Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements in vitro

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Received February 11, 1997; Revised and Accepted March 18, 1997

ABSTRACT

The mechanism by which retinoids, thyroid hormone (T3) and estrogens modulate the growth of breast cancer cells is unclear. Since nuclear type II nuclear receptors, including retinoic acid receptor (RAR), retinoid X receptor (RXR) and thyroid hormone receptor (TR), bind direct repeats (DR) of the estrogen response elements (ERE) half-site (5′-AGGTCA-3′), we examined the ability of estrogen receptor (ER) versus type II nuclear receptors, i.e., RARα, β and γ, RXRβ, TRα and TRβ, to bind various EREs in vitro. ER bound a consensus ERE, containing a perfectly palindromic 17 bp inverted repeat (IR), as a homodimer. In contrast, ER did not bind to a single ERE half-site. Likewise, ER did not bind two tandem (38 bp apart) half-sites, but low ER binding was detected to three tandem copies of the same half-site. RARα, β or γ bound both ERE and half-site constructs as a homodimer. RXRβ did not bind full or half-site ERs, nor did RXRβ enhance RARα binding to a full ERE. However, RARα and RXRβ bound a half-site ERE cooperatively forming a dimeric complex. The RARα–RXRβ heterodimer bound the Xenopus vitellogenin B1 estrogen responsive unit, with two non-consensus ERs, with higher affinity than one or two copies of the full or half-site ERE. Both TRα and TRβ bound the full and the half-site ERE as monomers and homodimers and cooperatively as heterodimers with RXRβ. We suggest that the cellular concentrations of nuclear receptors and their ligands, and the nature of the ERE or half-site sequence and those of its flanking sequences determine the occupation of ERs in estrogen-regulated genes in vivo.

INTRODUCTION

Retinoic acid is critical in mediating differentiation and development (1,2). Retinoids inhibit the initiation and promotion of mammary tumors in rats treated with 7,12-dimethylbenz(a)anthracene or N-methyl-nitrosourea (3). Similarly, treatment of MCF-7 human breast cancer cells with all-trans retinoic acid (tRA), 9-cis RA and other retinoids; vitamin D3 (VD3) or four VD3 analogs, inhibited E2-stimulated cell proliferation (4–6). However, the usefulness of retinoids is limited by their undesirable side effects and teratogenicity (reviewed in 7). These findings have generated a search for synthetic retinoids whose selectivity and efficacy might render them useful as chemopreventive or chemotherapeutic agents.

Retinoid action is mediated by nuclear receptor proteins, retinoic acid receptor (RARα, β and γ) and retinoid X receptor (RXRα, β and γ) (reviewed in 8), by cellular retinoic acid binding proteins I and II, and cellular retinol-binding proteins (reviewed in 9). Estrogen action is mediated by hormone binding to the estrogen receptor (ER), a transactivating enhancer protein that is a member of the ligand-activated steroid/nuclear receptor gene superfamily (8). RAR, ER and other nuclear receptors have two highly conserved regions: the ligand binding (E) and the DNA-binding (C) domains. Ligand binding initiates a series of steps leading to an ‘activated’, homodimeric E2–ER that binds with high affinity to estrogen response elements (ERE). Sequence analysis of the 5′ regulatory regions of numerous estrogen responsive genes revealed a 13 bp minimal palindromic ERE consensus sequence: 5′-GGTCAAnmTGACC-3′ (EREc). EREc conferred estrogen responsiveness to reporter genes analyzed by transfection assay (10,11). Some genes, e.g. Xenopus vitellogenin A2 (12) and the human oxytocin gene (13), contain a single perfect or sequence variant copy of EREc. Other estrogen responsive genes, e.g. Xenopus vitellogenin B1 (14), the rat progesterone receptor (PR) (15) and human c-fos (16), contain multiple copies of EREc, usually with one or more base changes that function synergetically to induce E2-dependent gene expression in vivo (15,17,18). Other estrogen regulated genes, e.g. ovalbumin and prolactin, contain multiple copies of the ERE half-site, but not the palindrome (19). These genes are also regulated by the type II nuclear receptors including RAR and RXR (19). Recent studies demonstrated that ER binds variously spaced direct repeats (DR) of the ERE half-site motif, albeit with significantly lower affinity than ER binding to EREc (19,20).

Once bound to DNA, the precise mechanism of transcriptional activation, or repression, by nuclear receptors is unknown. ER-mediated effects on transcription involve interaction between the DNA-bound ER and transcription factors, adaptor proteins, e.g. ERAP160, RIP140, SPT6, SRC-1, TIF1, SUG1 or components of the TATA binding complex including TFIIB and TBP (reviewed in 21).

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Table 1. Sequences of estrogen responsive elements

<table>
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<th>Name</th>
<th>Sequence</th>
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<tr>
<td>ERE&lt;sub&gt;c38&lt;/sub&gt;</td>
<td>5′-CAGGTCAGATGTACCTGAGCTAATAATACACATTGA-3′</td>
</tr>
<tr>
<td>ERE&lt;sub&gt;n(-)&lt;/sub&gt;</td>
<td>5′-CAGGTCACCTGACC-3′</td>
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<tr>
<td>1/2ERE&lt;sub&gt;c6&lt;/sub&gt;</td>
<td>5′-CAGGTCAGAGCTTCCTGAG-3′</td>
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<td>1ERE&lt;sub&gt;c38&lt;/sub&gt;</td>
<td>5′-CAGGTCAGGAGCTTTCCTGAGCTAATAATACACATTGA-3′</td>
</tr>
<tr>
<td>1ERE&lt;sub&gt;c38&lt;/sub&gt;</td>
<td>5′-CCTCAAGGAGTCACCCTTGAGCTAATAATACACATTGA-3′</td>
</tr>
<tr>
<td>VIT&lt;sub&gt;B1&lt;/sub&gt;</td>
<td>5′-CCTCCAGCTACTGACCCCAACCCCAAGTTATCATGCACCTCCTACACATTCG-3′</td>
</tr>
<tr>
<td>VIT&lt;sub&gt;B1&lt;/sub&gt;</td>
<td>5′-CCTCCAGTCACCTGACCCCAACCCCAAGTTATCATGCACCTCCTACACATTCG-3′</td>
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<tr>
<td>VIT&lt;sub&gt;B1&lt;/sub&gt;</td>
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<tr>
<td>ERE&lt;sub&gt;3ΔT&lt;/sub&gt;</td>
<td>5′-CAGGTCAGAATGTTCCTGAGCTAATAATACACATTGA-3′</td>
</tr>
<tr>
<td>δRAR&lt;sub&gt;E&lt;/sub&gt;</td>
<td>5′-CAGGTCAGACGTTCCTGAGCTAATAATACACATTGA-3′</td>
</tr>
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</table>

The underlined nucleotides correspond to the minimal EREc. The Xenopus Vitellogenin B1 (Vit B1) gene estrogen responsive unit (ERU) consists of two imperfect EREs (67). The δRAR<sub>E</sub> is a synthetic version of the mouse RAR type β gene (68).

The specificity of Type II nuclear receptor–DNA binding is determined by the number of bases separating the direct repeats (DR) or inverted repeats (IR) of the ERE half-site: 5′-AGGTCA-3′ (reviewed in 22). Initial studies showed no binding of TRα or TRβ–RXRα to an ERE (23), leading to the model that TR bound specifically to a DR with a 4 bp spacer (DR4) or an IR with no separation between the half-sites (IR0) (reviewed in 22,24).

However, type II nuclear hormone receptors including TR (25) and peroxisome proliferator-activated receptor (PPAR) bind EREs as heterodimers with RXR (26,27). RXR also forms heterodimers with RAR and vitamin D<sub>3</sub> receptor (VDR) resulting in DNA binding cooperativity (22,23,28,29). In contrast, RXR did not enhance binding of ER to the vitellogenin ERE or form a heterodimer with the ER (30,31).

It is still unclear what nucleotide arrangements, or those of sequences flanking the recognition element, are essential for nuclear receptor binding and transactivation. Studies are needed to determine whether specific nuclear receptors synergize with or antagonize one another and the mechanisms behind such activities. For example, co-expression of v-erbA in MCF-7 cells inhibited E<sub>2</sub>-mediated transactivation from an ERE–CAT reporter construct (32) and VDR and RAR were hypothesized to bind to the ERE or to indirectly impair ER–ERE binding (33). Recent work demonstrated that the ‘3, 4, 5 rule’ of DR element spacing specificity of type II receptors is relaxed with DR spaced ≥ 10 bp apart, resulting in ‘promiscuous elements’ that bind and respond to RAR–RXR, VDR–RXR and ER, but not to TR–RXR or to progesterone receptor (PR) (19). Together, these results indicate a new dimension of ‘cross talk’ between nuclear receptors, e.g. competition for binding to 5′-AGGTCA-3′. The dissection of the molecular basis by which ER and other nuclear receptors activate or inhibit gene expression from this common motif is important for elucidating mechanisms controlling estrogen-regulated gene transcription.

To determine whether type II nuclear receptors bind directly to EREs, we examined the binding of ER, TR, RAR and RXR to various EREs in vitro. The palindromic EREs used in these experiments include a perfect consensus ERE, featuring a 17 bp inverted repeat (IR) and flanked by a naturally-occurring AT-rich region (34), a 13 bp minimal ERE, an ERE half-site alone or in the presence of a naturally occurring AT-rich flanking region (35), and the estrogen response unit (ERU), featuring two imperfect EREs, of the Xenopus vitellogenin B1 gene (17). Our results demonstrate that individually RARα, β and γ, TRα or β and ER bind fully palindromic EREs as a homodimer and that RAR and TR bind full and half-site EREs as heterodimers with RXR. The results of our studies suggest that the cellular levels of the nuclear receptors and their ligands and the nature of the nucleotide sequence of the promoter region are important for determining receptor–DNA interaction.

**MATERIALS AND METHODS**

**Preparation of ERE containing plasmids**

The sequences of synthetic ERE oligonucleotides are given in Table 1. Double stranded ERE oligomers were cloned into pGEM-7Zf(+) (Promega, Madison, WI) as described (35–37).

**Preparation of estrogen receptor**

ER was partially purified from calf uterus by heparin agarose affinity chromatography (35). ER was liganded with either 17β-[2,4,6,7,16,17-3H]E<sub>2</sub> (142 Ci/mmol from NEN), (Z)-4-hydroxytamoxifen (4-OHT) (Research Biochemicals International, Natick, MA), or [ring-3H]-tamoxifen aziridine ([1H]TAz, 23 Ci/mmol from Amersham). The concentration of ER was determined by adsorption to hydroxyapatite (HAP) (47). All receptor concentrations refer to dimeric ER, i.e. with two molecules of ligand bound. All referrals to ‘partially purified’ ER indicate the post-heparin agarose ER. When using 4-OHT, the 4-OHT-ER was protected from exposure to light.

**Preparation of RARα, RARβ, RARγ, RXRβ, TRα and TRβ**

Full length human RARα, RARβ, RARγ and RXRβ were expressed in Sf9 insect cells using the baculovirus expression vector system (BEVS) (39). Sf9 cells in the log-phase of growth were inoculated with 10 p.f.u. of recombinant or wild-type baculovirus per cell and harvested 72 h post-infection. The cells were washed in 1× PBS and in 1× hypotonic buffer containing protease inhibitors. Following resuspension, the cells were allowed to swell in this buffer for 20 min at 4°C and lysed by Dounce homogenization. The cytoplasmic and nuclear pellet were separated by centrifugation. The nuclear pellet was resuspended in extraction buffer (0.05 M Tris–HCl pH 7.4, 10% glycerol, 0.01 M monothioglycerol, 1 mM EDTA, 1 mM PMSF and 2 µg/ml each of aprotinin, leupeptin and pepstatin), sonicated 3 × 10 s bursts, incubated 2–3 h (4°C) and sedimented at 100,000 g to obtain a soluble nuclear extract (NE) that was quickly frozen and stored at −80°C.
Preparation of TRα and TRβ

Purified, full length human recombinant TRα and TRβ, expressed in Sf9 insect cells using the baculovirus expression vector system, were obtained from Karo-Bio AB, Huddinge, Sweden.

Microtiter well plate assay of ER binding to plasmid DNA

The microtiter (well) plate assay for measuring [3H]liganded-ER binding to DNA has been previously described (40). This is an equilibrium binding assay that quantitates ER–ERE binding based on [3H]ligand and [35S]DNA retention in specially treated microtiter wells. For each of the experiments presented here, plasmid DNA was linearized with EcoRI. Aliquots of EcoRI-digested plasmid DNA were labeled by incorporation of [35S]dATP (>600 Ci/mmol, NEN) using the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs, Beverly, MA) and mixed with unlabeled EcoRI-digested DNA for the desired final concentration.

Briefly, for saturation binding analysis, various concentrations of partially purified [3H]E2-ER was preincubated with ~0.22 nM of [35S]DNA (plasmid DNA with or without ERE) for 2.5 h at 4°C, with shaking, in TDPK 100 buffer (TDPK buffer containing 100 mM KCl) containing 0.1% NP-40. Aliquots of 50 µl of the receptor–DNA equilibrium mixture were then incubated in histone/gelatin-coated microtiter wells for 2.5 h at 4°C with shaking. Wells were rinsed, and the radioactivity in the wells was counted using Ecoscint A (National Diagnostics, Atlanta, GA). Calculation of specific [3H]E2-ER, [3H]-OHT-ER, or [3H]TAz-ER binding to ERs was previously described, with binding to pGEM-7Zf(+) plasmid alone subtracted from binding to plasmid containing an ERE (35,40).

Gel mobility shift assay

Gel mobility shift assays were performed as described (41–42), with the following 32P-labeled DNA oligomers, obtained by EcoRI-BamHI digestion of insert-containing pGEM-7Zf(+):: EREc38, 1/2/EREc38 and 1/2ERE3′c38 (each 77 bp); EREEm(–) (54 bp); 1/2EREc38 (54 bp); Vit B1ERU (85 bp), VIT 1B1 (66 bp) or VIT 2B1 (65 bp). Typical binding reactions contained 10 fmol (25 000 d.p.m.) 32P-labeled DNA and ER and other reaction components indicated in the Figure legends. Reactions were incubated on ice for 2.5 h before 40 µl aliquots were loaded on 4% non-denaturing polyacrylamide gels and subjected to electrophoresis at 200 V in 0.5× TBE. Gels were dried and autoradiographed on Kodak X-OMAT-AR film (Eastman Kodak Co., Rochester, NY) with an intensifying screen (Lightning Plus from DuPont, Wilmington, DE).

ER antibody H222, a generous gift of Abbott Laboratories (Abbott Park, IL), was diluted 1:10 in TE. One µl was added to confirm the identity of ER in ER–ERE complexes. Monoclonal anti-ER antibodies AER304, AER314, AER308, AER315, AER303, AER310, AER311, AER317 and AER320 were a generous gift of Neomarkers (Fremont, CA).

Antibodies for RARα and TRβ. Synthesis and purification of the oligopeptides was as described previously (43). The antisera was tested by ELISA and those with a positive titer were tested by western blot analysis, sucrose density gradients and gel shift assays (39).

Two site-directed polyclonal antibodies to RARα were raised against two oligopeptides from the N-terminal region of RARα. Polyclonal antibody R1AB was raised against a polypeptide encompassing amino acids 63–77 (TQSSSSSEIVPSPPS), Polyclonal antibody NTB (also called NTAB) was raised against an oligopeptide corresponding to the residues between 55 and 68 (STPSPATIETQSSS) of RARα. These antibodies recognized RARα by western blot and sucrose density gradient analyses. The antibodies did not recognize ER, PR, GR or AR, suggesting receptor specificity (39). Polyclonal antibody γNTB also recognized RARβ and RARγ by western blot and gel shift assays. Monoclonal antibodies to RARα and γ were prepared by standard hybridoma technology using partially purified RARα or γ as the antigen inoculated into mice. Western blot analysis with authentic RARα or γ was used to insure specificity.

PPAR antibody was a gift from Dr Michel Daucu (Université de Nancy I, France). RXR polyclonal antisera was a generous gift from Dr Pierre Chambon (CNRS, INSERM, Université Louis Pasteur, Strasbourg, France). Monoclonal antibody (9A7) to the VDR was a generous gift from Dr Mark R. Haussler of the University of Arizona. Monoclonal antibodies to TRα and TRβ were purchased from Affinity BioReagents (Golden, CO). Polyclonal antisera to COUP-TF was a generous gift from Dr Sophia Y. Tsai of Baylor University (Houston, TX).

The amount of receptor–DNA complex formed and that of free DNA were determined by excision of the entire corresponding regions from the dried gels and measuring the radioactivity present. The fraction of total [32P]DNA in the receptor–DNA complex [F(t)] was calculated as follows (44): F(t) = (c.p.m. in the receptor–DNA complex)∕(total c.p.m. in the lane), where the (total c.p.m. in the lane) = (c.p.m. in free DNA) + (c.p.m. in the receptor–DNA complex).

RESULTS

ER binds EREc38 but not an ERE half-site in vitro

We examined the ability of partially purified E2-liganded ER (E2-ER) to bind to a consensus ERE, EREC38 derived from three (TGTGGTG) to bind to a consensus ERE, EREC38 derived from three triple repeats of ERE (EREc38). For a single copy of EREc38, the half-site, 1/2EREc38, was not bound by ER (Figure 1).
highly estrogen-responsive genes (34), versus a half-site ERE, 1/2EREc38 which lacked the 3′ERE half-site but was otherwise identical in sequence to EREc38 (Fig. 1, sequences in Table 1). We detected an ER–EREc38 binding complex of which 85% was supershifted by the ER-specific antibody H222. This result together with our previous work (35–37,41–43), in which we quantitated both the amount of receptor, based on [3H]ligand, and DNA, based on 35S incorporation, indicates that E2–ER binds EREc38 with high affinity and a stoichiometry of one E2–ER dimer per ERE.

Addition of equal amounts of NE containing RARα and RXRβ produced a complex, similar in mobility to that of ER–EREc38 (Fig. 2). Addition of a monoclonal antibody (MoAb) to RARα slightly inhibited the amount of complex formed. Addition of polyclonal RAR antibody R1AB supershifted and enhanced the amount of RARα homodimer bound to EREc38 (data not shown). Incubation of [32P]EREc38 with NE from null baculovirus infected Sf9 cells did not appear to bind EREc38. We detected an ER–EREc38 binding complex of which 85% was supershifted by the ER-specific antibody H222. This result together with our previous work (35–37,41–43,45,46), in which we quantitated both the amount of receptor, based on [3H]ligand, and DNA, based on 35S incorporation, indicates that E2–ER binds EREc38 with high affinity and a stoichiometry of one E2–ER dimer per ERE.

Addition of equal amounts of NE containing RARα and RXRβ resulted in the formation of a complex whose migration was slightly faster than that of the homodimeric RARα–EREc38 complex (Fig. 2A). The amount of RARα–RXRβ–EREc38 complex formed was equal to that of the RARα–EREc38 complex formed with partially purified E2–ER (100 fmol per lane) was incubated with 32P-oligomers (25 000 d.p.m. per reaction) containing EREc38 (lanes 1 and 2), 1/2EREc38 (lanes 3 and 4), 1/2EREc38 or RXRc38 (lanes 5 and 6). The specificity of COUP-TF–1/2EREc38 or EREc38 binding was demonstrated by competition with unlabeled EREc38, 1/2EREc38 and the 17 bp core ERE IR, but not by the AT-rich region that flanks the ERE IR in EREc38 or by the region in the vicinity of the ERE IR in EREc38 or by the region in the vicinity of the ERE IR in EREc38.
complex formed with the same amount of extract (Fig. 2B). The addition of a MoAb to RARα decreased the amount of complex formed. Addition of the R1AB RAR antisera, which did not itself bind ER, approximately doubled the amount of complex detected and supershifted by AER304 (data not shown). The relative binding affinity of ER for 3(1/2EREc38) is ~34% of ER binding to a single copy of EREc38 and 26% of that for ER binding to three tandem copies of EREc38 (data not shown). Based on our previous work (35–37, 41–43), this yields an estimated $K_d$ value of 0.92 versus 0.27 nM for ER–EREc38 interaction.

RARα, β and γ bind 1/2EREc38, but RXRβ does not

We next tested the ability of each RAR subtype, RXRβ, or combinations thereof, to bind a single ERE half-site in vitro. Although RARα was capable of binding 1/2EREc38 in a dose-dependent manner, more RARα was bound to ERc38 versus 1/2EREc38 (compare Figs. 2, 4 and 5). Thus both halves of the ERE IR are required for RARα homodimer binding. Interestingly, RARα–1/2EREc38 corresponded in migration to a dimeric receptor complex. Addition of R1AB antibody enhanced the amount of RARα–1/2EREc38 detected (Fig. 5, lane 2). Similar results were detected for RXRβ and RARγ (data not shown). The specificity of RARα binding was demonstrated by the ability of ERc38 and βARE to inhibit RARα–1/2EREc38 binding, by the ability of the MoAb to RARα to decrease the amount of complex formed, and by the supershift of RARα with R1AB. RXR antibody had no effect on RARα–1/2EREc38 binding (data not shown), indicating that the Sf9 NE did not contain an RXR related activity. Neither R1AB nor the RXR antibody bound EREc38 or 1/2EREc38 (data not shown).

RXRβ did not appear to bind 1/2EREc38. This result indicates that binding of either ERc38 or 1/2EREc38 by RXRβ was of lower affinity compared with RARα and is consistent with the role of RXR as a dimerization partner for type II nuclear receptors that binds only certain RXREs as a homodimer (28). Addition of RXR antibody enhanced and supershifted the RXRβ–1/2EREc38 complex (Fig. 5, lane 5).
Table 2. Comparison of binding of 4-OHT–ER and RARα + RXRβ to Vitellogenin B1 ERU

<table>
<thead>
<tr>
<th>Fraction of total (d.p.m.)</th>
<th>Vit B1 ERU</th>
<th>1B1</th>
<th>2B1</th>
<th>RARα + RXRβ</th>
<th>ER</th>
<th>RARα + RXRβ</th>
<th>ER</th>
<th>2EREc38</th>
<th>ER</th>
<th>RARα + RXRβ</th>
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<th>2EREc38</th>
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<tr>
<td>Bound</td>
<td>0.41</td>
<td>0.40</td>
<td>0.35</td>
<td>0.55</td>
<td>0.35</td>
<td>N.D.²</td>
<td>0.68</td>
<td>0.16</td>
<td>0.92</td>
<td>N.D.²</td>
<td>0.78</td>
<td>0.94</td>
</tr>
<tr>
<td>not SS by H222</td>
<td>0.24</td>
<td>0.26</td>
<td>0.17</td>
<td>0.07</td>
<td>0.07</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SS H222</td>
<td>0.48</td>
<td>0.22</td>
<td>0.24</td>
<td>0.78</td>
<td>0.78</td>
<td>0.94</td>
<td></td>
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</table>

Receptor–ERE binding was determined by gel mobility shift assay and the total amount of shifted complex is given as F(t) values from two assays as described in Materials and Methods (44). ER (7.39 nM) was liganded with 4-OHT (84.4 fmol ER dimer per gel lane). Incubation of 4-OHT–ER with H222 increased the amount of complex formed.

RXRα–RXRβ heterodimers bind cooperatively to 1/2EREc38, but not EREc38

Co-incubation of RARα and RXRβ with 1/2EREc38 produced a 5-fold increase in the amount of complex (Fig. 4, lanes 9–11 and Fig. 5, lanes 7–11). Similar results were obtained for RARα–RXRβ binding to 1/2EREc38 and to two or three tandem copies of 1/2EREc38 (data not shown). The specificity of the RARα–RXRβ heterodimer was confirmed by the ability of R1αB and RXR antisera to supershift the complex. In addition, inclusion of 10-fold molar excess unlabeled EREc38 decreased complex formation by 41% (Fig. 5, lane 11).

Incubation of either 1/2EREc38 or EREc38 with NE from Sf9 cells transfected with wild-type (wt) baculovirus formed two complexes whose migration was faster than that of the ER–ERE complex (Fig. 3), and faster than the migration of the TR monomer complex (data not shown). In results similar to those for RARα alone, the amount of binding of the RARα–RXRβ to EREc38 was twice that of binding to 1/2EREc38. Addition of unlabeled competitor EREc38 or RXR antisera, rNTAB and R1AB, supershifted the binding of RARα–RXRβ to EREc38 and 1/2EREc38, indicating binding specificity (data not shown).

RARα–RXRβ heterodimers bind naturally occurring EREs

We compared the binding of ER versus RARα or RXRβ to the Xenopus vitellogenin B1 estrogen responsive unit (ERU; Vit B1 ERU), consisting of two imperfect palindromic EREs, versus ER binding to each ERE alone (1B1 and 2B1) (sequences in Table 1). In contrast to the ability of two ER homodimers to bind to two tandem copies of EREc38, only a single homodimer of ER bound to the Vit B1 ERU (data not shown). The affinity of ER binding to the Vit B1 ERU was ~40% lower than binding to ER–VIT B1–ERU (data not shown). To quantitate the affinity of ER binding to the Vit B1 ERU versus EREc38, equilibrium binding experiments were performed (40). E2–ER bound Vit B1 ERU with considerably lower affinity than EREc38, Kd = 7.17 versus 0.24 or 0.27 nM, for Vit B1 ERU versus 1 or 2 tandem copies of EREc38 respectively (data not shown). The binding of ER to the individual EREs within the Vit B1 ERU, 1B1 and 2B1 was similar to that for ER binding to the Vit B1 ERU, i.e. one dimeric ER appeared to bind each construct. This is indicated by the proportion of [32P]DNA complex retarded by ER gel shift assays shown in Table 2. These data also show that incubation with antibody H222 increased the amount of complex formed.

RARα and RXRβ appeared to bind cooperatively to VIT B1–ERU since the total complex formed in the presence of both was significantly greater than the sum of each alone (data not shown). RXRβ did not bind the Vit B1–ERU, 1B1 or 2B1 alone. Interestingly, the total RARα/RXRβ–VIT B1–ERU complex formed was twice that of RARα/RXRβ–EREc38 or RARα/RXRβ–1/2EREc38. However, the total amount of RARα–RXRβ–EREc38 complex was significantly less than that for ER–EREc38 and slightly lower than that for ER–VIT B1–ERU. By performing gel shift experiments in which the concentration of input [32P]ERE was varied with a fixed volume of RAR NE, Kd values were calculated (data not shown). RARα bound EREc38 and 1/2EREc38 with Kd = 1.24 and 3.8 nM respectively. Similar values (Kd = 0.77 and 1.17 nM for EREc38 and 0.88 and 0.79 nM for 1/2EREc38) were obtained for RARβ and RXRγ respectively. In comparison, a Kd = 0.3 nM was estimated for RARα–RXRβ binding to VIT B1 ERU (data not shown). We conclude that the RARα–RXRβ heterodimer binds VIT B1 ERU with higher affinity than EREc38 or 1/2EREc38.

TRα and TRβ bind EREc38 as monomers, homodimers or as heterodimers with RARα and RXRβ

Since TR was reported to bind an ERE and inhibit E2-dependent transactivation (25) and a heterodimer of TRα and RXRβ was reported to bind to the vitellogenin A2 ERE in vitro (31), we evaluated the ability of recombinant baculovirus-expressed human TR to bind EREc38 individually or with the addition of RARα or RXRβ (Fig. 6). In contrast to our findings with ER, RARα and RXRα–RXRβ, two specifically retarded TR-containing bands were observed. The faster migrating band represents TR monomer and the more slowly migrating complex is TR dimer. The monomer complex appeared first with low concentrations of TR whereas both complexes were formed at higher TR concentrations (Figs 6 and 7 and data not shown). Thus, in the absence of T3 as ligand, both TRα and TRβ bound EREc38 as monomers as well as dimers. Inclusion of a small amount of either TRα- or TRβ-specific antibody did not affect the migration or amount of complex formed. Inclusion of higher antibody concentrations supershifted the complex (data not shown). Addition of RARα to the TRβ–EREc38 binding reaction decreased the amount of retarded complex detected by 20% (Fig. 6, quantitation not shown). One explanation for this result is that TRβ forms a non-productive heterodimer with RARα that is unable to bind DNA. Neither preincubation of RARα with the RARα MoAb, for 30 min at 4°C, nor inclusion of RARα MoAb with the ERE affected migration or amount of complex detected. As seen in all gel shift assays performed, RARα-specific MoAb did not supershift the DNA-bound RARα, but rather minimally inhibited RARα–DNA binding. In contrast, polyclonal RAR antisera, rNTAB and R1AB, supershifted the RARα–DNA binding.
complex. Since this RARα MoAb works well in Western blots with denatured RARα, we believe that the RARα MoAb recognizes an epitope that is inaccessible or buried in the RARα-DNA complex.

When TRβ was incubated with RXRβ and EREc38, the monomer TR complex disappeared and concomitantly an increased amount of dimeric complex was detected (Fig. 6, lanes 10–12). This indicates cooperative binding. Interestingly, a small amount, ∼13% of the total TRβ-RXRβ-EREc38 complex, migrated more slowly than the dimeric receptor (see the complex annotated by a star in Fig. 6, lanes 11 and 12). Addition of TRβ antibody reduced the intensity of this band and generated two supershifted complexes (Fig. 6, lane 11). The complex denoted by the star in Fig. 6, lanes 11 and 12). Addition of TRβ monomer, a less mobile complex appeared with addition of RXRβ (star). SS indicates the supershifted complexes.

antibodies to these type II nuclear receptors had no effect on ER-ERE binding. This indicates that bovine ER did not heterodimerize with any of these type II nuclear receptors.

The MoAb to TRβ supershifted ∼28% of the total TRβ-EREc38 complex and enhanced the total amount of TRβ-EREc38 detected by 41%. Addition of RXRβ to the TRβ + [32P]EREc38 reaction generated only the dimeric receptor binding form. Antibodies to either TRβ and RXR supershifted the complex. Addition of RARα to the TRβ + [32P]EREc38 binding reaction decreased the amount of complex detected by 26%. This indicates that interaction of RARα with TRβ either inhibited TRβ binding to 1EREc38 or competes away a co-factor required for TR–DNA binding. Addition of the MoAb to RARα inhibited the amount of TRβ-1EREc38 complex detected by 12%. This may be due to some cross-reactivity between the RARα MoAb and TRβ.

**DISCUSSION**

Because nuclear type II receptors bind various arrangements of the ERE half-site (reviewed in 41), we postulated that the inhibitory effects of retinoids (4-6) and the stimulatory effects of T3 (48,49) on breast cancer cell replication could be mediated, at least in part, by RAR, primarily through RARα (6), or TR binding to EREs. In this model, type II nuclear receptors compete with estrogen-liganded ER for ERE binding and thus preclude subsequent transactivation of genes essential for cell replication. As a first step in elucidating these
mechanisms, we compared the ability of ER, RAR, RXR and TR to bind to palindromic EREs or ERE half-sites in vitro.

ER did not bind to a single copy of the 1/2EREc38 or 1/2ERE3′c38 half-sites, regardless of ligand, even at concentrations of up to 200 fmol ER dimer in a binding reaction including 10 fmol ERE half-site oligomer. This confirms our previous results (35–37,40–42,45,46,50) and those of other investigators that the ER homodimer requires both arms of the ERE IR. We did detect the binding of COUP-TF, present in the partially, but not highly, purified bovine ER, to 1/2EREc38. COUP-TF is one of the best characterized of the orphan nuclear receptors that competes for 5′-AGGTCA-3′ elements with other nuclear receptors and inhibits gene expression (51).

Although it was long dogma that ER bound only to EREs containing an IR+3 sequence, recent work demonstrated ER binding to half-sites as DR (19,20). We did not observe ER binding to a single or two tandem (head-to-tail) copies of 1/2EREc38, but low ER binding was detected to three tandem copies of 1/2EREc38. Since ER was reported to bind DR half-sites of 1 or 6 nt (20) and up to 300 nt apart (19), the reason for the failure of ER to bind the second half-site in two tandem 1/2EREc38 is unclear. It is possible that the helical spacing between half-sites is important. When there are two tandem copies of 1/2EREc38, the distance between each 5′-AGGTCA-3′ motif is 38 nt. Assuming 10.5 bp/turn in B-form DNA (52), this indicates that the half-sites are 3.6 helical turns apart, on opposite faces of the DNA helix. When a third half-site is present, the first and third half-sites are located 72 nt, 6.9 helical turns, apart on the same face of the helix. This may enhance ER binding stability in a way analogous to the co-operativity of E2-ER binding to three, but not two, tandem copies of EREc38 (35–37,41,46). A model depicting this possibility is shown in Figure 8A.

Our results provide the first demonstration that RARα, β or γ bind as homodimers to an ERE half-site in the absence of ligand in vitro. It is unlikely that the RARα–1/2EREc38 complex detected was the result of heterodimerization between RARα and a protein in the Sf9 NE because addition of increasing amounts of NE from cells infected with wt baculovirus did not enhance RARα–DNA complex formation. Additionally, RXR antisera had no effect on the complex formed. Two previous reports showed RAR binding to a half-site. RARα bound a single half-site as a monomer (53).

Figure 8. Model of ER and type II nuclear receptor interaction with ERE and ERE half-sites. (A) The ER homodimer (grey ovals) binds only to the full palindromic ERE (EREc38, the consensus AT-rich region is indicated with the grey stripes) and not to a single half-site (1/2EREc38 or 1/2ERE3′c38). ER does not bind two head-to-tail tandem copies of 1/2EREc38 (2(1/2EREc38)), but low specific binding was observed to [3(1/2EREc38)]. We suggest that DNA bending may be involved in this event. If two half-sites occur on the same face of the DNA helix, each ER monomer in the dimeric ER can contact the two half-sites. The broad Xs indicate no binding. The single arrows between the ER homodimer and the various EREs indicate ER binding. The double arrows indicate that ER binds with lower affinity to that ERE. (B) RARα homodimers (open ovals) bind the indicated full ERE and half-sites, but not the 13 bp ERE, EREm(–). RXRβ (grey rectangles) does not bind any of these constructs. A RARα–RXRβ heterodimer binds EREc38, 1/2EREc38 and 1/2ERE3′c38, but not EREm(–). Symbols are identical to those in (A). (C) TRα or TRβ (indicated as striped ovals) as monomers or homodimers, or as heterodimers with RXRβ, bind full and half-site EREs as indicated, but not EREm(–). Symbols are identical to those in part A. (C) TRα and TRβ (indicated as striped ovals) as monomers or homodimers, or as heterodimers with RXRβ, bind full and half-site EREs as indicated, but not EREm(–). Our data suggest that the RXRβ–TRβ heterodimer also interacts with an additional RXRβ monomer to form a complex composed of three receptor monomers, i.e. two RXRβ monomers plus one TRβ monomer on the EREc38 element. This binding is dependent on the presence of the two half-sites within the ERE palindrome and the adjacent AT-rich region. Symbols are identical to those in (A).
The molecular species of RARα, β or γ bound to 5′-GTTCA-3′ in the zif268 gene promoter was not stated (54). In contrast to the binding of RAR homodimers to 1/2EREc38, RXRβ did not bind 1/2EREc38 in the absence of ligand in vitro. These binding data are modeled in Figure 8B.

Interestingly, the RARα–RXRβ heterodimer bound cooperatively to 1/2EREc38 and differs from previous reports suggesting that cooperative binding of RAR, TR and RXR occurred only on elements with specific spacing between the half-sites (55–56). RAR–RXR forms in solution and binds more efficiently than homodimers to a number of RA response elements in vitro (e.g. 23,24,29,30,49). Thus, our observation of cooperative binding of RARα–RXRβ to a single ERE half-site with an adjacent AT-rich region is unique. An earlier report predicted such binding (57), but this was, to our knowledge, not reported until now. Because we did not observe cooperative RARα–RXRβ binding to 1/2EREc, we believe that the proximity of AT-rich region and the half-site is responsible for enhancing binding. One possible mechanism for the AT-effect involves possible enhanced receptor-induced DNA bending. Both RAR and RXR bend DNA (reviewed in 58).

TR monomers and homodimers bound to 1/2EREc38. Similarly, a TR homodimer or TR–RXRβ heterodimer bound to a single half-site within the murine MHC Class I region II promoter (30). In contrast, only monomeric RAR or TR bound an ERE half-site (53) and TR required a HeLa cell protein, TRAP, to bind to an imperfect half-site (59). We suggest that sequences flanking the half-site influence the stability of dimeric TR binding. This is true for ER–ERE binding (35–37,41,46) and a recent report demonstrated that the sequences immediately flanking the half-site modulate RXR–TRα binding (60). Again, one possible mechanism involves TR-induced DNA bending (58).

While both TRα and TRβ bound 1/2EREc38 as monomers and homodimers in vitro, RARα inhibited TRβ–1/2EREc38 binding. Likewise, RARα inhibited TRβ–EREc38 binding. These results appear to correlate with the ability of RAR to inhibit T₃ responses from DR4 (26,61). One explanation may be competition between receptors for DNA binding. Alternatively, heterodimerization between RARα and TRβ may result in an ‘incorrect’ orientation of the receptor monomers. For both RXR–RAR and RXR–TR heterodimers, binding cooperativity depends on RXR bound 5′ to its cooperating partner (56). RXR stimulated the binding of TR to TRE (26,29) and, as shown here, TR–ERE or half-site binding. RARα, β and γ bound EREc38 as homodimers, but RXRβ did not bind to EREc38. In contrast, neither A/B-domain truncated RARα nor RXRβ bound to the vitellogenin ERE, but did bind the ERE as a heterodimer (62). This implicates sequences in the N-terminal region as necessary for the three-dimensional conformation of RARα for ERE binding. Neither RARα nor RXRβ bound EREN(−), indicating the importance of the 5′ A nucleotide of the 6 bp half-site. It is currently unclear whether the liganded RAR homodimer or RAR–RXR heterodimer transactivate gene expression from an ERE. One report showed that RAR bound an ERE, but activated transcription only from DR-3, thus indicating that RAR–DNA binding is not necessarily correlated with transcriptional activation (63). However, since ER has only an ∼4-fold higher affinity for binding EREc38 compared with the RARα–RXRβ heterodimer, our findings indicate that RAR–ERE binding could block ER binding and subsequent signal transduction depending on the cellular concentrations of each receptor and its ligands.

We observed greater binding of RARα–RXRβ heterodimers to the Vit B1 ERU compared with a single copy of EREc38. However, ER–Vit B1 ERU binding was greater than that of RARα–RXRβ. Similarly, Ozato’s group (30) reported that RXRβ bound the Vit B1 ERU with higher affinity compared with Xenopus Vit A1 (34–36). One reason for this result is that the Vit B1 ERU contains two imperfect EREs. Each ERE could bind one RARα–RXRβ heterodimer. However, using gel shift assays, neither Marks and co-workers (30) nor we have observed a complex comparable in mobility to that for two RARα–RXRβ heterodimers bound to the two EREs. On the other hand, methylation interference (30) and DNase I footprinting (64) showed that both EREs in the Vit B1 ERU were protected by TR and TR–RXR or ER. We did not detect the binding of two ER dimers to the Vit B1 ERU by either gel shift or plate assays. Differences in the sensitivity of the assay systems seem likely to account for these observations.

A small amount of the TRβ–RXRβ heterodimer–EREc38 binding complex, migrated more slowly than the dimeric receptor form (see the complex denoted by a star in Fig. 6). We believe this to be a unique observation, distinct from the larger complex formed between a JEG cell extract and the rat α-myosin heavy chain, malic enzyme or human α subunit elements (65). A similar complex was observed by chemical cross linking using a C-terminal truncated RXRβ co-incubated with TR and the region I of the murine MHC class I genes that contains no apparent perfect half-site (30). Similar to our results, region I did not bind RXR but did bind TR homodimer and the RXR–TR heterodimer.

Results from experiments using XRR and TR antisera indicate that the complex denoted by a star in Figure 6 represents the binding of an additional RXRβ monomer with the TRβ–RXRβ heterodimer to EREc38. The exact sequence specificity of this binding is unknown. However, this complex did not appear when we examined the binding of TRβ–RXRβ to 1/2EREc38 (Fig. 7) or to 1/2EREc (data not shown), indicating that its formation requires both halves of the ERE IR.

TR was reported to bind the ERE as a monomer and dimer (25). Later work showed binding of a TR monomer, but not the TR dimer, either to a single half-site or to an IR3 ERE. Previous reports indicated that RXRβ did not enhance TR binding to a RARE (23), but did enhance TR–TRE binding (23,28,29,48,55). We showed that TRβ–RXRβ–ERE binding was significantly greater than that of TRβ alone, indicating binding cooperativity for the heterodimeric form. These results corroborate and extend earlier reports on TR–ERE binding (49,66).

TR did not bind to ERE(–) (data not shown). Thus, in results identical to those for RAR, TR binding is dependent upon the presence of the 5′-A in the ERE half-site. It is important to note that co-incubation of ER with RARα, β and γ, TRα or TRβ, or RXRβ did not generate the appearance of complexes of different mobility than that detected for ER alone and that antibodies to these receptors had no effect on ER–ERE binding. This indicates that bovine ER does not heterodimerize with any of these receptors.

In conclusion, our results suggest that RARα, β and γ, TRα, TRβ and RXRβ, and likely other type II nuclear receptors compete for binding to full and half EREs and may thus contribute in an elaborate manner to control the expression of estrogen-regulated genes. Our observations suggest the mechanism accounting for the E2 antagonistic properties of retinoids, and possibly the stimulatory effect of T₃, is by binding competition between RAR, RXR, TR
and ER for fully palindromic EREs and may involve RAR, RXR and TR binding to half-site EREs in a number of naturally occurring estrogen-responsive genes.

ACKNOWLEDGEMENTS

We thank Abbott Laboratories for the gift of the H222 antibody; Dr Pierre Chambon (CNRS, INSERM, Université Louis Pasteur, France) for the gift of the RXR antibody; Dr Michel Daucé of the Université de Nancy I in France for the PPAR antibody; Dr Sophia France) for the gift of the RXR antisera; Dr Michel Daucé of the Dr Pierre Chambon (CNRS, INSERM, Université Louis Pasteur, France) for the gift of the RXR antibody; and Dr Sophia France) for the gift of the RXR antisera; Dr Michel Daucé of the University of Rochester for plasmids containing Vit BE, ER, and 2B1. Supported in part by NIH grant HD24459 to R. Hilf at the University of Rochester and NCI grant CA59530 to R.M.N.

REFERENCES