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Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species

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Abstract

The ability of 14 structurally diverse estrogenic compounds to induce reporter gene expression mediated by estrogen receptors (ERs) from different species was examined. MCF-7 cells were transiently transfected with a Gal4-regulated luciferase reporter gene (17m5-G-Luc) and Gal4-ER chimeric receptors containing the D, E and F domains of the human α (Gal4-hERαdef), mouse α (Gal4-mERαdef), mouse β (Gal4-mERβdef), chicken (Gal4-cERαdef), green anole (Gal4-aERαdef), *Xenopus* (Gal4-xERdef) or rainbow trout α ERs (Gal4-rtERαdef). The efficacy of 17β-estradiol (E2) in inducing reporter gene expression was similar among the different constructs overall, with EC_{50} values ranging from 0.05 to 0.7 nM. However, Gal4-rtERαdef had an EC_{50} value at 37 °C of 28 nM, though at 20 °C an EC_{50} value of 1 nM was observed. Despite a similar response to E2 treatment among the ERs, many differences were observed in the magnitude of the response to other structurally diverse chemicals. For example, coumestrol induced Gal4-mERβdef- and Gal4-aERdef-mediated reporter gene expression 164- and 8-fold greater, respectively, than mediated with the other Gal4-ERs. As well, in contrast to results with other Gal4-ERs, α-zearalenol consistently induced Gal4-rtERαdef-mediated reporter gene activity at lower concentrations than did E2. Overall, the results demonstrate that selected estrogenic compounds exhibit a differential ability to induce reporter gene activity mediated by ERs from different vertebrate species. These data also highlight the importance of incubation temperature when examining rtERα-mediated activity.

Keywords: Estrogen; Species; EED

1. Introduction

In recent years, there has been heightened concern that exposure to hormonally active chemicals, known as endocrine disrupting compounds (EDCs), may cause adverse health effects in humans and wildlife [1]. Suspected EDCs include a wide range of structurally diverse natural products and synthetic compounds that are commonly encountered in modern society (Fig. 1). The greatest attention has focused on estrogenic endocrine disruptors (EEDs) [2–5], although screening and testing protocols for androgenic and thyroid-like chemicals are also being developed. Many EEDs do not share any obvious structural similarity to the prototypical estrogen, 17β-estradiol (E2), which impedes the identification of estrogenic substances based solely on molecular structure [6]. Consequently, it has been proposed that chemicals can be more effectively screened for EED activity using estrogen receptor (ER)-based assays [4], although other mechanisms such as interactions with binding globulins [7,8], inhibition or induction of steroidogenic enzymes [9–11], and binding to membrane receptors [12,13] or other nuclear receptors [14,15] should not be ignored.

The ER is a member of the nuclear receptor (NR) superfamily, a large group of ligand- and non-ligand-regulated transcription factors that play critical roles in development, differentiation, and homeostasis. The two ER isoforms, ERα and ERβ, are each encoded by a distinct gene and display differential tissue distribution and ligand preference [16–18]. Despite the conserved physiological actions of the ER among different species, the amino acid sequences of

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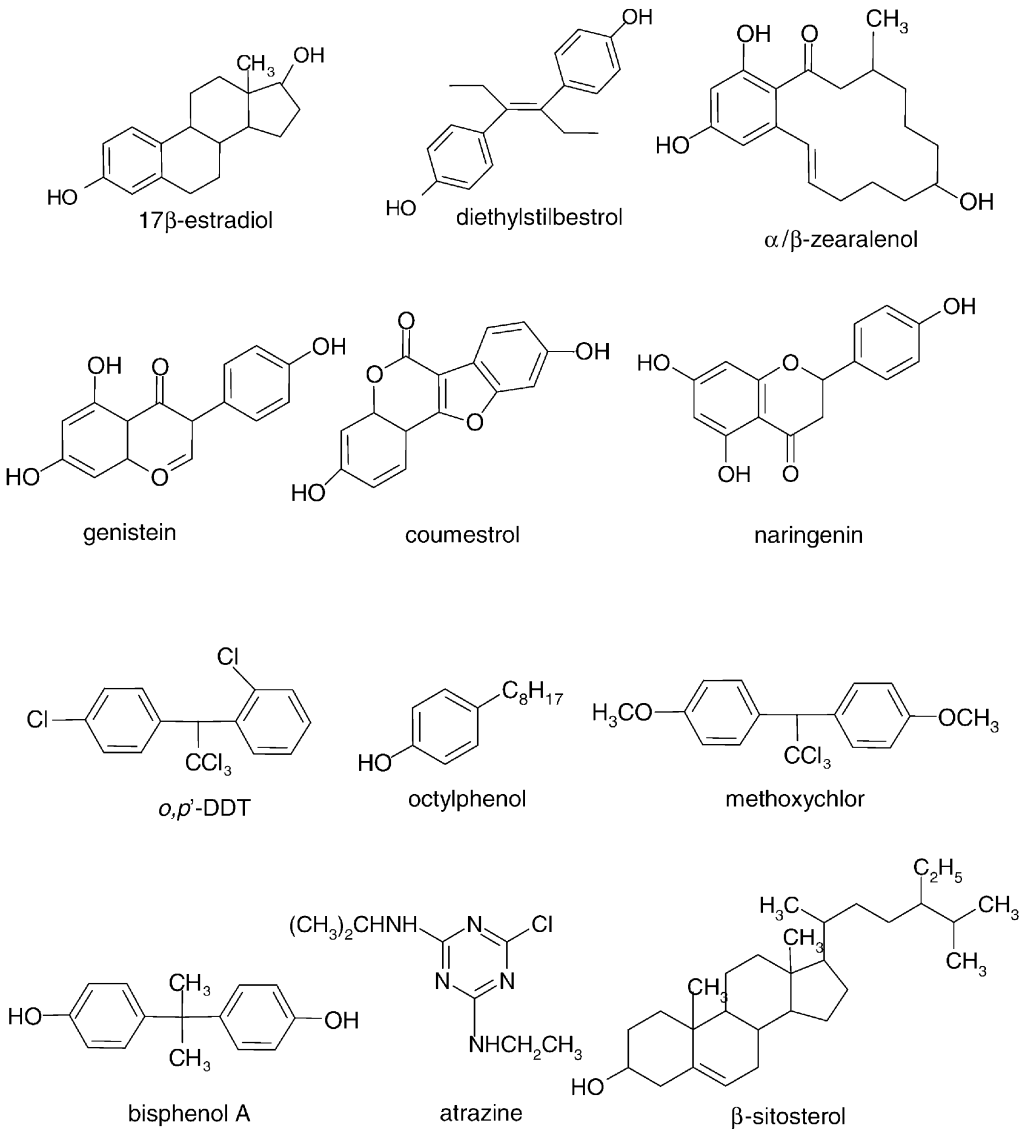

Fig. 1. Structures of selected suspected estrogenic endocrine disruptors (EEDs).

the regions involved in ligand binding are surprisingly variable [19,20]. This suggests that species may exhibit differential responses and sensitivities to EEDs, and that a single species may not be an appropriate surrogate for identifying and predicting responses in other distantly related species.

The transcriptional activity of the ER is mediated by both an NH_2-terminal ligand-independent activation function (AF-1) and a ligand-dependent activation function (AF-2) located in the ligand binding domain. Although the mechanism by which the AF-2 region transmits ligand signals to the basal transcriptional machinery is poorly understood, several proteins that interact with the AF-2 region in a ligand-dependent manner have been identified [21,22]. These proteins, collectively termed cofactors, function as coactivators or corepressors to induce or inhibit gene expression, respectively. Functional and structural studies have shown that coactivators interact with the AF-2 region via the short leucine rich motif LXXLL to transduce the ligand signal to the basal transcriptional machinery. Several structurally distinct classes of coactivator proteins have been identified. For example, the steroid receptor coactivators (SRC) or nuclear receptor coactivators (NCoA) [23] are a family of 160 kDa molecular weight proteins that interact with NRs in a ligand-dependent fashion. Members of this family include NCoA-1 (also known as p160/ERAP-160) [23,24], NCoA-2 (also known as TIF2/GRIP-1) [25,26], and NCoA-3 (also known as AIB1/ACTR/RAC-3/TRAM-1/pCIP) [27–30]. Recent studies suggest that the ability of the ER to recruit SRC coactivators and LXXLL peptides is dependent on the structural characteristics of the ligand [31–33].

Several studies have reported differences in relative binding affinity (RBA) of EEDs for ERs among vertebrate species and between ER isoforms [17,19,34–36]. Moreover,

the RBAs of several EEDs do not correlate with their transactivation ability [17,37–39]. For example, despite the apparent greater affinity of genistein for ERβ, genistein exhibits comparable transactivation ability mediated by either ERβ or ERα [17]. In addition, bisphenol A binds to ERβ with 10-fold greater affinity than to ERα, but only exhibits a 2-fold greater ability to induce gene expression mediated by either isoform [37]. Collectively, these results suggest that RBA can be a poor predictor of ER-mediated transactivation ability.

Previous studies in this laboratory have investigated the RBA of structurally diverse estrogenic chemicals for glutathione-*S*-transferase (GST) fusion proteins containing the ER D, E and F domains from several different species using in vitro competitive binding assays [19]. Qualitatively, the fusion proteins exhibited similar binding preferences and rank order of RBAs for the majority of EEDs examined. However, several significant quantitative differences were observed [19]. The present study further investigates these differences in RBAs by examining the ability of these EEDs to exhibit differential transactivation ability in MCF-7 cells transiently transfected with Gal4-ERdef chimeric receptor constructs consisting of the DNA binding domain of the yeast transcription factor Gal4 fused upstream of the D, E, and F domains from the human α, mouse α, mouse β, chicken, green anole, *Xenopus*, and rainbow trout α estrogen receptors. The correlation between RBA and transactivation ability was also examined.

2. Materials and methods

2.1. Chemicals and biochemicals

17β-Estradiol (1,3,5 [40]-estratriene-3,17β-diol), DHT (dihydrotestosterone, 5α-androstan-17β-ol-3-one), diethylstilbestrol (4,4′-(1,2-diethyl-1,2-ethene-diyl)-bisphenol), α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-undecyl] benzoic acid μ-lactone), β-zearalenol (2,4-dihydroxy-6-[6β, 10-dihydroxy-undecyl]benzoic acid μ-lactone), genistein (4′,5,7-trihydroxyisoflavone), naringenin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside), and β-sitosterol (22,23-dihydrostimasterol) were purchased from Sigma (St. Louis, MO). Coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Acros Organics (Pittsburgh, PA). Methoxychlor (1,1,1-trichloro-2, 2-bis(*p*-methoxphenyl)ethane) was provided by William Kelce (Monsanto, St. Louis, MO). *o,p′*-DDT (1,1,1-trichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethane) and *p,p′*-DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane) were purchased from AccuStandard (New Haven, CT). 4-*t*-octylphenol and bisphenol A (4,4′-isopropylidenediphenol) were obtained from Aldrich (Milwaukee, WI). Atrazine was purchased from Chem-Service (West Chester, PA).

Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) and medium supplements were purchased from Life Technologies (Gaithesburg, MD). Fetal bovine serum (FBS) and D-luciferin were obtained from Intergen (Purchase, NY) and Molecular Probes (Eugene, OR), respectively. All other chemicals and biochemicals were of the highest quality available from commercial sources.

2.2. Construction of plasmids

The plasmid pG4M-hERαdef (Gal4-HEG0) was a gift from Dr. P. Chambon (IGBMC CNRS-LGME, Illkirch Cedex C.U. de Strasbourg, France). The construction of pG4M-mERβdef has been described elsewhere [41,42]. The plasmid pG4M-rtERαdef was constructed by PCR amplifying amino acid residues 214-576 of the rainbow trout ERα as described previously [43]. The plasmid pG4M-mERαdef (a.a. 268–599) was generated by PCR amplification of the plasmid pJ3MOR containing the complete mouse ERα cDNA (provided by Dr. M.G. Parker, Molecular Endocrinology Research Laboratory, London, United Kingdom) using primers 5′-caaagaaattcatcgattggcggcatacggaaagaccgc-3′ (forward) and 5′-aaaagaattcgcggccgctcagatcgtgttggggaagccctc-3′ (reverse). The plasmid pG4M-cERdef (a.a. 257–599) was constructed by PCR amplification of using primers 5′-aaaactcgagccaaaggtggaatccggaaagac-3′ (forward) and 5′-aaaaagatctttatattgtattctgcatactctcctct-3′ (reverse). The plasmid pG4M-aERdef (a.a. 8–349) was generated by PCR amplification using primers 5′-aaaaggatccctcgagccggtggaattcggaaagaccgcag-3′ (forward) and 5′-aaaaggatccctcgagtcaaattgcttcctgctcatttccc-3′ (reverse), while the plasmid pG4M-xERdef (a.a. 248–586) was constructed by PCR amplification of the plasmid CMV5xER1 containing the complete *Xenopus* ER cDNA (provided by Dr. D. Shapiro, University of Chicago, Urbana, IL) using primers 5′-aaaactcgagccgggggcattcgaaaggatcgca-3′ (forward) and 5′-aaaaggtaccgagctctcatactgtgctttgtaagctcact-3′ (reverse). PCR fragments were digested with the appropriate restriction enzymes and ligated into the eukaryotic expression vector containing the DNA binding domain of the yeast transcription factor Gal4, pG4MpolyII (Dr. P. Chambon). The plasmid pGEXKG-ACTR (NCoA-3, also referred to as AIB1, RAC-3, TRAM-1 and pCIP) (a.a. 615–768) was a gift from Dr. L. Freedman (Memorial Sloan-Kettering Cancer Center, New York, NY), while the plasmid pGEX-TIF2 (NCoA-2, also referred to as GRIP-1) (a.a. 594–766) was constructed by PCR amplification of the plasmid pSG5-TIF2 (Dr. P. Chambon) using primers 5′-caaaggatccgaaggtacaactggacaagcagag-3′ (forward) and 5′-caaactcgagtcaatctgcttactgtccagtctctc-3′ (reverse). PCR fragments were digested with the appropriate restriction enzymes and ligated into pGEX6p3, a GST expression vector (Amersham Pharmacia, Piscataway, NJ).

All PCR amplification was performed as previously described [44]. The sequence of each construct was confirmed

by restriction enzyme digest and ABI/Prism automated sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA).

2.3. Cell culture and transient transfection assays

MCF-7 human breast cancer estrogen receptor positive cells (obtained from Dr. L. Murphy, University of Manitoba, Canada) were maintained in phenol red-free DMEM supplemented with 3.7 g/l $NaHCO_3$, 2 mM L-glutamine, 10% FBS, 10 mM HEPES, 500 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. Cells were cultured in a humidified environment at 37 °C with 5% CO_2.

Transactivation assays were performed using the Gal4-ERdef/17m5-G-Luc chimera in order to investigate whether amino acid variation within the estrogen receptor ligand binding domain among species not only affects relative binding affinity [19,45] but also transactivation activity. The advantages and limitations of the Gal4-ERdef/17m5-G-Luc transactivation assay have been reviewed [46] and its utility previously demonstrated [37,41–43,47]. Briefly, MCF-7 cells were seeded at approximately 50% confluency in six-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to settle for 7 h. MCF-7 cells were transiently transfected by the calcium phosphate precipitation method with (1) 1.5 μg of 17m5-G-Luc (provided by Dr. P. Chambon (IGBMC CNRS-LGME, Illkirch Cedex C.U. de Strasbourg, France), (2) 0.2 μg of either pG4M-hERαdef, pG4M-mERαdef, pG4M-mERβdef, pG4M-rtERαdef or 0.5 μg of pG4M-cERdef, pG4M-cERdef, or pG4M-xERdef, and (3) 0.05 μg of pCMV-lacZ (β-galactosidase expression vector used to control for transfection efficiency between wells) per well. Transiently transfected cells were washed 16 h later with sterile phosphate buffered saline and fresh medium was added to each well.

Transiently transfected cells were exposed to final concentrations ranging from 10^{-12} to 10^{-5} M of test compound dissolved in dimethyl sulfoxide. Final concentrations were obtained by adding 2 μl of test chemical to 2 ml of medium. After 24 h incubation with test compound, cells were harvested and assayed for luciferase and β-galactosidase activity according to standard methods.

Each treatment was performed in duplicate and two samples were assayed from each replicate. Each experiment was repeated three times. Values are reported as a percentage relative to the maximum induction observed with E2.

2.4. Reverse transcriptase polymerase chain reaction

Total RNA from MCF-7 cells was isolated using the Trizol reagent (Life Technologies) according to manufacturer's instructions. In brief, cells were lysed with two successive washes of 325 μl of Trizol. The lysates were pooled and incubated with 300 μl of chloroform for 10 min at room temperature. The mixture was separated by centrifugation at 12,000 × *g* for 15 min at 4 °C and the aqueous layer (1 ml) containing the RNA was removed and the RNA precipitated with 500 μl of isopropanol and 2 μl of linear acrylamide (10 mg/ml). The RNA was pelleted by centrifugation at 12,000 × *g* for 10 min at 4 °C and the resulting pellet was washed with 75% ethanol. The pellet was air dried and resuspended in 100 μl of 3 M sodium citrate. The RNA was stored at −20 °C until use.

Total RNA (5 μg) was incubated for 10 min at 70 °C with 0.5 μg oligo dT primer (5′-ttttttttttttttttttttvn-3′). The reaction was then chilled on ice for 2 min and the mRNA was reverse transcribed in a 20 μl reaction mixture containing PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), 10 mM DTT, 500 μM dNTPs and 200 IU of SuperScript II reverse transcriptase for 60 min at 42 °C. The reaction was terminated with 15 min incubation at 70 °C. A total of 1 μl of the reverse transcription reaction was used in the subsequent PCR reactions using the following primers for β-actin: 5′-aaaagcatccaagcttctgaagtaccccattgaacatggca-3′ (forward) and 5′-aaaactcgaggcggccgctgtcacgcacgatttccctctcag-3′ (reverse), NCoA-1: 5′-caaaccatggatccagacagtaaatactctcaaaccagtc-3′ (forward) and 5′-caaactcgagtcaatcaggctcgacagacaaagtgg-3′ (reverse), NCoA-2: 5′-caaaggatccagaaggtacaactggagaagcagag-3′ (forward) and 5′-caaagaattctcagtgatggtgatggtgatgatctgtcttactgtccagtctctc-3′ (reverse), and NCoA-3: 5′-gaaagtaaggagagcagtgttgag-3′ (forward) and 5′-gtcagaactagtcagatcaccaag-3′ (reverse). After the addition of template, the samples were incubated at 94 °C for 3 min and then amplified for 25 cycles. Each cycle included: 45 s denaturation at 94 °C, 45 s annealing at 60 °C and 1 min elongation at 72 °C. PCR products were separated by 2% agarose electrophoresis and visualized with ethidium bromide staining.

2.5. GST pull-down assays

The expression and purification of GST fusion proteins was performed as described previously [19]. Glutathione–Sepharose beads (Amersham Pharmacia) were prewashed in NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol). Crude bacterial extracts containing the fusion proteins (GST-NCoA-2 and GST-NCoA-3) were purified onto the prewashed beads (10 μl beads +20 μl crude in 500 μl total reaction) by incubation at 4 °C for 1.5 h on a rotary mixer. The beads (loaded with fusion protein) were collected by centrifugation and washed three times with NETN buffer. GST fusion proteins or GST alone were incubated overnight in 1.5 ml centrifuge tubes with 490 μl of NETN buffer containing 1% bovine serum albumin and 5 μl of the in vitro translated ^{35}S-labeled rtERα, in the presence of 5 μl of test compound or vehicle alone. Beads were washed four times with NETN, and dried under vacuum for 10 min. The dried beads were resuspended in 25 μl of 3× protein loading buffer (reducing),

incubated for 5 min at 95 °C, and the entire sample was separated by 12% SDS-PAGE. The gels were fixed, dried, and the ^{35}S-labeled protein was visualized by fluorography. The amount of bound ^{35}S-labeled protein was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Ability of 17β-estradiol to induce gene expression mediated by Gal4-ERdef constructs from several different species

E2 and 14 structurally diverse EEDs, several of which exhibit preferential RBA for select vertebrate ERs [19], were examined for their ability to induce ER-mediated gene expression by measuring treatment-induced luciferase activity in transiently transfected MCF-7 cells. In order to focus on differences in transactivation ability among different vertebrate ERs that reside within their respective ligand binding domains, Gal4-ERdef fusion proteins containing the D, E, and F domains of each receptor were used in order to ensure that differential effects imposed by other domains, such as the DNA binding domain, would not influence the results [48]. Comparable levels of expression in transiently transfected MCF-7 cells were observed with the different constructs ([19] and data not shown). Gal4-ERdef fusion proteins have been previously shown to exhibit comparable E2 transactivation ability when compared to

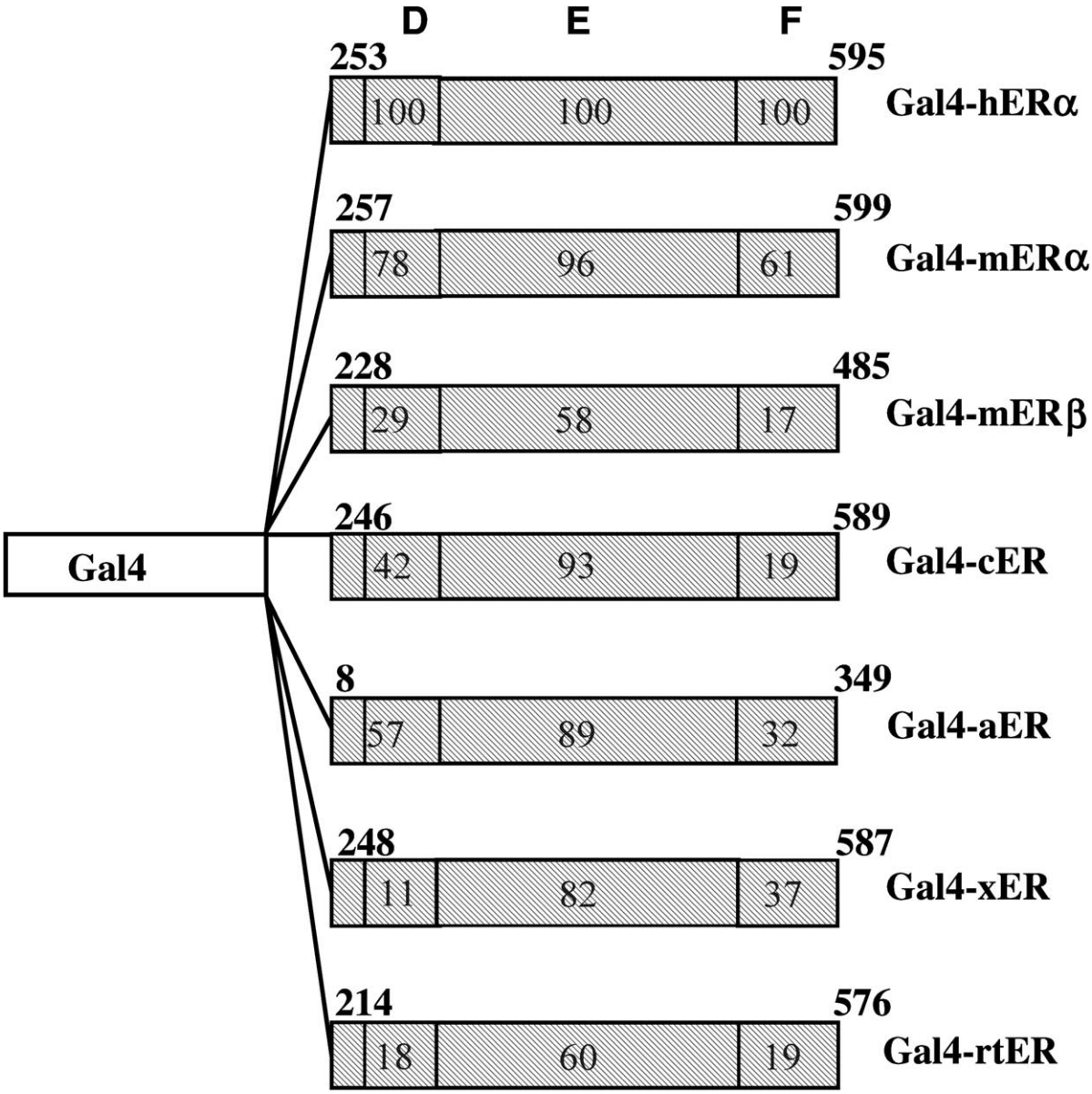

Fig. 2. Schematic representation of Gal4-ER chimeric receptors that were transiently transfected into MCF-7 human breast cancer cells. The chimeric receptors consisted of the DNA binding domain of the yeast transcription factor Gal4, linked upstream of the last 11 amino acid residues of the C domain and the entire D, E, and F domains of ERs from several vertebrate species. Numbers provided above the boundary of the domains identify the amino acids used in the construction of the expression vectors. The numbers within the domains indicate the percent amino acid sequence identity compared to hERα.

full-length ERs [49,50] and the ER domains used are the same as those in the GST-ERdef constructs used to examine RBA differences [19], thus, allowing direct comparison between relative ligand binding and gene expression. The precise amino acid residues of the ER D, E, and F domains used to generate the Gal4-ERdef fusion proteins Gal4-hERαdef (human α), Gal4-mERαdef (mouse α), Gal4-mERβdef (mouse β), Gal4-cERdef (chicken), Gal4-aERdef (anole), Gal4-xERdef (*Xenopus*), and Gal4-rtERαdef (rainbow trout) and a comparison of their sequence identities to hERα are shown in Fig. 2.

E2 treatment was shown to cause a concentration-dependent increase in luciferase activity, as shown in Fig. 3, with EC_{50} values ranging from 0.05 to 0.7 nM among the chimeric receptors. However, the E2-induced response mediated by Gal4-rtERαdef was two orders of magnitude lower, with an EC_{50} value of 28 nM when transfected cells were maintained at 37 °C (Fig. 3A). Interestingly, the EC_{50} value was reduced to 1 nM when the cells were incubated at 20 °C (Fig. 3B), which approaches a more physiological relevant temperature for rainbow trout. Two conservative amino acid residue substitutions that line the hormone binding pocket and interact with E2 have been shown to partially contribute to this temperature sensitive phenotype [43]. Neither the *Xenopus* nor the anole ERs exhibited compromised transactivation ability as evidenced by their ability to induce reporter gene expression at 37 °C when compared to Gal4-hERαdef (Fig. 3A and Table 1).

3.2. rtERαdef interaction with NCoA coactivators

GST pull-down assays were performed in order to determine if differences in the responsiveness of Gal4-rtERαdef to E2 involved an inability to interact with human coactivators. Reverse transcriptase polymerase chain reaction analysis of total RNA isolated from MCF-7 cells revealed that the mRNA expression level of NCoA-3 was approximately 5-fold greater than for NCoA-1 and NCoA-2. GST pull-down assays verified that rtERαdef was capable of interacting with human coactivators (Figs. 4 and 5).

3.3. Ability of EEDs to induce Gal4-ERdef mediated-reporter gene expression

A summary of the EC_{50} values for the 14 EEDs examined is presented in Table 1. The transactivation profiles for these EEDs varied not only in EC_{50} values, but also in the maximum response level achieved at the highest doses examined. For example, the maximum response induced by E2 ranged from 20- to 100-fold among the Gal4-ERdef constructs compared to vehicle treatment alone. Due to the variability in fold induction, the data are reported as percent luciferase activity relative to the maximal activity induced by E2 with each receptor, which was set to 100% for comparative purposes. As a result, the following classification scheme was adopted in Table 1: (1) for EEDs unable to induce 50% of the maximal activation induced by E2, the percent activation at the highest dose (10 μM) was reported, (2) EEDs

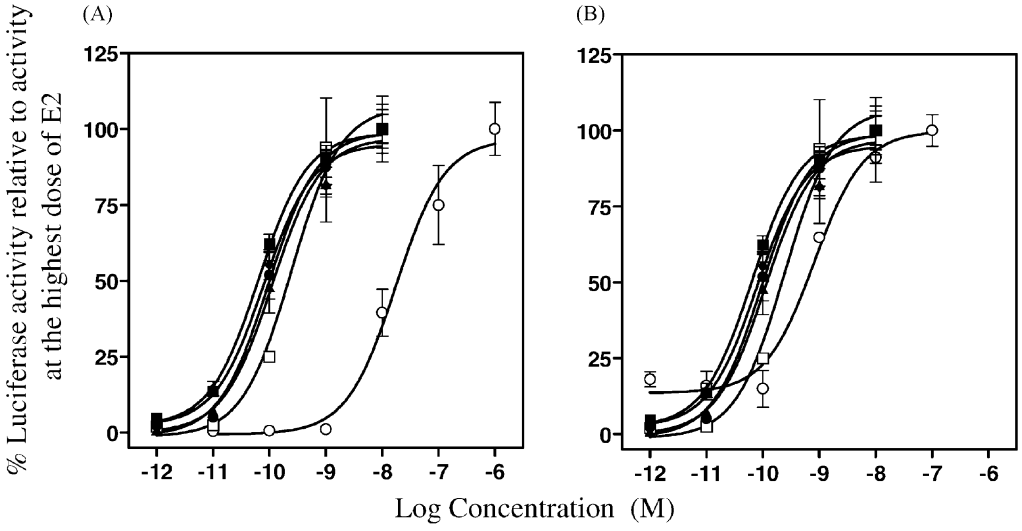

Fig. 3. Comparison of the E2-induced transactivation ability mediated by Gal4-ERdef chimeric receptors at 37 °C and 20 °C. MCF-7 human breast cancer cells were transiently transfected with Gal4-hERαdef (●), Gal4-mERβdef (■), Gal4-rtERαdef (○), Gal4-cERdef (▲), Gal4-aERdef (◆), or Gal4-xERdef (□), as well as 1.5 μg of the Gal4 regulated-luciferase reporter gene, 17m5-G-Luc, and 0.1 μg of pCMV-lacZ as described in Section 2. In (A) all transfected cells were treated with E2 and incubated at 37 °C. In (B) all transfected cells were treated with E2 and incubated at the 37 °C with the exception of cells transfected with Gal4-rtERαdef which were incubated at 20 °C, resulting in the E2-induced Gal4-rtERαdef-mediated dose response curve shifting to the left. Note that the maximum response induced by E2 ranged from 20- to 100-fold among the Gal4-ERdef constructs compared to vehicle treatment alone. Due to the variability in fold induction, the data are reported as percent luciferase activity relative to the maximal activity induced by E2 with each receptor, which was set to 100% for comparative purposes.

Table 1
EC_{50} values of luciferase reporter gene activity mediated by the ERs from several different species following a 24 h exposure to EEDs at 37 °C, unless stated otherwise

	EC_{50} (M)[a]							
	Gal4-hERαdef	Gal4-mERαdef	Gal4-mERβdef	Gal4-cERdef	Gal4-aERdef	Gal4-xERdef	Gal4-rtERαdef (37 °C)	Gal4-rtERαdef (20 °C)
17β-Estradiol	$9.5 \pm 9.2 \times 10^{-11}$	$6.2 \pm 3.5 \times 10^{-11}$	$7.9 \pm 2.7 \times 10^{-11}$	$2.8 \pm 0.8 \times 10^{-10}$	$4.5 \pm 2.6 \times 10^{-11}$	$4.9 \pm 8.4 \times 10^{-10}$	$2.8 \pm 0.8 \times 10^{-8}$	$1.0 \pm 0.5 \times 10^{-9}$
DES	$1.8 \pm 0.7 \times 10^{-10}$	$3.2 \pm 2.6 \times 10^{-11}$	$6.6 \pm 1.2 \times 10^{-11}$	$1.9 \pm 1.0 \times 10^{-10}$	$4.9 \pm 3.8 \times 10^{-11}$	$1.8 \pm 0.8 \times 10^{-10}$	$1.5 \pm 0.8 \times 10^{-8}$	$9.3 \pm 4.3 \times 10^{-10}$
α-Zearalenol	$7.8 \pm 3.2 \times 10^{-10}$	$1.0 \pm 0.1 \times 10^{-10}$	$4.4 \pm 0.6 \times 10^{-10}$	$9.6 \pm 3.3 \times 10^{-10}$	$5.6 \pm 3.2 \times 10^{-9}$	$2.5 \pm 2.1 \times 10^{-9}$	$2.2 \pm 1.2 \times 10^{-8}$	$2.1 \pm 0.7 \times 10^{-10}$
β-Zearalenol	$7.0 \pm 2.6 \times 10^{-9}$	$1.8 \pm 1.5 \times 10^{-9}$	$2.2 \pm 1.0 \times 10^{-9}$	$3.3 \pm 0.5 \times 10^{-8}$	$5.3 \pm 2.7 \times 10^{-8}$	$7.5 \pm 4.0 \times 10^{-9}$	$6.0 \pm 2.9 \times 10^{-8}$	$9.8 \pm 0.1 \times 10^{-9}$
DHT	$3.8 \pm 3.0 \times 10^{-8}$	$7.5 \pm 7.4 \times 10^{-7}$	$1.9 \pm 0.9 \times 10^{-8}$	$1.0 \pm 1.4 \times 10^{-7}$	$2.1 \pm 0.7 \times 10^{-8}$	$4.4 \pm 1.1 \times 10^{-7}$	15%	$1.0 \pm 0.7 \times 10^{-7}$
4-*t*-Octylphenol	$1.0 \pm 1.5 \times 10^{-7}$	$1.1 \pm 1.2 \times 10^{-6}$	$2.5 \pm 0.4 \times 10^{-8}$	40%	50%	30%	20%	$7.1 \pm 3.8 \times 10^{-7}$
Coumestrol	$6.1 \pm 3.7 \times 10^{-7}$	$1.0 \pm 0.5 \times 10^{-8}$	$3.7 \pm 1.8 \times 10^{-9}$	$6.3 \pm 5.1 \times 10^{-7}$	$7.5 \pm 0.6 \times 10^{-8}$	$7.2 \pm 2.8 \times 10^{-7}$	50%	$5.1 \pm 2.1 \times 10^{-7}$
Genistein	$4.0 \pm 4.2 \times 10^{-7}$	$7.6 \pm 2.0 \times 10^{-7}$	$4.2 \pm 0.6 \times 10^{-9}$	$2.0 \pm 1.1 \times 10^{-7}$	$1.5 \pm 0.7 \times 10^{-7}$	$9.8 \pm 5.9 \times 10^{-7}$	$3.3 \pm 0.3 \times 10^{-6}$	$2.3 \pm 1.4 \times 10^{-7}$
BPA	$2.7 \pm 0.2 \times 10^{-6}$	$6.6 \pm 4.5 \times 10^{-7}$	$3.9 \pm 1.2 \times 10^{-7}$	25%	30%	30%	50%	$3.3 \pm 2.6 \times 10^{-7}$
Naringenin	$3.0 \pm 0.2 \times 10^{-5}$	$9.0 \pm 1.2 \times 10^{-6}$	$4.1 \pm 0.8 \times 10^{-7}$	$1.6 \pm 0.8 \times 10^{-5}$	$2.1 \pm 1.6 \times 10^{-6}$	ni	20%	$4.2 \pm 2.5 \times 10^{-6}$
Methoxychlor	wi[b]	wi	wi	ni	ni	ni	ni	ni
o,*p*′-DDT	40%[c]	$2.9 \pm 2.5 \times 10^{6}$	25%	40%	ni	ni	ni	ni
p,*p*′-DDT	wi	nd[e]	ni	ni	ni	ni	ni	ni
β-Sitosterol	ni[d]	nd	ni	ni	ni	ni	ni	ni
Atrazine	ni	nd	ni	ni	ni	ni	ni	ni

[a] EC_{50} values were determined using the sigmoidal dose–response function in GraphPad 3.0 and represent the mean and standard deviation from three independent experiments.
[b] Denotes a weak inducer (wi) since treatment resulted in less than a 20% increase in reporter gene activity.
[c] Refers to percent maximal luciferase activity at the highest dose examined (10 μM).
[d] Denotes non inducer (ni) since less than a 10% induction of reporter gene activity was observed at the highest dose examined (10 μM).
[e] Not defined.

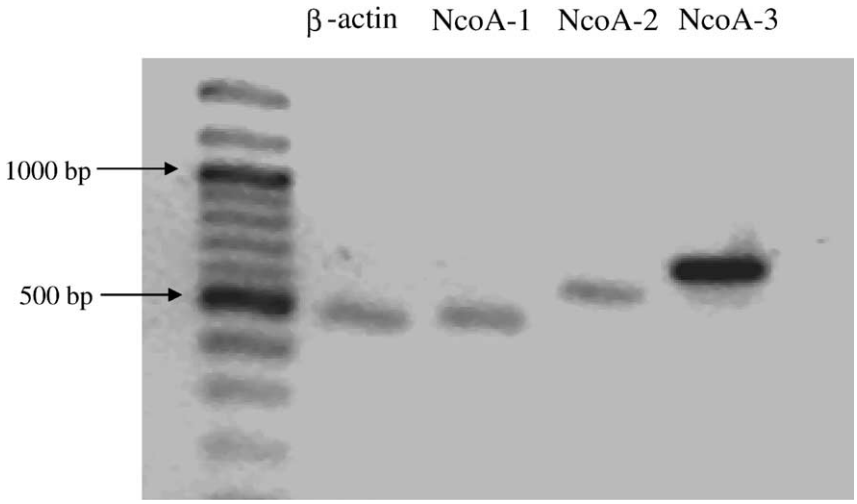

Fig. 4. Differential expression levels of NCoA coactivators in MCF-7 cells. Total RNA from MCF-7 cells was reverse transcribed with an oligo dT primer, followed by a 25 cycle PCR amplification with gene specific primers. The samples were separated on a 2% agarose gel and visualized with ethidium bromide staining.

able to induce only 10–20% of the maximal E2 response were classified as weak inducers (wi), and (3) EEDs that induced <10% of the maximal E2 response were considered non-inducers (ni). Gal4-rtERαdef mediated transactivation studies were conducted at both 37 °C and at 20 °C. EC_{50} values obtained from studies at 20 °C were consistently lower than values obtained at 37 °C.

Overall the potency of E2 induced reporter gene expression was similar among the ERs, with the exception of Gal4- rtERαdef. However, several EEDs exhibited differences in both the rank order of their EC_{50}s and in the absolute transactivation responses to EEDs. DES and E2 exhibited comparable transactivation ability across all Gal4-ERdef species, and in general were the most potent compounds examined. Interestingly, α-zearalenol consistently induced Gal4-rtERαdef-mediated reporter gene expression at lower concentrations than those required by E2 or DES, which was not observed in other Gal4-ERs, but is consistent with the higher RBA of α-zearalenol for GST-rtERαdef [19].

Overall, the rank order of phytoestrogen binding strength was similar among the ERs (coumestrol = genistein ≫ naringenin), though significant differences in potency were observed. An exception, however, was observed with phytoestrogen induction mediated by Gal4-mERβdef. Genistein and naringenin preferentially induced reporter gene expression mediated by Gal4-mERβdef at concentrations 95- and 70-fold lower than that mediated by the other receptors, respectively (Fig. 6). Moreover, coumestrol and genistein were able to induce a full agonist Gal4-mERβdef-mediated response at concentrations comparable to physiological hormone levels (10–100 nM) despite being classified as weak agonists in other assays. In contrast to reports of estrogenic activity [51] but consistent with reported competitive binding results [19], β-sitosterol did not induce reporter gene expression mediated by any of the Gal4-ERdef constructs examined.

Significant differences in potency and efficacy among Gal4-ERdef constructs were also observed for industrial chemicals. Of the compounds examined, 4-*t*-octylphenol was the most potent inducer of luciferase activity with EC_{50}values ranging from 0.01 to 0.1 μM for hERα, mERα, mERβ and rtERα (Fig. 7). Bisphenol A (BPA) EC_{50} values ranged from 0.3 to 3 μM, with the greatest transactivation activity induced by Gal4-mERβdef and Gal4-rtERαdef and minimal induction by the chicken, anole, and *Xenopus* Gal4 constructs. The pesticide *o,p′*-DDT exhibited the most variable potency across species, with reporter gene expression EC_{50} values of 0.8 ± 0.1 and 2.9 ± 2.5 μM observed for Gal4-cERdef and Gal4-mERαdef, respectively. By contrast, only a 40% maximal response was observed with Gal4-hERαdef, and negligible luciferase induction was mediated by Gal4-mERαdef, Gal4-cERdef, Gal4-xERdef and Gal4-rtERαdef (Fig. 7). Methoxychlor, *p,p′*-DDT, and atrazine failed to significantly induce reporter gene expression through any Gal4-ERdef.

4. Discussion

Differences in the relative and absolute binding affinities of several structurally diverse EEDs have been previously reported among ERs from different species. This differential binding has been partially attributed to conservative amino acid sequence substitutions within the ligand binding pocket of ERs from different species [19,43]. Amino acid residue substitutions in other nuclear receptors have also been shown to alter relative binding affinities for selected ligands and to attenuate transactivation ability [52–54]. The present study

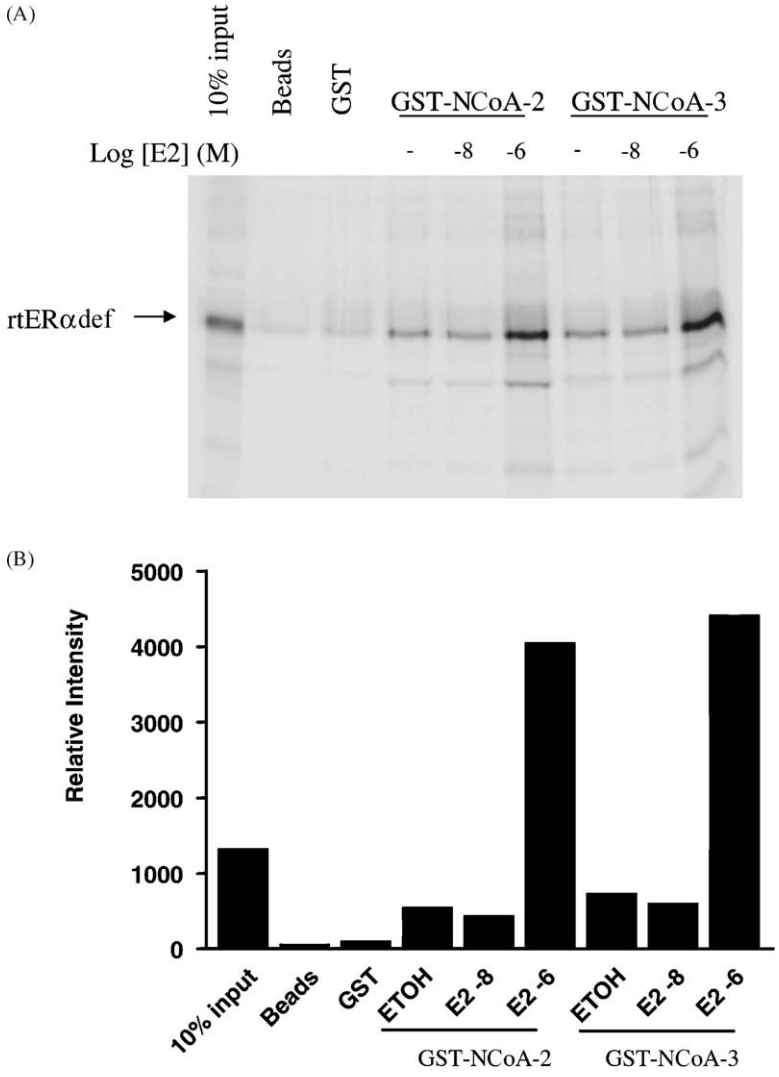

Fig. 5. rtERαdef binds human NCoA-2 and NCoA-3. (A) GST fusion proteins coupled to Sepharose beads were incubated with in vitro translated, [^{35}S]methionine-labeled rtERαdef, in the presence or absence of 17β-estradiol (E2). After extensive washing, samples were separated by 12% SDS-polyacrylamide gels. Gels were fixed, dried and visualized by fluorography. (B) Pixel intensity of the dried gels were quantified using a PhosphorImager.

extends these findings by determining the correlation between RBAs of EEDs to GST-ERdef constructs from various species and the transactivation ability of the corresponding Gal4-ERdefs.

It has been previously reported that the rtERα exhibits temperature sensitive characteristics [36,43]. Although the ability of Gal4-rtERαdef to induce reporter gene expression improved at 20 °C compared to 37 °C, basal luciferase activity was significantly increased at the lower temperature, resulting in an overall reduction in fold induction. The elevated baseline and consequent lower fold induction was also observed when cells transfected with Gal4-hERαdef were incubated at 20 °C compared with 37 °C; however, no significant differences in EC_{50} values were observed [43]. The reduced fold induction observed for the rtERα at the lower temperature may be due to a more stable Gal4-rtERαdef protein that, like other ERs, binds DNA in the absence of ligand. In addition, the lower incubation temperature may result in a larger proportion of ERs occupying the promoter as well as a reduction in the on and off cycling of the ER from the promoter [55], causing greater basal luciferase activity.

Overall the gene expression data correlate with the results of the competitive ligand binding studies [19]. For example, the mycotoxin, α-zearalenol, which bound with greater affinity than E2 to GST-rtERαdef, induced reporter gene expression mediated by Gal4-rtERαdef at lower concentrations than were required for E2. Similarly, the phytoestrogens coumestrol, genistein and naringenin, which exhibited an affinity preference for GST-aERdef [19], also exhibited a Gal4-aERdef-mediated luciferase reporter gene reduction preference when compared to the other Gal4-ERdef constructs. A graph of the log transformed IC_{50} values [19] versus EC_{50} values for the 14 EEDs reveals a Pearson r value of 0.86 and a slope of 0.82, indicating a high degree

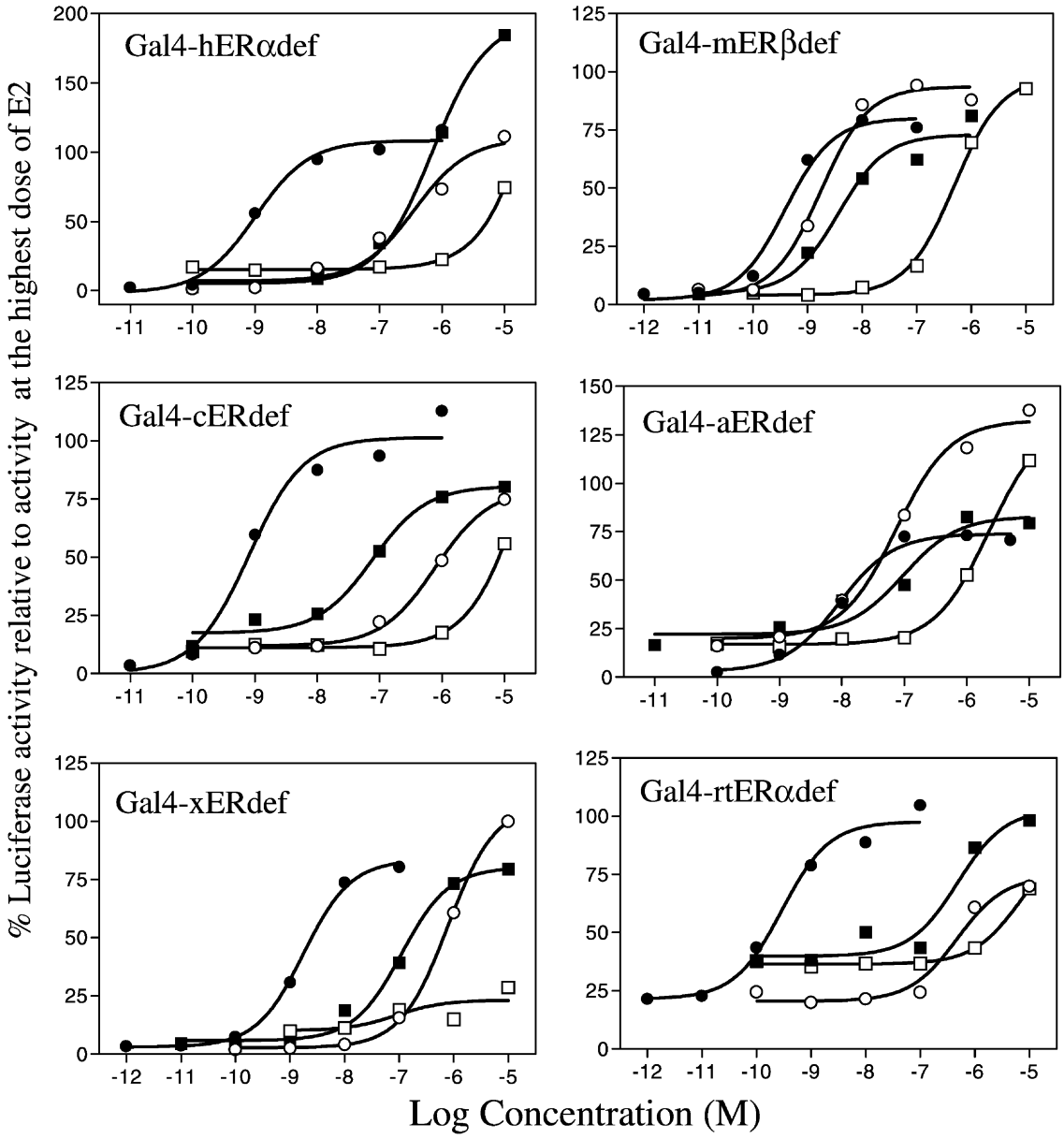

Fig. 6. Mycotoxins and phytoestrogens differ in their ability to induce gene expression mediated by each ER isoform and by ERs from different vertebrate species. This figure summarizes the ability of α-zearalenol (●), coumestrol (■), genistein (○), and naringenin (□) to induce gene expression mediated by Gal4-hERαdef, Gal4-mERβdef, Gal4-cERdef, Gal4-aERdef, Gal4-xERdef, and Gal4-rtERαdef. MCF-7 human breast cancer cells were transiently transfected with 0.2–0.5 μg of Gal4-ERs, 1.5 μg of 17m5-G-Luc, and 0.1 μg of pCMV-lacZ as described in Section 2. Cells transfected with Gal4-rtERαdef were incubated for 24 h at 20 °C after dosing with test compound. Cells transfected with the other Gal4-ER chimeric receptors were incubated for 24 h at 37 °C. Note that the maximum response induced by E2 ranged from 20- to 100-fold among the Gal4-ERdef constructs compared to vehicle treatment alone. Due to the variability in fold induction, the data are reported as percent luciferase activity relative to the maximal activity induced by E2 with Gal4-hERαdef, which was set to 100% for comparative purposes. Standard deviations for points on the graph ranged from 5 to 15% of the mean.

of correlation between ligand binding and transactivation ability mediated by human α, mouse α, chicken, anole and rainbow trout (at 20 °C) Gal4-ERdef constructs (Fig. 8). These results also suggest that fewer occupied receptors are required to induce an EC_{50} response compared to receptor occupancy requirements to inhibit [^{3}H]E2 binding by 50%. Moreover, when examining the RBA and reporter gene induction relationship within a specific species, the correlations exhibited comparable Pearson *r* values, indicating that all GST-/Gal4-ERdef constructs possessed similar relative ligand binding and reporter gene transactivation ability correlations (data not shown).

Despite the strong overall and within species correlations, there were several notable examples of discordance between RBA and transactivation ability between species. In particular, phytoestrogens exhibited an increased ability to induce gene expression mediated by Gal4-mERβdef. However, the magnitude of the differential activity correlated poorly with

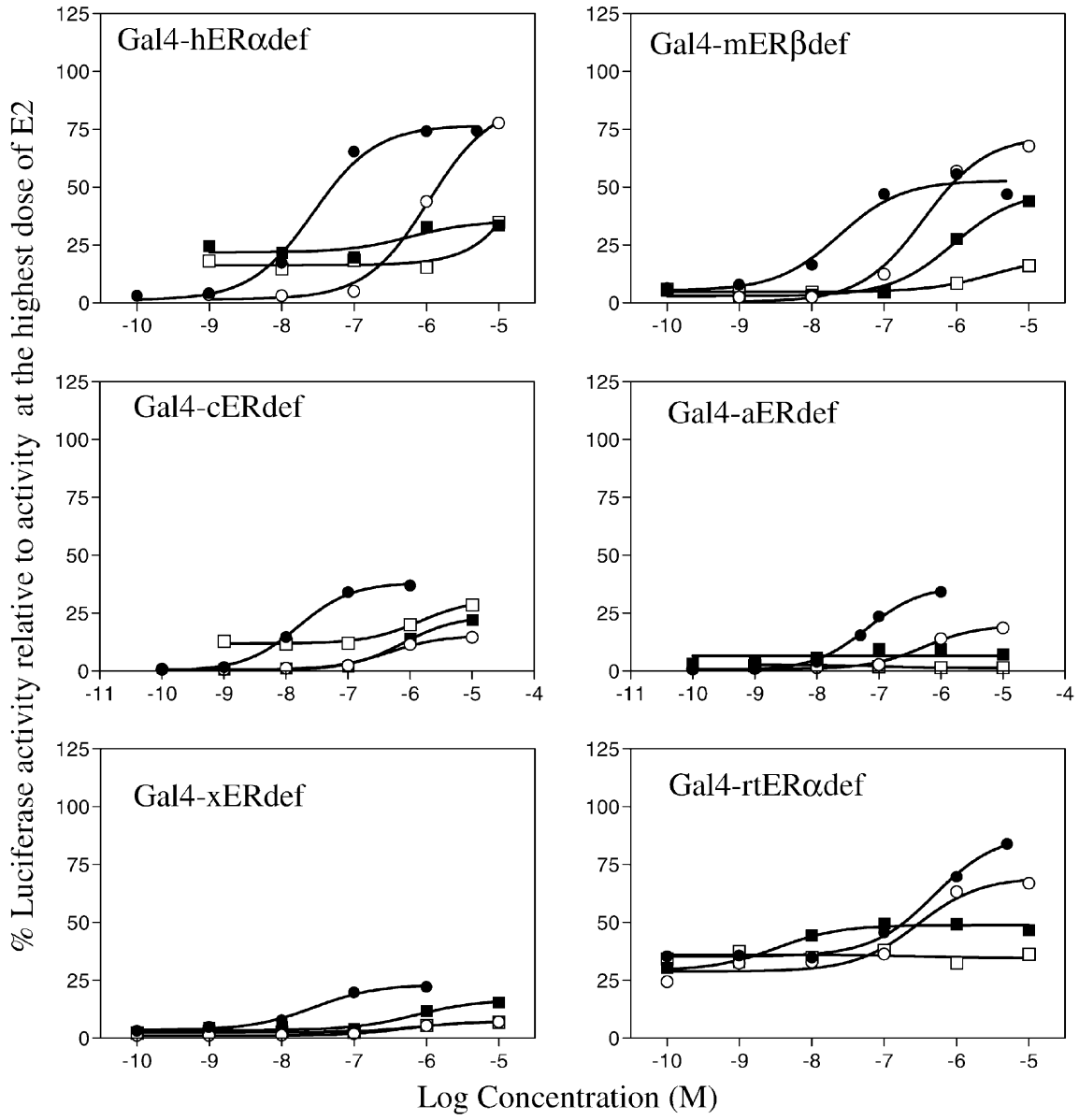

Fig. 7. Xenobiotics differ in their ability to induce gene expression mediated by both ER isoforms and ERs from different vertebrate species. This figure summarizes the ability of 4-*t*-octylphenol (●), bisphenol A (■), *o,p′*-DDT (○), and methoxychlor (□) to induce gene expression mediated by Gal4-hERαdef, Gal4-mERβdef, Gal4-cERdef, Gal4-aERdef, Gal4-xERdef, or Gal4-rtERαdef. MCF-7 human breast cancer cells were transiently transfected with Gal4-ERs as described in Section 2. Cells transfected with Gal4-rtERαdef were incubated for 24 h at 20 °C after dosing with test compound. Cells transfected with the other Gal4-ERdef chimeric receptors were incubated for 24 h at 37 °C. Note that the maximum response induced by E2 ranged from 20- to 100-fold among the Gal4-ERdef constructs compared to vehicle treatment alone [55]. Standard deviations for points on the graph ranged from 5 to 15% of the mean.

the differences observed in RBAs. For example, genistein exhibited a 30-fold greater RBA for ERβ compared to ERα, while there was only a 3-fold lower EC_{50} value for ERβ compared to that of ERα [56]. This is substantially less than the 95-fold lower EC_{50} value of genistein for mERβ compared to that for hERα, as reported in the present study (Table 1). The discrepancy may be due to differences in the cell-based assay conditions, since the previous studies used human embryonic kidney 293 cells transiently transfected with an ERE driven reporter gene, and full length hERα and hERβ [56]. In addition, despite significant improvements in E2 potency at 20 °C mediated by Gal4-rtERαdef, negligible reporter gene induction was observed for xenobiotics, despite the fact that they effectively competed with [^{3}H]E2 for binding to GST-ERαdef. Methoxychlor, *o,p′*-DDT and *p, p′*-DDT had IC_{50} values of 3.5, 0.8 and 2.0 μM, respectively [19], yet were classified as non inducers due to <10% induction of reporter gene activity when compared to the maximal response observed with 10 nM E2. One possible explanation for the discrepancy between RBA and

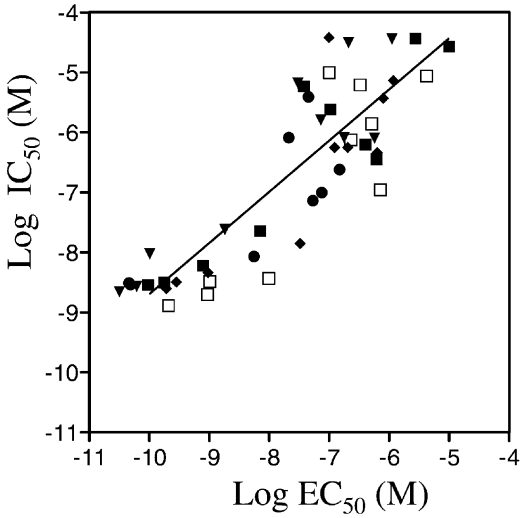

Fig. 8. Comparison of IC_{50} and EC_{50} values among vertebrate ERs. Correlation analysis of the log transformed IC_{50} values [19] vs. EC_{50} values (Table 1) of several EEDs for hERαdef (●), mERαdef (▼), cERdef (■), aERdef (□), and rtERαdef (◆) reveals an overall linear correlation with a Pearson *r* value of 0.86.

transactivation ability may be metabolism of the parent compound. MCF-7 cells have been previously shown to possess a number of metabolic activities (reviewed in [46]), which can significantly alter the activity of a chemical. For example, benzo[a]pyrene and other polyaromatic hydrocarbons have been shown to exhibit estrogenic activity only after hydroxylation at specific sites [41,42,57], while the methoxychlor metabolite, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), can behave both as an ERα agonist and as an ERβ and androgen receptor antagonist [58,59]. Consequently, the lack of correlation between RBA and transactivation ability may be due to metabolic alteration of the parent compound.

Differences in liganded receptor interactions with coactivators may also contribute to discrepancies between RBA and transactivation ability. EEDs bound to ERα and ERβ have been shown to affect the recruitment of coactivator proteins and LXXLL-containing peptides [31–33]. The hydroxylated PCB, 2′,3′,4′,5-tetrachlorobiphenyl, has been shown to exhibit a similar RBA for both ERα and ERβ, and to facilitate SRC1 and 2 coactivator interactions with ERβ, yet failed to initiate recruitment to ERα [33]. Moreover, GST pull-down assays have shown that genistein is more effective at recruiting GRIP1 to ERβ than to ERα [31]. Interestingly, X-ray crystallographic data of the genistein-ERβ complex reveals that the critical helix 12, which contains the AF-2 region, is in an intermediate position between agonist and antagonist conformations, suggesting that the complex may not adequately interact with coactivators [38]. Genistein, however, is reported to cause a 12,000- and 33-fold greater ability of ERβ to recruit NCoA-1 and NCoA-2, respectively, when compared to ERα [33], consistent with the increased ability of genistein to induce ERβ-mediated luciferase reporter gene activity in this study. Differential expression levels of coactivators in cell lines combined with the observation that members of the NCoA family of coactivators interact with nuclear receptors via both the AF-1 and AF-2 regions may also account for some of the discrepancies observed among the different studies. In addition, some of the differences in EC_{50} values, as well as in EED efficacy, may be the result of differential ability of ERs from different species to interact with key coactivators.

Although reporter gene assays have greater sensitivity than competitive binding assays and can provide information regarding the agonist and antagonist activity of the tested chemical, this information is limited to the particular assay and can not be extrapolated to all other responses. It is well established that the gene expression activity of a substance is promoter-, cell-, tissue- and species-dependent (reviewed in [46]). Furthermore, with the ability to monitor the expression of thousands of genes simultaneously using microarrays, studies have shown that E2 and EEDs have complex responses involving both the induction and inhibition of gene expression, thus rendering simple agonist and antagonist ligand designations obsolete [60].

Currently the United States Environmental Protection Agency, as well as European and Asian regulatory agencies, are establishing screening and testing programs for EEDs.[2] Several high throughput approaches such as quantitative structure activity relationships, competitive binding assays, and in vitro reporter gene assays are being considered to assist with the prioritization and screening of the estimated 80–90,000 chemicals in commerce for estrogenic, androgenic and thyroid-like activities. Results from the present study report subtle quantitative differences in reporter gene induction activity among ERs from different species that rarely varied by greater than an order of magnitude when the assays were performed under appropriate conditions (e.g. Gal4-rtERαdef at 20 °C). Furthermore, transactivation ability correlated well with previously reported RBA data [19], indicating that the two assays provide comparable results, although the transactivation assay exhibited greater sensitivity. These results suggest that the use of a single surrogate ER may be sufficient to qualitatively screen chemicals to identify chemicals that warrant further in vivo testing when considered with other short term testing results. Notably, however, previous studies examining correlations between in vitro assays have led to conflicting conclusions [39,61].

More important is the question of how well transactivaction assays predict in vivo biological responses, since it is clear that in vitro assays do not adequately account for pharmacokinetic and pharmacodynamic interactions that occur in animals. Initial studies in rodents [61], rainbow trout ([62], Celius, manuscript in preparation) and *Xenopus* (Huang, manuscript in preparation) indicate a poor correlation between in vitro and in vivo assays, which may be attributed to pharmacokinetic effects, particularly metabolism

[2] http://www.epa.gov/scipoly/oscpendo/, http://endocrine.ei.jrc.it/gedri/pack_edri.All_Page%20.

of the compound. Consequently, although competitive binding and receptor-mediated reporter gene assays may be suitable for screening to identify chemicals that interact with the ER, their utility for determining adverse in vivo responses and assessing risk is questionable, thus, limiting their use in prioritization and screening steps while not drawing conclusions on possible endocrine disrupting activity in the absence of in vivo data. However, other in vitro assays that are more comprehensive, such as microarray assays, may prove to have greater utility and predictive value.

Acknowledgements

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