interacts with COUP-TF and ERRα-1, we expressed recombinant human ERα in insect cells. A nuclear extract of these cells, containing rhERα, was pre-incubated with E_2 or 4-OHT and used in GST-pull-down assays (Fig. 4c). As

was seen for bovine ER, 4-OHT-ERα showed a stronger interaction with COUP-TFI and ERRα compared to E_2-ERα. Interestingly, we found that 4-OHT-ERα also showed stronger interaction with the C-terminal NR interaction domain of the co-repressor SMRT compared to E_2-ERα. Thus, the interaction of ER with COUP-TF and ERRα1 is conserved between bovine and human ERα.

3.7. ER and COUP-TF co-immunoprecipitate in MCF-7 and MDA-MB-231 cell extracts

To address the role of ligand on ER interaction with COUP-TF in vivo, whole cell extracts were prepared from MCF-7 cells treated for with ethanol (control), 1 nM E_2, or both 1 nM E_2 and 100 nM 4-OHT. When the cell extracts were immunoprecipitated with COUP-TF antisera and probed with ER antibody AER320, a 66 kDa ERα band was detected (Fig. 5A). Repeated experiments performed using different cell extracts showed that E_2 significantly ($p < 0.05$) increased the amount of ERα co-precipitated with COUP-TF antiserum (Fig. 5B), likely reflecting E_2-induced ERα expression. These data indicate that endogenous ERα and COUP-TF interact in co-immunoprecipitation experiments using cell extracts from MCF-7 cells.

When the cell extract was immunoprecipitated with ER antisera and probed for COUP-TF, a single COUP-TF band of approx. 50 kDa was detected (Fig. 5A right panel). Repeated experiments using different cell extracts showed that treatment of cells with E_2 and 4-OHT significantly ($p < 0.05$) decreased the amount of COUP-TF that co-precipitated with ER antibody (Fig. 5C). Since the level of ERα in MCF-7 cells treated with E_2 and 4-OHT was not significantly altered, a possible interpretation of the data is that 4-OHT decreases COUP-TF expression. The factors regulating COUP-TF transcription are unknown.

We also examined the interaction of ER with COUP-TF in whole cell extracts of ER negative MDA-MB-231 cells transiently transfected with an expression vector for ERα (Fig. 5D). As seen in Fig. 5A, ERα was co-immunoprecipitated with COUP-TF antiserum. Thus, over-expression of ERα permits endogenous COUP-TF to form a complex with ERα. There was no significant difference in the amount of ER co-precipitated with COUP-TF antiserum in cells under different treatments (quantitation not shown). Thus, three different assays used to assess the interaction between ER and COUP-TF and between ER and ERRα1 indicate a stronger interaction between each orphan receptor and antiestrogen-liganded ER compared to E_2-ER.

3.8. DNA binding decreases direct ER-COUP-TFI interaction

Since EMSA showed that E_2-ER, but not 4-OHT- or TAz-liganded ER, increased COUP-TF-DNA binding and vice versa (Figs. 1–3), we examined how addition of EREc38 affected the interaction between liganded ER and COUP-TFI. The reactions were incubated under equilibrium conditions in which ER-EREc38 binding is expected to reach saturation [33,44,45]. The amount of E_2-ER interaction with GST-COUP-TFI was decreased by the addition of EREc38 (Fig. 6). Similar results were obtained with TAz-ER (data not shown). Likewise, addition of EREc38 decreased E_2-ER retention by the affinity resin in the presence of GST-ERRα1. These data suggest that DNA binding decreases the interaction between ER and COUP-TF or ER and ERRα1.

3.9. COUP-TFI interacts with ERα through the ligand binding domain

To examine the region of ER necessary for interaction with ER, GST pull-down experiments were performed using rhERα in the presence of the following domain-specific ER antibodies: AER304 (A/B domain)

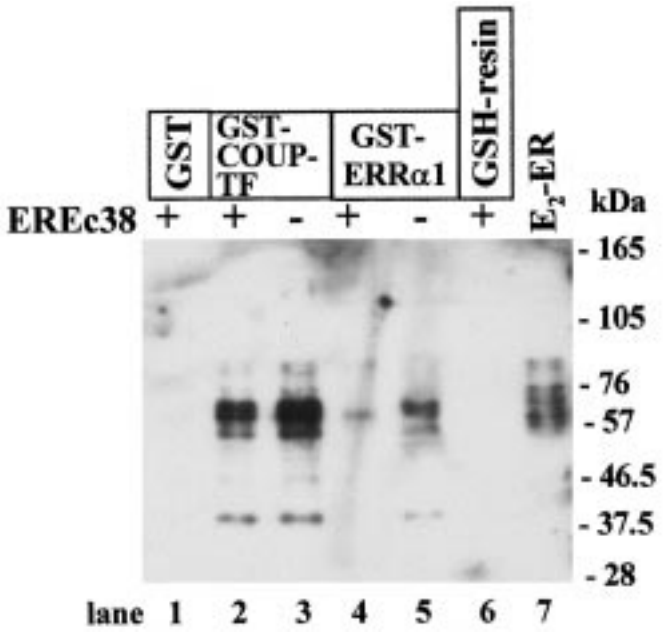

Fig. 6. DNA binding decreases ER-COUP-TF and ER-ERRα1 interaction. Heparin agarose-purified [^{3}H]E_2-ER (1.13 nM) was incubated with the indicated purified GST fusion protein in the presence or absence (indicated by the + or - at the top of the gel) of EREc38. A GST-pulldown assay was performed as described in Materials and Methods. Lane 7 was loaded with 20% of the amount of [^{3}H]E_2-ER used in the preincubation step. The membrane was probed with H222 antibody to ER. The sizes of protein MW markers are indicated in kDa.

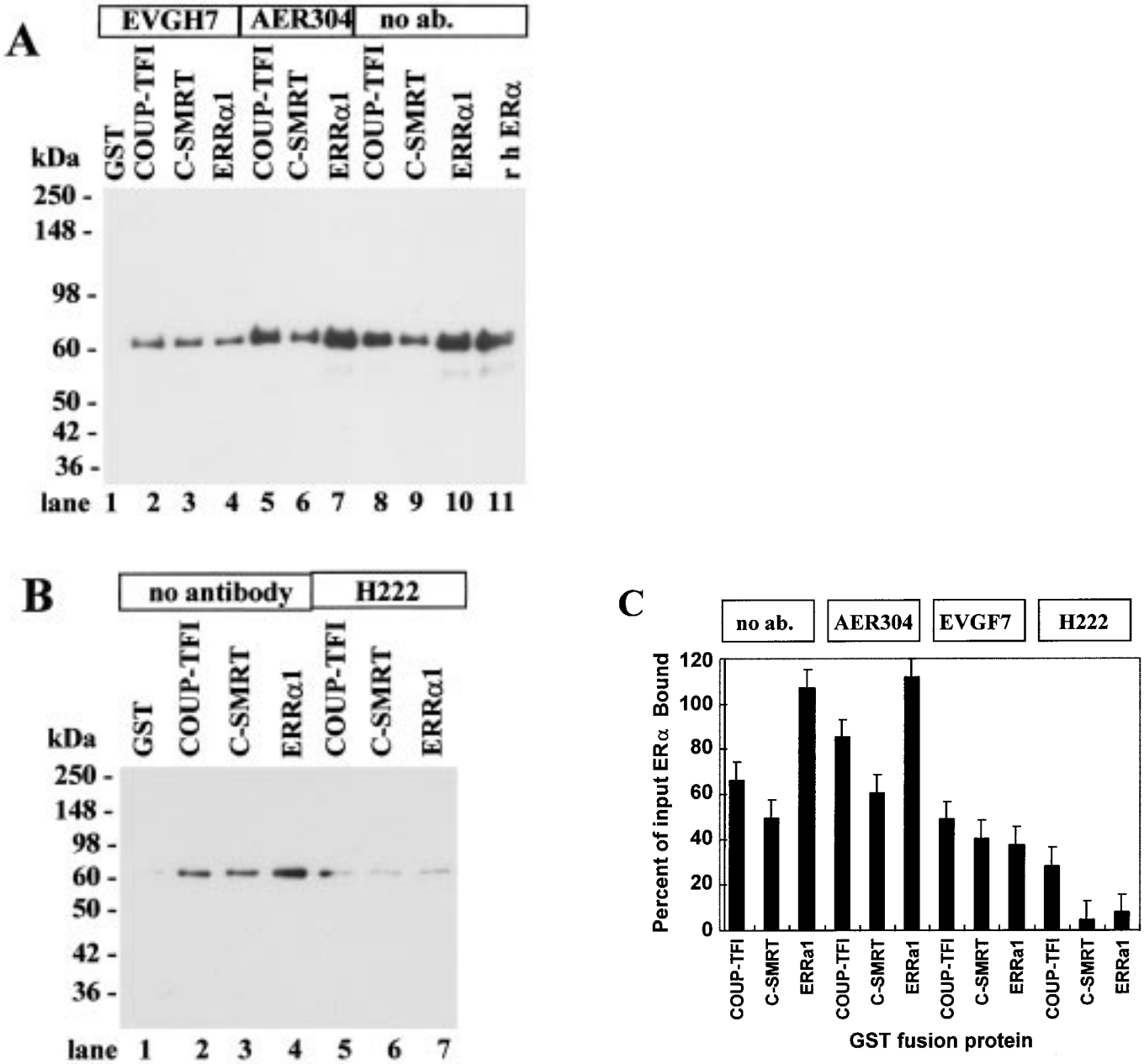

Fig. 7. Antibodies to the DBD and LBD of ERα decrease ERα interaction with COUP-TF, SMRT and ERRα1. (A and B) A nuclear extract of rh ERα (144 fmol ER/reaction) was incubated either in the absence of antibodies (indicated by the box at the top of the blot), or the following domain-specific ER antibodies: AER304 (A/B domain) [39], EVGH7 (C/D domain) [40], or H222 (E/F domain) [47]. The ERα-antibody reaction mixture was then incubated with GST fusion proteins for COUP-TFI, SMRT and ERRα1 and GST pull-down assays were performed [26]. The PVDF membranes were probed with AER320 ER antibody. The interacting proteins were visualized by chemiluminescence [26]. MW markers are indicated in kDa at the left of the blots. (C) The data from two separate GST pull-down assays were quantitated by densitometric scanning and converted to the percent of input rh ERα bound to the same membrane.

[39], EVGH7 (C/D domain) [40], or H222 (E/F domain) [47]. The ERα-antibody reaction mixture was then incubated with GST fusion proteins for COUP-TFI, SMRT and ERRα1 and GST pull-down assays were performed [26]. AER304 had no significant effect on the amount of rh ERα retained (Fig. 7A). In contrast, EVGH7 and H222 decreased rh ERα interaction with COUP-TF, C-SMRT and ERRα1 (Fig. 7a,b). The data from two separate GST pull-down assays were quantitated and are presented in Fig. 7C.

3.10. COUP-TF does not stimulate basal luciferase expression

Although COUP-TF is expressed in MCF-7 cells [26], we wanted to address the possible biological sig-

nificance of COUP-TF by evaluating estrogen-stimulated reporter gene expression in response to increased levels of COUP-TF. Estradiol did not induce luciferase activity from the parental pGL3-Promoter plasmid (Fig. 8A). Co-treatment of cells with E_2 and 4-OHT or with 4-OHT alone did not alter basal luciferase activity. Likewise, transfection of cells with pRSV-COUP-TFI did not stimulate basal luciferase activity. However, cells treated with E_2 and co-transfected with 0.5 or 1 μg pRSV-COUP-TFI showed a small (0.5-fold), but statistically significantly increase ($p < 0.05$) basal luciferase activity. Corrections were made for this slight increase in basal expression in subsequent transfections. Increased COUP-TF expression in the

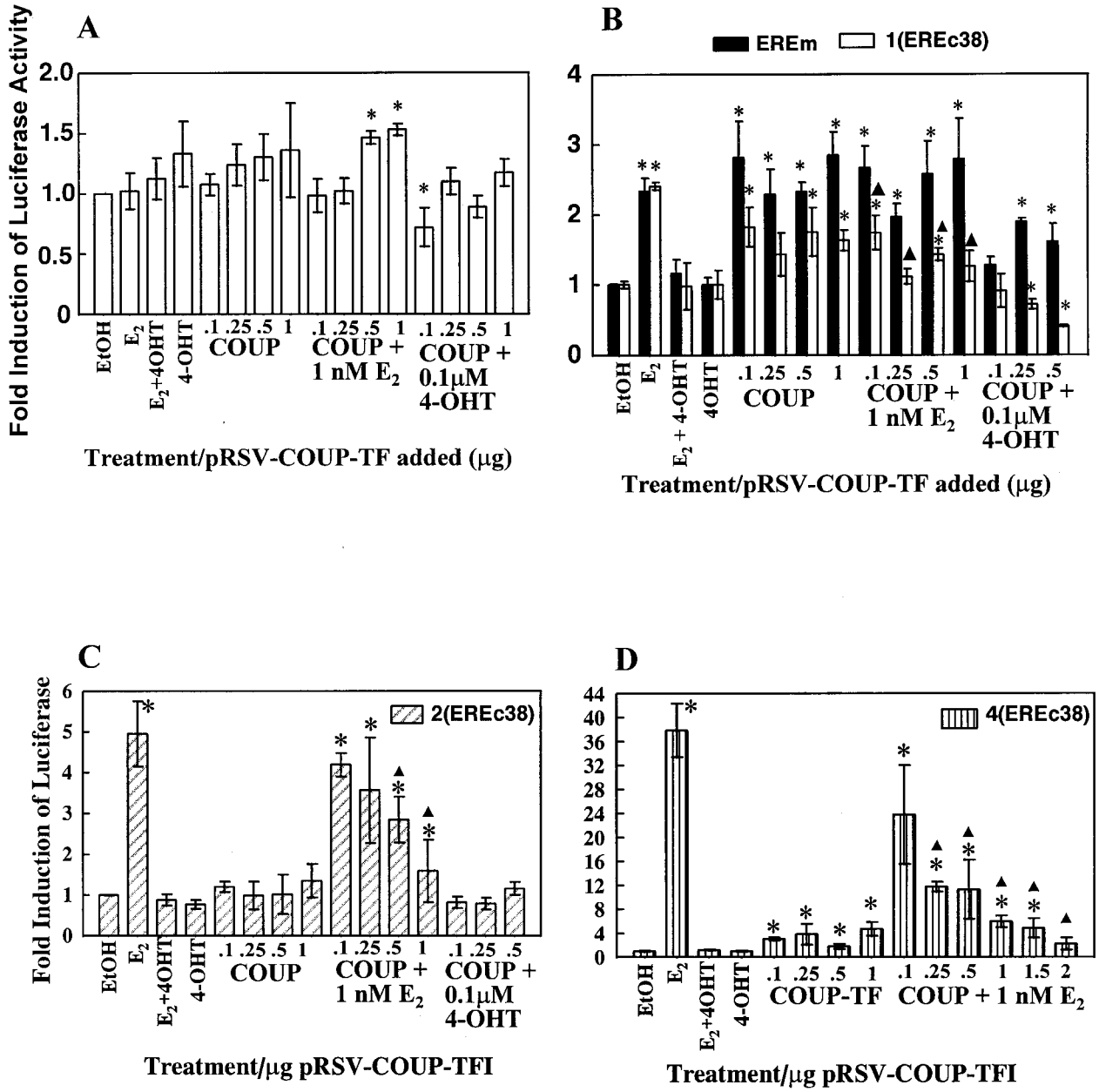

Fig. 8. Modulation luciferase reporter gene expression by COUP-TFI. MCF-7 cells were co-transfected with pGL3-pro (A); pGL3-pro-EREm or pGL3-pro-1(EREc38) (B); pGL3-pro-2(EREc38) (C); pGL3-pro-4(EREc38) (D). In each case the amount of pGL3-pro reporter plasmid was 0.6 μg and 0.4 μg pCMV-βgal was co-transfected as a control. Some cells were co-transfected with the indicated amounts (in μg) of pRSV-COUP-TFI. Four hours after plating, the cells were treated with ethanol (EtOH), 1 nM E_2, 1 nM E_2 plus 100 nM 4-OHT, or 100 nM 4-OHT, as indicated. The cells were harvested 24 h after treatment and the cell extracts were assayed for luciferase and β-gal activities. The fold induction of luciferase activity was normalized for β-gal and is expressed as the ratio of RLU between treatment groups and the ethanol control. Data are the mean ± SEM from four, six, five and three different experiments in a, b, c and d, respectively. The asterisk (*) and the closed triangle indicate values that are significantly different from control (basal) and E_2-stimulated luciferase expression from the same plasmid ($p < 0.05$).

MCF-7 cells co-transfected with pRSV-COUP-TFI was confirmed by Western blotting of slot blots of whole cell extracts [26].

3.11. COUP-TF stimulates luciferase transcription from single EREs

E_2-induced a 2.4-fold induction of luciferase activity from a single copy of the minimal EREm and from 1(EREc38) (Fig. 8B). The equivalent induction of luciferase by E_2 from each of these EREs was not anticipated since the affinity of E_2-ER binding to EREm was 4-fold lower than the binding to EREc38 [45]. The E_2-induced luciferase activity was blocked by 4-OHT, indicating that ER is responsible for the activation of reporter expression. 4-OHT alone did not induce luciferase expression from either construct.

Co-transfection with pRSV-COUP-TFI significantly increased luciferase expression from both EREm and 1(EREc38). One possible explanation for the lack of dose-response relationship between the amount of COUP-TF co-transfected and the induction of luciferase is that MCF-7 cells contain limited amounts of a particular co-activator or ligand required for the agonist activity of COUP-TF. COUP-TFI co-transfection did not affect E_2-induced luciferase activity from EREm. In contrast, COUP-TFI inhibited E_2-induced luciferase activity from 1(EREc38). Treatment of COUP-TFI-transfected cells with 4-OHT suppressed the luciferase activity induced from both EREm and 1(EREc38), with stronger inhibition for 1(EREc38). In fact, 4-OHT suppressed luciferase below basal levels in cells transfected with 0.5 μg pRSV-COUP-TFI.

3.12. COUP-TFI inhibits E_2-induced luciferase activity from two or four tandem EREc38

Co-transfection with COUP-TFI inhibited E_2-stimulated luciferase activity from three-tandem copies of EREc38 [26]. Since E_2 synergistically activates reporter gene expression from three or four, but not two, tandem copies of EREc38 [48], we tested the effect of COUP-TF co-expression on E_2-induced reporter activation from two and four tandem copies of EREc38 ((2(EREc38) and 4(EREc38)). E_2 induced a 5- and a 41-fold increase in luciferase expression from 2(EREc38) and 4(EREc38), respectively (Fig. 8c,d). 4-OHT inhibited E_2-induced luciferase expression to basal levels and 4-OHT alone did not affect basal luciferase. Treatment of cells co-transfected with pRSV-COUP-TF with 4-OHT did not affect luciferase activity from 2(EREc38).

Co-transfection with pRSV-COUP-TFI inhibited E_2-stimultated luciferase activity from both 2(EREc38) and 4(EREc38) in a concentration-dependent manner (Fig. 8c,d). Over-expression of COUP-TFI generated no significant increase in luciferase above basal activity from 2(EREc38), but stimulated a significant increase in luciferase activity from 4(EREc38). Again, no concentration-response relationship was apparent in the stimulated luciferase.

3.13. COUP-TFI stimulates luciferase activity from single half-site EREs

Since COUP-TFI binds to single ERE half-sites, but ER does not [26], we examined how E_2 treatment and over-expression of COUP-TFI affected reporter gene expression from the two different half-site EREs. E_2 induced a 1.4- and 1.8-fold increase in luciferase activity from 1/2EREc38 and 1/2ERE3′c38, respectively, in transiently transfected MCF-7 cells (Fig. 9). The E_2-stimulated activity was inhibited by 4-OHT, indicating that ER is responsible for E_2-activation. Treatment of the cells with 4-OHT alone did not affect luciferase activity from either half-site. The stimulation of luciferase activity from these single half-sites with E_2 was surprising, since we did not detect ER binding to these sequences in EMSA (Fig. 1 and [49]). This result indicates that endogenous cellular factors may contribute to E_2-induced activity from a single half-site ERE.

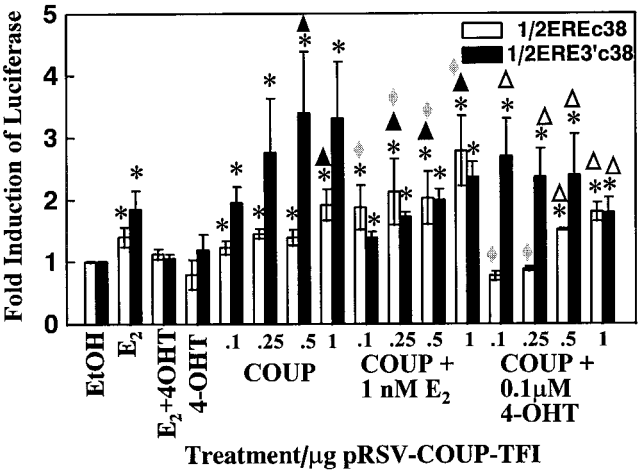

Fig. 9. COUP-TFI stimulates luciferase reporter gene expression from ERE half-sites. MCF-7 cells were co-transfected with pGL3-1/2EREc38 (open bars) or pGL3-pro-1/2ERE3′c38 (black bars) and treated with vehicle (EtOH) or ER ligands as described in Fig. 7. The fold induction of luciferase activity was normalized for β-gal and is expressed as the ratio of RLU between treatment groups and the ethanol control. Data are the mean ± SEM from four different experiments. The asterisk (*) and the closed triangles indicate values that are significantly different from control (basal) and E_2-stimulated luciferase expression from the same plasmid, respectively ($p < 0.05$). The open triangles indicate values from the same plasmid in COUP-TF-transfected cells that are significantly different from those detected in cells treated with 4-OHT alone ($p < 0.05$). The grey diamonds indicate values from the same plasmid that are significantly different from those in cells co-transfected with COUP-TF and treated with ethanol ($p < 0.05$).

Co-transfection of MCF-7 cells with pRSV-COUP-TFI increased luciferase expression above basal levels from each of the half-site constructs. Over-expression of COUP-TFI induced higher luciferase activity from 1/2ERE3′c38, compared to 1/2EREc38 (Fig. 9b). This finding reflects the observed higher binding affinity of COUP-TFI for 1/2ERE3′c38 versus 1/2EREc38 (Fig. 1). Interestingly, COUP-TFI significantly enhanced E_2-induced luciferase activity from 1/2EREc38. Thus, endogenous COUP-TFI may contribute to the E_2-induced activation of luciferase from 1/2EREc38 detected in MCF-7 cells. COUP-TFI did not significantly increase E_2-induced luciferase activity from 1/2ERE3′c38. Since COUP-TF binds this half-site with higher affinity than 1/2EREc38 and since COUP-TF binding to EREs inhibits its interaction with E_2-ER, one possible explanation for the observed lack of enhanced luciferase in the presence of E_2 is altered COUP-TF protein conformation that suppresses functional synergy. 4-OHT repressed COUP-TFI-stimulated luciferase activity from 1/2EREc38 at the lowest levels of co-transfected COUP-TFI. Higher levels of COUP-TFI appeared to overcome this inhibition, resulting in levels of luciferase activity that were significantly above basal expression, but not different from the activity elicited by COUP-TFI alone. In contrast, 4-OHT did not alter COUP-TFI-stimulated luciferase activity from 1/2ERE3′c38.

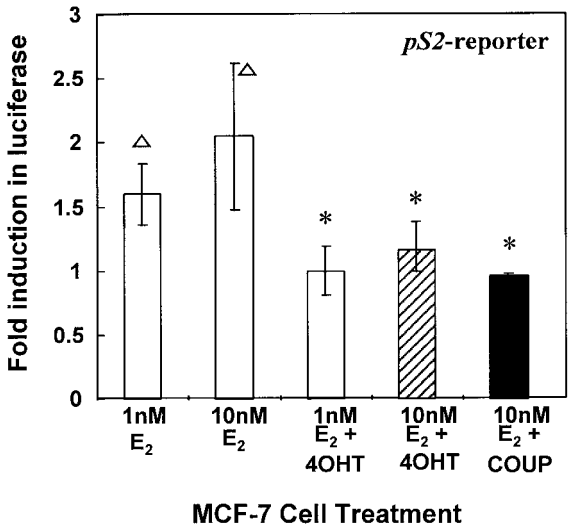

Fig. 10. COUP-TF inhibits reporter gene expression from a natural ERE in transiently transfected MCF-7 cells. MCF-7 cells were co-transfected with pGL3-pro-pS2, pCMV-βgal and treated with ligand(s) at the indicated concentration. Where indicated, cells were co-transfected with pRSV-COUP-TFI (0.4 μg). Data are the mean ± S.E.M. from seven different experiments. Asterisks indicate values that are statistically different ($p < 0.05$) from the luciferase activity in the presence of the identical (1 or 10 nM E_2) treatment. Open triangles indicate values that are statistically different ($p < 0.05$) from basal luciferase activity.

3.14. COUP-TF inhibits E_2-activated reporter gene expression from pS2

The pS2 protein is an estrogen-responsive, human breast cancer prognostic marker [50]. Here we tested how expression of COUP-TFI affected E_2-induced activity from the natural, imperfect ERE in the pS2 promoter in transiently transfected MCF-7 cells. E_2 alone stimulated a 1.6 to 2.1-fold induction in luciferase activity from the pS2 ERE (Fig. 10). Co-treatment with 4-OHT blocked the E_2-stimulated luciferase activity, indicating the specificity of the induction by ER. Co-transfection with COUP-TFI significantly inhibited E_2-stimulated luciferase activity from pS2.

4. Discussion

COUP-TF, an orphan receptor member of the steroid/nuclear receptor gene superfamily, is highly conserved in evolution and is thought to be involved in diverse biological functions in accordance with the observed lethality of COUP-TFI and COUP-TFII gene disruption [51,52]. We recently reported that ER interacts directly with COUP-TF [26]. Here we provide evidence that the direct interaction between ER and COUP-TF is influenced by ER ligand, i.e., interaction is stronger when ER is liganded by the antiestrogens 4-OHT and TAz compared to E_2. The stronger interaction between antiestrogen-liganded ER and COUP-TF was independent of the ER source, since both bovine and recombinant human ERα showed identical results. In accordance with the marked conformational differences detected in the ERα LBD liganded by E_2 versus the antiestrogen raloxifene [10], we suggest that the distinct ER conformation(s) induced by 4-OHT and TAz enhance direct ER-COUP-TF interaction.

In contrast to the stronger direct interaction between 4-OHT- or TAz- ER and COUP-TF detected in the GST pull-down assays, COUP-TFI did not affect the ERE binding of highly purified 4-OHT- or TAz-ER in EMSA. We and others have reported that highly purified ER binds EREs with lower affinity than partially purified ER [45,53,54]. Here we observed that COUP-TF enhanced the total ERE binding of highly purified E_2-ER, suggesting a possible role for COUP-TF in facilitating the ERE binding of E_2-ER, but not antiestrogen-liganded ER in vivo. Conversely, E_2-ER, but not TAz-ER, increased COUP-TFI binding to a single ERE half-site. This binding is only attributable to COUP-TF since ER does not bind to single ERE half-sites under any assay conditions employed [26,33,49,55] and ER antibodies that supershift ER in EMSA do nor alter COUP-TF-half-site binding even in the presence of added ER.

To reconcile the stronger direct interaction between

TAz-ER and COUP-TF with the inability of COUP-TF to enhance TAz-ER- or 4-OHT-ER- ERE binding, we note that ERE binding alters ER conformation in a ligand-specific manner characterized by distinct migrational differences between E_2- versus TAz- or 4-OHT-ER in EMSA [5,44,46,56,57]. We postulate that these ligand-specific conformations of the ER-ERE complex impact ER-COUP-TF interaction. Consistent with this hypothesis, we show that addition of ERE to the GST-pulldown reactions decreased ER-COUP-TF interaction.

We suggest that the ERE-bound conformation of E_2-ER permits direct COUP-TF interaction, resulting in the formation of a higher order E_2-ER-EREc38-COUP-TF complex seen as a second band of reduced mobility in EMSA. The exact composition of the two E_2-ER-EREc38-COUP-TF complexes is unknown. The lower band may consist of an ER homodimer and the upper band may include ER and COUP-TF-homodimers. We did not detect any intermediate bands between these complexes, suggesting that COUP-TF does not form a heterodimer with an ER monomer under the assay conditions employed.

In contrast, the conformation of DNA-bound 4-OHT- or TAz-ER appears to impede ER-COUP-TF interaction. A precedent for this observation is that the nur77 orphan receptor interacts with COUP-TF in vitro, but not when COUP-TF is bound to RARE [58]. Similar to the finding that the ERα LBD interacts directly with the I box of hepatocyte nuclear factor 4 (HNF4), TR, RAR, ERβ and RXR [24], GST pull-down assays performed in the presence of epitope specific antibodies indicate that COUP-TF interacts with ER through the ER LBD and DBD.

Since COUP-TF and ER interact with co-repressors NCoR and SMRT [18,59,60], one possible physiological consequence of COUP-TF-ER interaction would be to recruit or stabilize co-repressor interaction with the ER. This may result in transcriptional repression. The observation that 4-OHT, but not E_2, relieved transcriptional repression by RU486-liganded PR in vivo, presumably by competing for co-repressor binding, but that ER interaction with NCoR [17] and SMRT [18] in vitro was ligand-independent indicates that cellular factors in addition to ligand influence ER-co-repressor interaction. COUP-TF may play such a role. We noted a stronger interaction between 4-OHT-ERα and SMRT than between E_2-ERα and SMRT in GST-pull-down experiments, consistent with a role for this co-repressor in antiestrogen action.

Another unique observation reported here is that purified GST-COUP-TFI generated the appearance of two specific DNA-bound complexes with either a minimal 13 bp perfectly palindromic ERE, i.e. EREm, or a consensus ERE, i.e. EREc38, containing a 17 bp palindromic ERE of the *Xenopus* vitellogenin A2 gene plus an AT-rich region immediately flanking the ERE that is a consensus derived from the ERE flanking regions of the *Xenopus* vitellogenin A2, chicken vitellogenin II and chicken very low density apolipoprotein II EREs [32]. Because purified GST did not bind these EREs, co-purifying bacterial proteins are not responsible for the slower migrating band. The lack of COUP-TF interaction with the AT-rich region in EREc38 supports the possibility that COUP-TF multimers bind to a single minimal ERE and exclude COUP-TF binding to a 'cryptic' site in EREc38, unless that cryptic site is formed subsequent to COUP-TF binding the ERE. We speculate that COUP-TF binds palindromic EREs minimally as a dimer and that the upper complex may include three or more COUP-TF monomers. We also note that purified GST-ERRα1 formed only a single complex with EREc38.

A precedent for the formation of multimers of a nuclear receptor is the report that RXR forms a tetramer on certain reiterated half-sites [61]. Recently a novel protein-protein interaction surface including three tandem phenylalanine residues was identified in the LBD of RXR [62]. This array of phenylalanines is not found in RAR, vitamin D receptor, TR, or peroxisome proliferator-activated receptor but is conserved in COUP-TFI and other orphan receptors [62]. This phenylalanine array could allow multimeric COUP-TF interactions, thus supporting the possibility that COUP-TFI forms multimers on an ERE.

4.1. Effects of COUP-TF and ER on activation of reporter gene expression from EREs

Depending on the gene promoter and cell context, COUP-TF stimulates or inhibits gene expression [63,64]. Here we observed that the effect of COUP-TF overexpression on ERE-driven reporter gene expression in transiently transfected MCF-7 cells varied with the ERE sequence in a manner for which no simple rules can be formulated. These results confirm the complexity of estrogen regulated gene expression in mammalian cells [65].

COUP-TF up-regulated luciferase expression from EREm and EREc38, with higher activation from EREm. Since COUP-TF has been shown to activate gene expression via interaction with Sp-1 [64], the COUP-TF-activated luciferase expression may be mediated by interaction with Sp-1 bound to the SV-40 promoter in the pGL3 vector. However, COUP-TF alone did not stimulate luciferase from the pGL3-promoter vector lacking EREs. COUP-TF-Sp1 interaction may play a role in the increased basal luciferase from the pGL3-promoter vector detected in E_2-treated MCF-7 cells cotransfected with COUP-TF; however, since ER also synergizes with Sp1 to transactivate gene expression [23], the exact mechanism remains unclear.

Given the higher affinity of COUP-TF binding to EREc38 versus EREm, the greater luciferase activity induced by COUP-TF from EREm indicates that COUP-TF binding affinity does not correlate with transcription. Binding of endogenous, unliganded, i.e. transcriptionally inactive, ER to EREc38, may block COUP-TF binding. Support for this suggestion comes from promoter interference studies in intact cells showing that ER binds EREs in the absence of ligand [66]. An alternative possibility is that the consensus AT-rich region in EREc38 inhibits transactivation by COUP-TF, perhaps by altering DNA structure or by binding an endogenous protein(s). AT-rich DNA flanks EREs in genes whose transcription is highly induced by E_2 [67] and may be involved in DNA bending which also plays a role in gene expression [68,69].

Similarly, in contrast to our expectation that the lower affinity of ER for EREm would result in reduced E_2-induced luciferase, E_2 induced comparable activity from EREm and EREc38. We interpret these results as indicating that endogenous cellular factors in addition to ER may contribute to the E_2-induced transactivation from these EREs.

Co-transfection of COUP-TFI suppressed E_2-stimulated luciferase activity from a single copy of EREc38 and from pS2, but not from EREm. These data indicate that the ERE sequence influences COUP-TF repression. Repression may be caused by competition for EREc38 binding between COUP-TF and E_2-ER. Mechanisms accounting for differences in COUP-TF activity at EREm versus EREc38 or the pS2 ERE may involve proteins that bind transiently to EREs, e.g., HMG-1 [70], or ER-induced or intrinsic DNA conformational differences between these EREs.

Most estrogen responsive genes contain one or more imperfect EREs and/or multiple ERE half-sites [71]. The inclusion of tandem EREs mimics natural estrogen responsive gene promoters that contain multiple enhancer elements recognized by different proteins. Consistent with our report that COUP-TF inhibited E_2-induced luciferase activity from three tandem copies of EREc38 [26], here we show that overexpression of COUP-TFI inhibited E_2-induced luciferase from two or four tandem copies of EREc38.

Here we observed that COUP-TFI stimulated reporter gene expression from single half-site EREs. Reflecting the higher binding affinity of COUP-TF for 1/2ERE3′c38, more luciferase activity was induced by COUP-TF from 1/2ERE3′c38 versus 1/2EREc38. Since only the sequences immediately adjacent to the half-sites are different between these half-site construct, we conclude that adjacent sequences are important for COUP-TF binding and transactivation. Flanking sequence identity impacts the affinity of ER [33,45,57,67], TR [72] and RAR/RAR binding to their response elements [73]. Together, these results indicate that sequences beyond those physically occupied by nuclear receptors are likely to mediate the promoter occupancy and offers an additional mechanism to regulate specific target genes.

The stimulation of luciferase activity from the single half-sites by E_2 was surprising, since ER did not directly bind these sequences (Fig. 1 and [33,49]) or protect either half-site from DNase I digestion [55]. Since purified ERα DBD binds as a monomer to the perfect half-site of an imperfect ERE from the human *pS2* gene [74], one possible interpretation of our results is that an E_2-ER monomer binds and activates transcription from these half-sites. However, because our experiments use intact dimeric ERα [44], an alternative explanation is that one monomer of the ER dimer interacts with a single half-site with low affinity, i.e., below the detection limit of EMSA or DNase I footprinting. We suggest that low affinity interactions between ER and single half-sites may be increased by nuclear proteins, e.g., COUP-TF, indicated by the observation that co-transfection of cells with COUP-TFI enhanced E_2-induced luciferase from 1/2EREc38. Notably, since COUP-TFI did not increase E_2-induced activity from 1/2ERE3′c38, the ERE half-site sequence must play a role in transcriptional activation as well.

4.2. 4-OHT inhibits COUP-TF-induced reporter gene activity in a DNA sequence-dependent manner

The amount of repression of COUP-TF-stimulated luciferase activity from EREs by 4-OHT varied with the DNA sequence, reflecting differences in DNA binding affinity between 4-OHT-ER and COUP-TF. Interestingly, the transient transfection data appear to reflect the stronger interaction between antiestrogen-liganded ER and COUP-TF seen in GST-pull-down assays rather than the lack of stimulation of antiestrogen-liganded ER-ERE binding by COUP-TF in EMSA. It is possible that EMSA conditions de-stabilize COUP-TF-antiestrogen-ER interaction. Further studies are needed to ascertain the role of COUP-TF in antiestrogen action.

Both COUP-TF and 4-OHT-ER interact with co-repressors N-CoR and SMRT in vitro [17,18,31]. Interactions between N-CoR, SMRT, COUP-TF and 4-OHT-ER-ERE may reinforce repressed chromatin structure, thus accounting for the suppression of gene expression by 4-OHT. Cells in which 4-OHT acts as a complete antagonist may have higher levels of COUP-TF, NCoR and SMRT than cells in which 4-OHT acts as a partial agonist.

In summary, our findings add further support to the model that ER works with multiple partners, including COUP-TF, to determine the transcriptional response of estrogen target genes to estrogens and antiestrogens. Importantly, our data suggest that the ERE sequence

and the nucleotides immediately surrounding the ERE, modulate ER and COUP-TF binding affinity and the magnitude of transcriptional activation by E_2 and may contribute to the selectivity of gene activation. Defining the specific role of COUP-TF in modulating ER action is expected to lead to a better understanding of the tissue- and gene-specific effects of estradiol and antiestrogens.

Acknowledgements

We thank Dr. Kulwant Kaur for preparing the cell extracts for co-immunoprecipitation experiments and Bethany Silver for performing some of the transient transfection assays. We thank Drs. B.S. Katzenellenbogen, R. Evans, J. Mertz, S.Y. Tsai for providing plasmids and antisera. We thank Abbott Labs for the gift of H222. We thank Drs. B.J. Clark, P.C. Kulakosky and R.A. Prough for their suggestions on this manuscript. Supported by NIH R01 DK 53220, N.I.E.H.S. 1P20 ES06832-12, a University of Louisville Research Initiation Grant and Veterans Administration Center for the Study of Environmental Hazards to Reproductive Health Grant 0006, Dept. of Veterans Affairs Medical Center, Louisville, KY. to C.M.K.

References

[1] B.S. Katzenellenbogen, K.S. Korach, Editorial: A new actor in the estrogen receptor drama-enter ERβ, Endocrinology 138 (1997) 861–862.
[2] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.
[3] M. Beato, A. Sanchez-Pacheco, Interaction of steroid hormone receptors with the transcription initiation complex, Endocrine Rev. 17 (1996) 587–609.
[4] V.C. Jordan, Tamoxifen treatment for breast cancer: concept to gold standard. Oncology (1997) 7–13.
[5] C.M. Klinge, R.A. Bambara, R. Hilf, What differentiates anti-estrogen-liganded versus estradiol-liganded estrogen receptor action?, Oncology Res. 4 (1992) 1073–1081.
[6] D.W. Robertson, L.L. Wei, J.R. Hayes, K.E. Carlson, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Tamoxifen aziridines: Effective inactivators of the estrogen receptors, Endocrinology 109 (1981) 1298–1300.
[7] T.E. Spencer, G. Jenster, M.M. Burcin, C.D. Allis, J. Zhou, C.A. Mizzen, N.J. McKenna, S.A. Onate, S. Tsai, M.J. Tsai, B.W. O'Malley, Steroid receptor coactivator-1 is a histone acetyltransferase, Nature 389 (1997) 194–198.
[8] K. Horwitz, T. Jackson, D. Bain, J. Richer, G. Takimoto, L. Tung, Nuclear receptor coactivators and corepressors, Mol. Endocrinol. 10 (1996) 1167–1177.
[9] B.S. Katzenellenbogen, M.M. Montano, K. Ekena, M.E. Herman, E.M. McInerney, Antiestrogens: mechanisms of action and resistance in breast cancer, Breast Cancer Res. Treat. 44 (1997) 23–38.
[10] A.M. Brzozowski, A.C. Pike, Z. Dauter, R.E. Hubbard, T. Bonn, O. Engstrom, L. Ohma, G.L. Greene, J.A. Gustafsson, M. Carlquist, Molecular basis of agonism and antagonism in the oestrogen receptor, Nature 389 (1997) 753–758.
[11] I. Zamir, H.P. Harding, G.B. Atkins, A. Horlein, C.K. Glass, M.G. Rosenfeld, M.A. Lazar, A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains, Mol. Cell. Biol. 16 (1996) 5458–5465.
[12] L. Nagy, H.-Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, R.M. Evans, Nuclear receptor repression mediated by a complex containing SMRT, mSin3A and histone deacetylase, Cell 89 (1997) 373–380.
[13] S.-H. Hong, G. David, C.-W. Wong, A. Dejean, M.L. Privalsky, SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor alpha (RARalpha) and PLZF-RARalpha oncoproteins associated with acute promyelocytic leukemia, Proc. Natl. Acad. Sci. USA 94 (1997) 9028–9033.
[14] B.C. Ling, S.-H. Hong, S. Krig, S.M. Yoh, M.L. Privalsky, A conformational switch in nuclear hormone receptors is involved in coupling hormone binding to corepressor release, Mol. Cell. Biol. 17 (1997) 6131–6138.
[15] H. Li, C. Leo, D.J. Schroen, J.D. Chen, Characterization of receptor interaction and transcriptional repression by the corepressor SMRT, Mol. Endocrinol. 11 (1997) 2025–2037.
[16] M. Soderstrom, A. Vo, T. Heinzel, R.M. Lavinsky, W.M. Yang, E. Seto, D.A. Peterson, M.G. Rosenfeld, C.K. Glass, Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes, Mol. Endocrinol. 11 (1997) 682–692.
[17] X. Zhang, M. Jeyakumar, S. Petukhov, M.K. Bagchi, A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor, Mol. Endocrinol. 12 (1998) 513–524.
[18] C.L. Smith, Z. Nawaz, B.W. O'Malley, Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen, Mol. Endocrinol. 11 (1997) 657–666.
[19] P. Webb, G.N. Lopez, R.M. Uht, P.J. Kushner, Tamoxifen activation of the estrogen receptor/AP-1 pathway, Mol. Endocrinol. 9 (1995) 443–456.
[20] R.M. Uht, C.M. Anderson, P. Webb, P.J. Kushner, Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element, Endocrinology 138 (1997) 2900–2908.
[21] K. Paech, P. Webb, G.G. Kuiper, S. Nilsson, J. Gustafsson, P.J. Kushner, T.S. Scanlan, Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites, Science 277 (1997) 1508–1510.
[22] W. Porter, F. Wang, W. Wang, R. Duan, S. Safe, Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression, Mol. Endocrinol. 10 (1996) 1371–1378.
[23] W. Porter, B. Saville, D. Hoivik, S. Safe, Functional synergy between the transcription factor Sp1 and the estrogen receptor, Mol. Endocrinol. 11 (1997) 1569–1580.
[24] S.-K. Lee, H.-S. Choi, M.-R. Song, M.-O. Lee, J.W. Lee, Estrogen receptor, a common interaction partner for a subset of nuclear receptors, Mol. Endocrinol. 12 (1998) 1184–1192.
[25] S.D. Johnson, X. Liu, F. Zuo, T.L. Eisenbraun, S.R. Wiley, R.J. Kraus, J.E. Mertz, Estrogen-related receptorα1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements, Mol. Endocrinol. 11 (1997) 342–352.
[26] C.M. Klinge, B.F. Silver, M.D. Driscoll, G. Sathya, R.A. Bambara, R. Hilf, COUP-TF interacts with estrogen receptor,

binds to estrogen response elements and half-sites and modulates estrogen-induced gene expression, J. Biol. Chem. 272 (1997) 31465–31474.
[27] S. Seol, B. Hanstein, M. Brown, D.D. Moore, Inhibition of estrogen receptor action by the orphan receptor SHP (short heterodimer partner), Mol. Endocrinol. 12 (1998) 1551–1557.
[28] E. Enmark, J.-A. Gustafsson, Orphan nuclear receptors-the first eight years, Mol. Endocrinol. 10 (1996) 1293–1307.
[29] P.B. Joel, J. Smith, T.W. Sturgill, T.L. Fisher, J. Blenis, D.A. Lannigan, pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167, Mol. Cell. Biol. 18 (1998) 1978–1984.
[30] S.Y. Tsai, M.-J. Tsai, Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): Coming of age, Endocrine Rev. 18 (1997) 229–240.
[31] H. Shibata, Z. Nawaz, S.Y. Tsia, B.W. O'Malley, M.-J. Tsai, Gene silencing by chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is mediated by transcriptional corepressors, nuclear receptor-corepressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT), Mol. Endocrinol. 11 (1997) 714–724.
[32] F.V. Peale, L.B. Ludwig, S. Zain, R. Hilf, R.A. Bambara, Properties of a high-affinity DNA binding site for estrogen receptor, Proc. Natl. Acad. Sci. USA 85 (1988) 1038–1042.
[33] C.M. Klinge, F.V. Peale Jr, R. Hilf, R.A. Bambara, S. Zain, Cooperative estrogen receptor interaction with consensus or variant estrogen responsive elements in vitro, Cancer Res. 52 (1992) 1073–1081.
[34] F.V. Peale Jr, Y. Ishibe, C.M. Klinge, S. Zain, R. Hilf, R.A. Bambara, Rapid purification of the estrogen receptor by sequence-specific DNA affinity chromatography, Biochemistry 28 (1989) 8671–8675.
[35] E.J. Pavlik, P.B. Coulson, Hydroxylapatite "batch" assay for estrogen receptor: Increased sensitivity over present receptor assays, J. Steroid Biochem. 7 (1976) 357–368.
[36] J.D. Obourn, N.J. Koszewski, A.C. Notides, Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor, Biochemistry 32 (1993) 6229–6236.
[37] C.M. Klinge, A.L. Studinski-Jones, P.C. Kulakosky, R.A. Bambara, R. Hilf, Comparison of tamoxifen ligands on estrogen receptor interaction with estrogen response elements, Mol. Cell. Endocrinol. 143 (1998) 79–90.
[38] A.J. Cooney, S.Y. Tsai, B.W. O'Malley, M.J. Tsai, Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone and retinoic acid receptors, Mol. Cell. Biol. 12 (1992) 4153–4163.
[39] C. Abbondanza, A. deFalco, V. Nigro, N. Medici, I. Armetta, A.M. Molinari, B. Moncharmont, G.A. Puca, Characterization and epitope mapping of a new panel of monoclonal antibodies to estradiol receptor, Steroids 58 (1993) 4–12.
[40] P.B. Joel, A.M. Traish, D.A. Lannigan, Estradiol-induced phosphorylation of Serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase, J. Biol. Chem. 273 (1998) 13317–13323.
[41] R.J. Lin, L. Nagy, S. Inoue, W. Shao, W.H. Miller Jr, R.M. Evans, Role of the histone deacetylase complex in acute promyelocytic leukaemia, Nature 391 (1998) 811–814.
[42] J.C. Reese, B.S. Katzenellenbogen, Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells, Nucleic Acids Res. 19 (1991) 6595–6602.
[43] N.J. Horscroft, P. Roy, Thermal denaturation of proteins for SDS-PAGE analysis by microwave irradiation, Biotechniques 22 (1997) 224–226.
[44] C.M. Klinge, A.M. Traish, R.A. Bambara, R. Hilf, Dissociation of 4-hydroxytamoxifen, but not estradiol or tamoxifen aziridine, from the estrogen receptor when the receptor binds estrogen response element DNA, J. Steroid Biochem. Molec. Biol. 57 (1996) 51–66.
[45] J.H. Anolik, C.M. Klinge, C.L. Brolly, R.A. Bambara, R. Hilf, Stability of the ligand of estrogen response element-bound estrogen receptor depends on flanking sequences and cellular factors, J. Steroid Biochem. Molec. Biol. 59 (1996) 413–429.
[46] C.M. Klinge, A.M. Traish, M.D. Driscoll, R. Hilf, R.A. Bambara, Site-directed estrogen receptor antibodies stabilize 4-hydroxytamoxifen ligand, but not estradiol and indicate ligand-specific differences in the recognition of estrogen response element DNA in vitro, Steroids 61 (1996) 278–289.
[47] G.L. Greene, N.B. Sobel, W.J. King, E.V. Jensen, Immunochemical studies of estrogen receptors, J. Steroid Biochem. 20 (1984) 51–56.
[48] Klinge, C.M., in press. Estrogen receptor binding to estrogen response elements slows ligand dissociation and synergistically activates reporter gene expression. Mol. Cell. Endocrinol. 145.
[49] C.M. Klinge, D.L. Bodenner, D. Desai, R.M. Niles, A.M. Traish, Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements in vitro, Nucleic Acids Res. 25 (1997) 1903–1912.
[50] B.E. Gillesby, M. Stanostefano, W. Porter, S. Safe, Z.F. Wu, T.R. Zacharewski, Identification of a motif within the 5′ regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression, Biochemistry 36 (1997) 6080–6089.
[51] P. Kastner, M. Mark, P. Chambon, Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life?, Cell 83 (1995) 859–869.
[52] Y. Qiu, V. Krishnan, F.A. Piereira, S.Y. Tsai, M.-J. Tsai, Chicken ovalbumin upstream promoter-transcription factors and their regulation, J. Steroid Biochem. Molec. Biol. 56 (1996) 81–85.
[53] C.C. Landel, P.J. Kushner, G.L. Greene, The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins, Mol. Endocrinol. 8 (1994) 1407–1419.
[54] V. Boonyaratanakornkit, V. Melvin, P. Prendergast, M. Altmann, L. Ronfani, M.E. Bianchi, L. Taraseviciene, S.K. Nordeen, E.A. Allegretto, D.P. Edwards, High-Mobility Group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells, Mol. Cell. Biol. 18 (1998) 4471–4487.
[55] M.D. Driscoll, C.M. Klinge, R. Hilf, R.A. Bambara, Footprint analysis of estrogen receptor binding to adjacent estrogen response elements, J. Steroid Biochem. Molec. Biol. 58 (1996) 45–61.
[56] C.M. Klinge, R.A. Bambara, R. Hilf, Antiestrogen-liganded estrogen receptor interaction with estrogen responsive element DNA in vitro, J. Steroid Biochem. Molec. Biol. 43 (1992) 249–262.
[57] J.H. Anolik, C.M. Klinge, R.A. Bambara, R. Hilf, Differential impact of flanking sequences on estradiol- versus 4-hydroxytamoxifen-liganded estrogen receptor binding to estrogen responsive element DNA, J. Steroid Biochem. Molec. Biol. 46 (1993) 713–730.
[58] Q. Wu, Y. Li, R. Liu, A. Agadir, M.-O. Lee, Y. Liu, X.-k. Zhang, Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization, EMBO J. 16 (1997) 1656–1669.
[59] H. Shibata, T.E. Spencer, S.A. Onate, G. Jenster, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action, Recent Prog. Horm. Res. 52 (1997) 141–164.

[60] Z. Zhang, J. Laping, S. Glasser, P. Day, J. Mulholland, Mediators of estradiol-stimulated mitosis in the rat uterine luminal epithelium, Endocrinology 139 (1998) 961–966.
[61] B.C. Lin, C.-W. Won, H.-W. Chen, M.L. Privalsky, Plasticity of tetramer formation by retinoid X receptors: An alternative paradigm for DNA recognition, J. Biol. Chem. 272 (1997) 9860–9867.
[62] S. Kersten, P.R. Reczek, N. Noy, The tetramerization region of the retinoid X receptor is important for transcriptional activation by the receptor, J. Biol. Chem. 272 (1997) 29759–29768.
[63] E. Schaeffer, F. Guillou, D. Part, M.M. Zakin, A different combination of transcription factors modulates the expression of the human transferrin promoter in liver and Sertoli cells, J. Biol. Chem. 268 (1993) 23399–23408.
[64] O. Rohr, D. Aunis, E. Schaeffer, COUP-TF and Sp1 interact and cooperate in the transcriptional activation of the human immunodeficiency virus type 1 long terminal repeat in human microglial cells, J. Biol. Chem. 272 (1997) 31149–31155.
[65] H. Gronemeyer, Control of transcription activation by steroid hormone receptors, FASEB J. 6 (1992) 2524–2529.
[66] J.C. Reese, B.S. Katzenellenbogen, Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens, Mol. Cell. Biol. 12 (1992) 4531–4538.
[67] J.H. Anolik, C.M. Klinge, R. Hilf, R.A. Bambara, Cooperative binding of estrogen receptor to DNA depends on spacing of binding sites, flanking sequence and ligand, Biochemistry 34 (1995) 2511–2520.
[68] T.K. Kerppola, T. Curran, The transcription activation domains of Fos and Jun induce DNA bending through electrostatic interactions, EMBO J. 16 (1997) 2907–2916.
[69] A.M. Nardulli, C. Grobner, D. Cotter, Estrogen receptor-induced DNA bending: orientation of the bend and replacement of an estrogen response element with an intrinsic DNA bending sequence, Mol. Endocrinol. 9 (1995) 1064–1076.
[70] L.E. Romine, J.R. Wood, L.A. Lamia, P. Prendergast, D.P. Edwards, A.M. Nardulli, The high mobility group protein 1 enhances binding of the estrogen receptor DNA binding domain to the estrogen response element, Mol. Endocrinol. 12 (1998) 664–674.
[71] S. Kato, J.H. Sasaki, M. Suzawa, S. Masushige, L. Tora, P. Chambon, H. Gronemeyer, Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors, Mol. Cell. Biol. 15 (1995) 5858–5867.
[72] D.P. Olson, R.J. Koenig, 5-Flanking sequences in thyroid hormone response element half-sites determine the requirement of retinoid X receptor for receptor-mediated gene expression, J. Biol. Chem. 272 (1997) 9907–9914.
[73] S. Mader, P. Leroy, J.Y. Chen, P. Chambon, Multiple parameters control the selectivity of nuclear receptors for their response elements. Selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors, J. Biol. Chem. 268 (1993) 591–600.
[74] J.R. Wood, G.L. Greene, A.M. Nardulli, Estrogen response elements function as allosteric modulators of estrogen receptor conformation, Mol. Cell. Biol. 18 (1998) 1927–1934.