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Transcriptional Activation of the Human Estrogen Receptor by DDT Isomers and Metabolites in Yeast and MCF-7 Cells

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ABSTRACT. In this study, we determined whether the DDT isomers *p,p'*-DDT [1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane], *o,p'*-DDT [1,1,1-trichloro-2(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane], and their metabolites *p,p'*-DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane], *o,p'*-DDD [1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane], *p,p'*-DDE [1,1,-dichloro-2,2-bis(*p*-chlorophenyl)ethylene], *o,p'*-DDE [1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethylene], and *p,p'*-DDA [2,2-bis(*p*-chlorophenyl)acetic acid], could bind to and transcriptionally activate the human estrogen receptor (hER). Novel results from competitive binding assays showed that *o,p'*-DDD, *o,p'*-DDE, and *p,p'*-DDT, as well as the established environmental estrogen *o,p'*-DDT, were able to bind specifically to the hER with approximately 1000-fold weaker affinities for the hER than that of estradiol. In contrast, only *o,p'*-DDT, but not *p,p'*-DDT, bound to the rat estrogen receptor. Moreover, two yeast expression-reporter systems, constructed to test if the DDT isomers and metabolites could transcriptionally activate the hER, demonstrated that an *o,p'*-DDT metabolite could transactivate the hER or LexA-hER fusion protein with just a 140- to 300-fold weaker potency than that of estradiol. The DDT isomers and metabolites that bound the hER *in vitro* triggered estrogen receptor-mediated transcription of the *lacZ* reporter gene in the yeast systems. Furthermore, the DDT isomers and metabolites that transactivated the hER elicited an additive response when given together or with estradiol. The DDT isomers and metabolites that triggered transcription of the yeast expression-reporter systems also stimulated two estrogenic endpoints in estrogen-responsive MCF-7 cells: the induction of the progesterone receptor and the down-regulation of the hER. Thus, in MCF-7 cells and in yeast expression-reporter systems, certain DDT isomers and metabolites act directly as agonists and transactivate the hER at concentrations found in human tissues. BIOCHEM PHARMACOL 53;8:1161–1172, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. DDT isomers and metabolites; competitive binding; yeast; transcriptional activation; MCF-7 cells; human estrogen receptor

The insecticide DDT is still prevalent in our ecosystem today, even though it was banned in many countries, e.g the United States, in the 1970s. This organochlorine is very chemically stable, lipophilic, and resistant to metabolism [1], explaining its persistence in the environment. Technical grade DDT contains 20% of the *o,p'*-DDT and 80% of the *p,p'*-DDT isomer. *In vivo*, these two isomers are dechlorinated slowly to DDD, DDE, and DDA by cytochrome P450. DDE is the most stable metabolite formed [2].

The DDE metabolite has been found along with DDT in the sera of Americans at concentrations ranging from 2 to 538 ng/mL, i.e. 6.3 to 1690 nM [3]. In adipose tissues, these organochlorine compounds accumulated to levels approximately 1000-fold higher than in sera [4], while in other tissues DDT isomers and metabolites were measured at levels 200- to 300-fold higher than those in sera [5].

The main toxic effects associated with DDT isomers and

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‖ After completion of this article, Angelo C. Notides passed away; this article is dedicated in fond memory to him.

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§ *Abbreviations:* *o,p'*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; *o,p'*-DDD, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; *o,p'*-DDE, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethylene; *p,p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; *p,p'*-DDD, 1,1-dichloro-2,2,-bis(*p*-chlorophenyl)ethane; *p,p'*-DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; *p,p'*-DDA, 2,2-bis(*p*-chlorophenyl)acetic acid; ER, estrogen receptor; hER, human estrogen receptor; ICI, ICI 164,384; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride membrane; ONPG, o-nitrophenyl β-D-galactopyranoside; and ERE, estrogen response element.

metabolites include reproductive, developmental, neurologic, and hepatic alterations [1]. One postulated mechanism of action for DDT is the binding and activation of the ER, leading to the observed perturbations of reproductive functions, including constant estrus, lack of ovulation, and uterotrophic response in immature rats [6]. Early investigations by Bitman *et al.* [7] showed that certain DDT isomers and metabolites were estrogenic. In particular, it was demonstrated that the *o,p'*-DDT isomer, and not the *p,p'*-DDT isomer, bound to the rat ER [8].

Further evidence reveals that DDT, like estradiol may act as a promoter of carcinogenesis *in vivo*. This organochlorine can induce the formation of mammary tumors in male rats pretreated with an initiator. Since male rodents are rather resistant to breast cancer, the induction of mammary tumors may indicate that DDT is a strong promoter [9]. In addition, work done with two different mammary adenocarcinoma cell lines showed separately that: (1) *o,p'*-DDT injection supported tumor growth *in vivo* at a rate comparable to 17β-estradiol [9, 10] and (2) high *p,p'*-DDT or *o,p'*-DDT exposures elicited a proliferative effect on cell division *in vitro* similar to that of estradiol [11].

This body of evidence suggests that DDT isomers and metabolites, the putative environmental estrogens, may be able to transactivate the ER. A yeast hER expression-reporter system has many advantages for testing this hypothesis [12–15]. The fundamental transcriptional machinery is conserved in eukaryotes from yeast to humans. Therefore, the hER produced in yeast can interact with the basic transcriptional machinery of this eukaryote. Thus, it is possible to investigate the expression and function of human transcription factors through reporter expression in yeast cells. Another system commonly used to measure estrogen response is the human mammary MCF-7 cell line. Expressed levels of proteins, such as the progesterone receptor or the estrogen receptor itself, are often used as endpoints for measuring estrogenic response [16–18].

The present study elucidates whether DDT isomers and metabolites can transcriptionally activate the hER, and, if so, how potent the DDT isomers and metabolites are in inducing transcriptional activation. We demonstrated that the DDT isomers and certain metabolites bind the hER *in vitro*, cause specific transcriptional activation of a yeast cellular hER expression-reporter system, and induce estrogenic responses in MCF-7 cells.

MATERIALS AND METHODS

Chemicals

[^{3}H]Estradiol (50 Ci/mmol) was purchased from Dupont–NEN (Boston, MA). The DDT isomers and metabolites were obtained from the Aldrich Chemical Co. (Milwaukee, WI), and HPLC grade DDT isomers and metabolites from AccuStandard (New Haven, CT). The DDT isomers and metabolites include the two isomers of DDT, *o,p'*-DDT and *p,p'*-DDT, and the major metabolites of these compounds, e.g. *o,p'*-DDD, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDA. ICI 164,384, an estrogen antagonist, was donated by Dr. A. E. Wakeling from Zeneca Pharmaceuticals (Mereside, England) and tamoxifen citrate was from Stuart Pharmaceuticals (Wilmington, DE). Glass beads 0.5 mm in diameter, for breaking yeast cell walls, were purchased from Biospec (Bartlesville, OK).

Yeast Strains and Plasmids

The phER expression plasmid was designed by subcloning wild-type hER cDNA from the pUC19 hER plasmid [19] into the *Eco*RI site of pSCW231 [20]. The pSCW231 plasmid was provided by Dr. E. Phizicky, University of Rochester (Rochester, NY). Plasmid YRPE2, from Dr. B. O'Malley, Baylor College of Medicine (Houston, TX), contains two copies of a consensus estrogen responsive element (ERE) that are upstream from a CYC promoter linked to a *lacZ* reporter gene [14]. The yeast strain 939 ([*MATa prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52*]/[*MATα prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52*]) [21] provided by Dr. F. Sherman, University of Rochester, was transformed with phER and YRPE2 plasmids using the one-step transformation method [22]. Successfully transformed strains were then selected by growth on synthetic dextrose minimal medium plates [23] supplemented with leucine.

Another expression plasmid, the pLexA-hER, was constructed by subcloning the cDNA of hER from the pUC19 hER plasmid [19] into the *Eco*RI site of pBTM116. The strain CTY10-5d (*MATa ade2 trp1-901 leu2-3112 his3-200 gal4 gal80 URA3::lexA op-lacZ*) was transformed with pLexA-hER using a one-step transformation method [22]. Both pBTM116 and CTY10-5d were provided by Dr. E. Phizicky (University of Rochester). The transformed yeast was grown on minimal medium plates supplemented with adenine, leucine, histidine, and methionine [23].

Yeast Culture and Media

For the yeast protein extracts, the yeast strains were grown up in YPD (yeast extract, peptone, dextrose) medium. This complex medium consists of 1% Bacto-yeast extract, 2% Bacto-peptone from Difco (Detroit, MI), and 2% glucose. Overnight cultures were grown at 30° on a shaker. For transactivation assays, the yeast cells were grown up in a synthetic dextrose minimal medium containing 0.67% Bacto-yeast nitrogen base without amino acids, and 2% glucose. In addition, the medium for each of the yeast strains was supplemented with the necessary amino acids. The medium for the yeast strain expressing hER contained 100 mg/L leucine, while the medium for the strain expressing LexA-hER contained 100 mg/L leucine and 20 mg/L adenine, histidine, and methionine.

Competitive Binding Assays

Rat uterine whole cell extract containing rat ER was generated by freezing rat uteri in liquid nitrogen and pulverizing them with mortar and pestle. The sample was then

placed on ice, and a Polytron homogenizer was used to further shear the tissue. Protease inhibitors were added to a final concentration of 1 mM leupeptin, 25 μg/mL chymostatin, 25 μg/mL pepstatin, and 300 μM PMSF. This mixture was centrifuged for 20 min at 0° and 27,000 *g*. The supernatant was centrifuged for 35 min at 0° and 159,000 *g*. The high speed supernatant was then precipitated with a 40% final concentration of ammonium sulfate. After 30 min of incubation with the ammonium sulfate, the solution was distributed into microfuge tubes and centrifuged for 10 min at maximum. The supernatants were removed, and the pellets were frozen quickly in a dry ice bath at −79°.

Sf9 insect cells overexpressing the wild-type hER through the baculovirus system [19] were lysed and whole cell extract was obtained as previously described [19]. The high speed cytosol from the cell extract was precipitated with 40% ammonium sulfate. The ammonium sulfate precipitates of both the rat uterine and the Sf9 insect cell extracts were each dissolved in buffer containing 40 mM Tris, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol (DDT), 50 mM KCl, and 10% glycerol. In addition, 10 mg/mL γ-globulin, 0.2 mM leupeptin, and 500 μM PMSF were added. The amounts of ER in the ammonium sulfate precipitates of both the rat uterine and the Sf9 extracts were quantified by radiolabeled hormone binding analysis [19]. A dilution of the extracts containing 3 nM hER was incubated with different concentrations of unlabeled estradiol or DDT isomers and metabolites and 5 nM [^{3}H]estradiol at 4° for 18 hr. Next, 100 μL of 1% charcoal-0.1% dextran in the Tris buffer mentioned above was added to the hER and incubated for 10 min. The charcoal-dextran mixture is able to adsorb free and loosely bound hormone. The tubes were centrifuged, 100 μL of the supernatant was counted, and the [^{3}H]estradiol specifically bound to the hER was determined. Each value represents the mean and range of two determinations. All IC_{50} values were quantified by curve fitting using the logistic function equation [24].

$$y = a/[1 + e^{b\,(x\,-\,c)}] + d,$$

where x is the concentration of unlabeled competitor, and y is the B_c/B_o, or ratio of mean specific binding at a given concentration of competitor (B_c) to the maximum total specific binding value (B_o). The variable a is the range for the y values, b is the slope coefficient, c is the x value at the point of inflection, and d is the minimum mean y value. To find the IC_{50}, y is set to 0.5 and the x value is calculated.

Yeast Protein Extracts and Western Blot

Yeast cells were lysed and protein was extracted as described previously [23]. Briefly, approximately 1.8×10^6 cells were taken from culture and grown in YPD medium to a 0.5 to 1.0 optical density at 600 nm ($O.D._{600}$). They were then washed and centrifuged with 2 mL of ice-cold buffer that contained 50 mM Tris (pH 7.5) and 10 mM NaN_3. The wash was removed, and cells were suspended in 30 μL of a buffer consisting of 2% SDS, 80 mM Tris (pH 6.8), 10% glycerol, 1.5% DTT, and 0.1 mg/mL bromophenol blue. This mixture was transferred quickly to a microfuge tube and heated to 100° for 3 min. Subsequently, 0.1 g of 0.5-mm glass beads was added and vortexed for 2 min. An additional 70 μL of the aforementioned buffer was added, vortexed, and heated to 100° for 1 min. From this step, the extracts were loaded onto a 10% acrylamide gel and resolved by SDS–PAGE by applying 30 mA for 6 hr with tap water cooling. The proteins were transferred onto a PVDF membrane at a constant voltage of 20 V for 12 hr, with cooling, using a Tris-glycine based buffer containing 15% methanol and 0.025% SDS. Then the PVDF membrane was treated with a blocking solution containing 20 mM Tris, 0.5 M NaCl, 0.1% Tween-20, pH 7.4, and 10% nonfat dry milk for 1 hr. Next, the hER was probed by incubating the membrane with a 1:1000 dilution of affinity purified primary antibody 6 [19] (1 mg/mL) in blocking solution for 40 min. After washing the membrane in the Tris-based buffer without added proteins, the membrane was reblocked for 30 min, and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG for 40 min. After extensive washing of the membrane with buffer, the hER bands were visualized by chemiluminescence (Amersham, Skokie, IL).

Transactivation Assays

Yeast cells were grown in minimal medium overnight at 30° until the cells reached a density of 0.4 $O.D._{600}$, and then were distributed into test tubes. Ligands dissolved in dimethyl sulfoxide were added to the test tubes containing 2 mL of the yeast strains transformed with expression-reporter systems, and grown with a chosen ligand for 4–12 hr. The final concentration of solvent was never above 1%. The cell density at $O.D._{600}$ was then measured for each tube, and assayed for β-galactosidase expression [23]. The values for β-galactosidase activity were expressed in Miller units according to the following equation:

$$\text{Miller units} = \frac{1000 \times O.D._{420}}{([t][v][O.D._{600}])}$$

where $O.D._{420}$ is the optical density of the *o*-nitrophenol at 420 nm; t is the elapsed time, in minutes of incubation with the ONPG substrate; v is the volume, in milliliters, of the culture used for the assay; and $O.D._{600}$ is the optical density of 1 mL of the culture used.

The transcriptional activation concentration–response curves were fitted to the following equation [24].

$$y = a/[1 + e^{b\,(x\,-\,c)}] + d$$

where x is the concentration of ligand used, and y is the percent maximal response. The variable a represents the range for the y values, b is the slope coefficient, c is the x value at the point of inflection, and d is the minimum mean y value. From this equation, the EC_{50} was calculated for the

concentration–response curves by setting y equal to 50 and solving for x.

Treatment of MCF-7 Cells with DDT Isomers and Metabolites

MCF-7 cells were plated into 150-mm plates and grown to near confluence in Eagle's modified minimal essential medium, without phenol red (Sigma Chemical Co., St. Louis, MO). This medium was supplemented with 110 mg/L sodium pyruvate, 289 mg/L L-glutamine, 2.2 g/L bicarbonate, 20 μg/mL gentamycin, and 5% bovine calf serum that had been stripped with charcoal–dextran to remove estrogens contained in the medium [25]. The cells were grown under air, 5% CO_2 and 95% humidity at 37°. When the cells were close to confluence, the medium was brought to a 10 μM concentration of the DDT isomers and metabolites or 10 nM estradiol. After incubation for 2 days, the medium was removed, fresh medium was added with the appropriate compounds, and the cells were grown for an additional 2 days. Then whole cell extracts were prepared.

Protein Extraction from MCF-7 Cells

The MCF-7 cells were washed once with 25 mL of ice-cold Hanks' balanced salt solution, scraped into 1.5 mL of hypotonic buffer containing 20 mM HEPES, 1 mM EDTA, 20 mM Na_2MoO_4, 1 mM leupeptin, 25 μg/mL chymostatin, 25 μg/mL pepstatin, and 0.3 mM PMSF. Subsequently, the cells were lysed by freeze–thawing three times in the previously mentioned hypotonic buffer at temperatures of −70° and 25°, respectively [19]. Then the cells were incubated on ice for 30 min after adding 0.5 M NaCl. The extract was centrifuged for 45 min at 78,000 g and 4° to obtain the high speed supernatant.

Assay for Progesterone Receptor Induction

Aliquots (100 μL) of high speed supernatants from MCF-7 cell extracts were incubated in duplicate on ice for 4 hr with 20 nM [^{3}H]R5020 or 20 nM [^{3}H]R5020 plus 2 μM radioinert progesterone for the experiment shown in Fig. 6. Similarly, for the experiment displayed in Fig. 7, 100-μL aliquots of MCF-7 whole cell extracts were incubated with 2 nM [^{3}H]R5020 with or without 800 nM unlabeled progesterone. Free hormone was removed by adding a final concentration of 0.3% charcoal–0.03% dextran to the samples. The charcoal–dextran solution can remove free hormone from the mixture. The radioactivity of the bound receptor was determined by scintillation counting. Specifically bound counts were determined by subtracting the counts for radiolabeled R5020 with unlabeled progesterone from the counts with labeled R5020 alone.

Down-Regulation of the ER by DDT Isomers and Metabolites

Twenty-microliter samples of high speed supernatants from MCF-7 cell extracts were denatured by the addition of 10 μL of SDS sample buffer, containing 0.35 M Tris, pH 6.8, 10.3% SDS, 30% glycerol, 9.3% DTT, and 0.012% bromophenol blue. The samples were heated subsequently at 100° for 5 min and resolved on a 10% acrylamide gel for 6 hr, and a western blot analysis was carried out with the anti-hER antibody 6 [19]. The intensity of the hER protein bands from the western blot was compared by computer scanning.

Statistical Analysis

The data for each experiment are expressed as means ± SEM (or range if only two samples were used). Statistical significance was determined by conducting one-way ANOVA using the Tukey test.

RESULTS

Competition of DDT Isomers and Metabolites for Binding to Recombinant hER

An *in vitro* competitive binding assay using recombinant hER, [^{3}H]estradiol, and unlabeled estradiol or DDT isomers and metabolites showed binding of the *p,p'*-DDT and *o,p'*-DDT metabolites to the hER (Fig. 1B). The relative affinities of *o,p'*-DDT, *o,p'*-DDD, *o,p'*-DDE, and *p,p'*-DDT for the receptor were similar. *p,p'*-DDA was the only DDT metabolite tested in this study that did not have any affinity for the hER. In contrast, when a competitive binding assay was conducted with uterine rat ER (Fig. 1A), only the *o,p'*-DDT, and not the *p,p'*-DDT bound to the receptor. This was in agreement with previous work with rodent and avian models [6, 7].

For the rat ER, the IC_{50} for estradiol was 65.4 nM, whereas that of *o,p'*-DDT was 275 μM. These IC_{50} values indicate that *o,p'*-DDT has approximately a 4000-fold weaker affinity for binding to the rat ER than does estradiol. The hER showed an IC_{50} of 25.6 nM for estradiol and an IC_{50} of 29.9 μM for *o,p'*-DDT; from these values, it appears that for the hER *o,p'*-DDT has an affinity that is about 1000-fold weaker than estradiol.

Expression of hER in Yeast

Two expression-reporter systems were constructed in yeast to explore potential transcriptional activation of the hER by DDT isomers and metabolites. One system expressed hER and the other expressed LexA-hER. The hER expression-reporter system produced recombinant hER that bound two EREs upstream of a *lacZ* reporter (Fig. 2A). This strain transformed with phER and plasmid YRPE2 allows quantification of both the DNA binding and transcriptional activation function of the hER, since the reporter is estrogen-induced and binds its own response element.

To determine whether the hER was being expressed correctly, a western blot of extracts from the phER transformed yeast was incubated with anti-hER antibody. The western blot confirmed the presence of the hER, a 66-kDa protein.

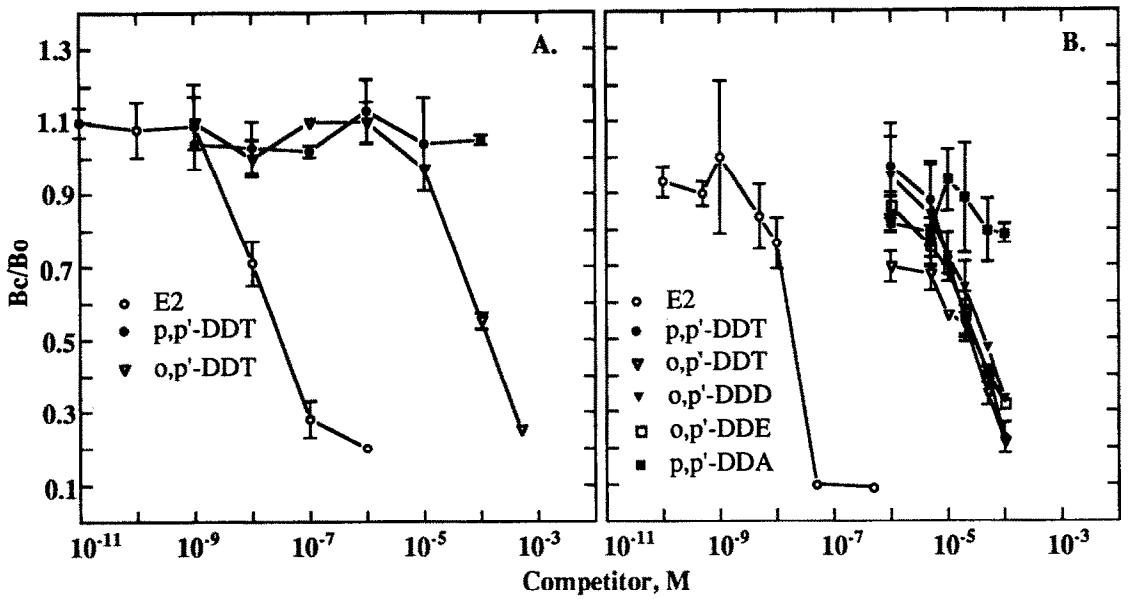

FIG. 1. Competitive binding of the DDT isomers and metabolites to the rat estrogen receptor (A) and the recombinant hER (B). A 3 nM concentration of either rat estrogen receptor from rat uterine cell extracts or the baculovirus expressed recombinant hER from Sf9 extracts was incubated with 4 nM [^{3}H]estradiol and the indicated concentrations of unlabeled estradiol (E2), or DDT isomers and metabolites for 18 hr at 0°. The [^{3}H]estradiol bound to ER was measured by treating the samples, in duplicate, with charcoal–dextran. A single representative experiment is displayed. The B_c/B_o was obtained by dividing the mean specific binding value at each given concentration of competitor (B_c) by the maximum total specific binding value (B_o). The range for two samples is shown by the error bars.

This protein was specific for the strain expressing hER, since it was not present in the yeast extracts, which had the reporter plasmid and the expression plasmid without the hER cDNA (data not shown).

The other yeast expression-reporter system produced a fusion protein, LexA-hER, that bound four LexA binding sites upstream of a *lacZ* reporter gene in the chromosome of the strain (Fig. 2B). In this strain expressing LexA-hER, the transcriptional activation is measured apart from the DNA binding function of the hER, since the fusion protein binds to LexA sites, not EREs, on the DNA.

To show that yeast cells with pLexA-hER were expressing the full-length fusion protein, we probed whole cell extracts from this strain on a western blot with an anti-hER antibody. A band of 89-kDa was present in the yeast expressing LexA-hER, and not in yeast extracts expressing LexA alone. The 89-kDa band observed represents the fusion protein LexA-hER that possesses both LexA (23 kDa) and hER (66 kDa) domains (data not shown).

Transcriptional Activation of hER by DDT Isomers and Metabolites in Yeast

Transcriptional activation assays were conducted using strains containing either hER or LexA-hER. Increasing concentrations of either estradiol or DDT isomers and metabolites were incubated with the strains expressing hER or LexA-hER overnight. These cells were then treated by the method of Kaiser *et al.* [23] and a concentration–response curve was obtained.

For the yeast strains containing the phER, the maximal transactivation was with 100 nM estradiol (Fig. 3A). The average value and standard error for transcriptional activation at this concentration was 4020 ± 335 Miller units. The

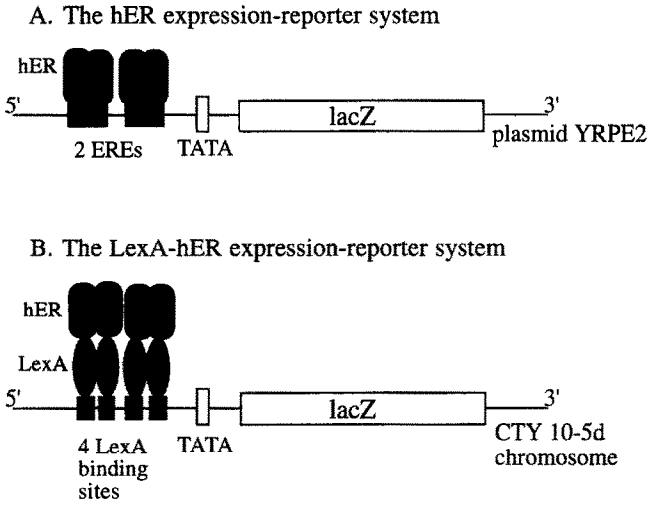

FIG. 2. Two hER expression-reporter systems. The 939 strain was transformed with the plasmid phER and plasmid YRPE2 (A). The plasmid phER is an expression vector producing the hER, and plasmid YRPE2 contains two ERE sites upstream of the *lacZ* reporter gene. Transcriptional activation in LexA-hER expression-reporter system (B). The CTY10-5d yeast strain was transformed with plasmid pLexA-hER. This plasmid produces the LexA-hER fusion protein. Integrated into its chromosome, the CTY10-5d has the *lacZ* reporter gene downstream of four LexA binding sites.

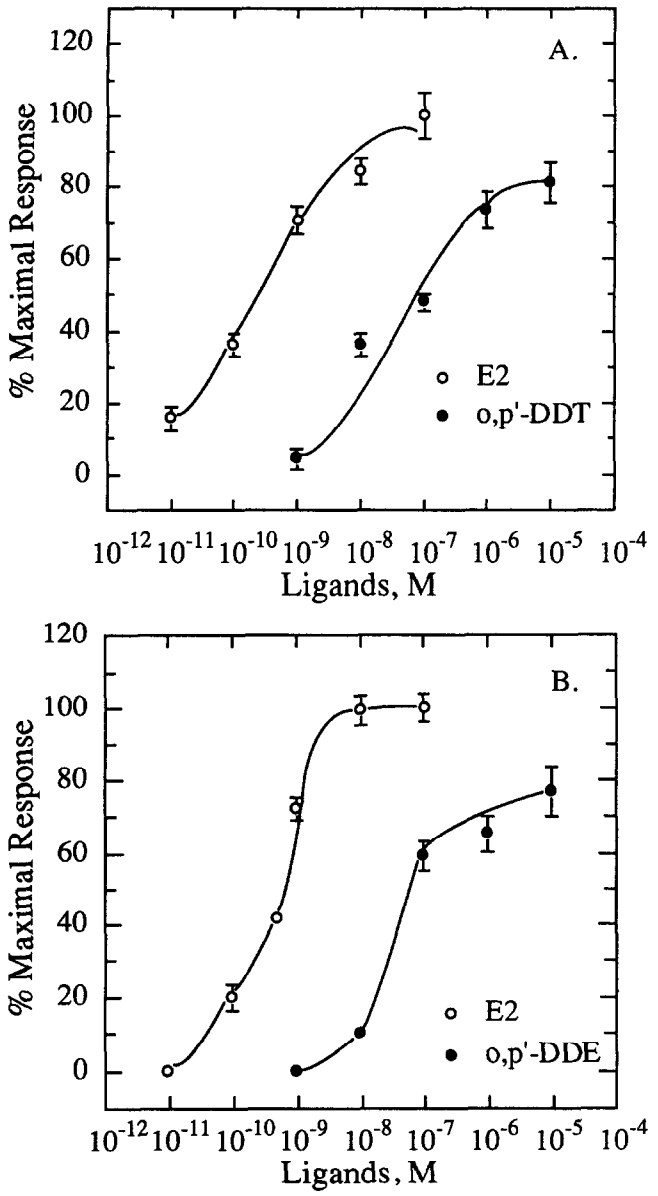

FIG. 3. Transactivation of the hER in yeast with estradiol (E2) and *o,p'*-DDT (A). Transactivation of the LexA-hER in yeast with estradiol (E2) and *o,p'*-DDE (B). The yeast strains expressing either hER or LexA-hER were grown for 12 hr with one of the specified ligands at the concentrations noted. The cells were lysed, and transcriptional activation mediated by either the hER or the LexA-hER was measured by the β-galactosidase assay. The percent maximal response was obtained by dividing the activities, expressed in Miller units, by the maximal response obtained with estradiol-treated (100 nM) cells. These data are expressed as the means ± SEM for 4–6 samples from two separate experiments.

EC_{50} value for estradiol was 0.60 nM, while that for *o,p'*-DDT was 193 nM. Therefore, *o,p'*-DDT is only about 320-fold less potent in triggering the transcriptional activation of the ER than estradiol.

The concentration–response of the strain expressing LexA-hER showed that the maximum transactivation with estradiol was approximately 200-fold higher than the background (Fig. 3B). The EC_{50} value for estradiol was 0.67 nM, while that of *o,p'*-DDE was 91 nM, so DDT isomers and metabolites were approximately 140-fold less potent than estradiol in this LexA-hER expressing yeast.

For the strain containing phER, *o,p'*-DDT, *o,p'*-DDE, *o,p'*-DDD, and *p,p'*-DDT were able to activate the hER at 1 μM (Table 1); however, *p,p'*-DDE and *p,p'*-DDA did not activate the receptor. Furthermore, estrogen antagonists, such as ICI 164, 384 and tamoxifen citrate were able to partially block the transactivation response. A concentration of 0.5 nM estradiol plus 100 nM ICI 164, 384 gave a response of 18.4%, as opposed to the response of 43.6% for estradiol alone. Tamoxifen citrate at 100 nM was able to partially inhibit the 0.5 nM estradiol response to 36.4%. The percent activities in all the transactivation assays were expressed relative to the response for 100 nM estradiol that yielded a maximal or 100% response. Ligands for other steroid hormone receptors, such as testosterone, dihydrotestosterone, and dexamethasone, did not activate the hER.

Similarly, ER antagonists and DDT isomers and metabolites were tested on the LexA-hER system to identify whether transcriptional activation was specific for the hER (Table 2). A 1 μM concentration of the estrogen antagonist, ICI 164, 384, was able to inhibit the activation of 1 nM estradiol by about 33%. In addition, dexamethasone and R5020, ligands specific for the other steroid receptors, did not activate the LexA-hER. The *o,p'*-DDT, *o,p'*-DDE, *o,p'*-DDD, and *p,p'*-DDT compounds were able to tran-

TABLE 1. Transactivation of the hER in the 939 yeast strain with the DDT isomers and metabolites and selected ligands

Ligand	Concentration (nM)	% Activity*
Control	0	0
Estradiol	100	100 ± 11
Estradiol	0.5	43.6 ± 1.1
ICI	100	0.3 ± 0.3
Estradiol + ICI	0.5 + 100	18.4 ± 1.5
Tamoxifen	100	0
Estradiol + Tamoxifen	0.5 + 100	36.4 ± 1.7
Testosterone	100	1.4 ± 0.3
Dihydrotestosterone	100	3.4 ± 1.0
Dexamethasone	100	0
o,p'-DDT	50	39.6 ± 1.7
o,p'-DDT	1000	65.5 ± 3.0
o,p'-DDE	1000	67.5 ± 6.0
o,p'-DDD	1000	58.1 ± 5.0
p,p'-DDT	1000	49.5 ± 2.0
p,p'-DDE	1000	0
p,p'-DDA	1000	0

The yeast cells expressing hER were grown with the indicated ligands at the concentrations noted. Agonists for other steroid hormone receptors, such as dexamethasone, testosterone, and dihydrotestosterone, were tested in addition to estrogen agonists. ICI 164, 384 (ICI) and tamoxifan citrate (tamoxifan), estrogen antagonists, were also used. After 12 hr of incubation, the cells were lysed and transactivation was measured by the β-galactosidase assay.

* Percent activity was obtained by dividing the mean ± SEM activity for 3–6 samples, expressed in Miller units, by the maximum value for estradiol (100 nM).

TABLE 2. Transactivation of the LexA-hER fusion protein in the CTY10-5d yeast strain with the DDT isomers and metabolites and selected ligands

Ligand	Concentration (nM)	% Activity*
Control	0	0
Estradiol	100	100 ± 10
Estradiol	1	98 ± 3
ICI	1000	0
Estradiol + ICI	1 + 1000	65 ± 7
R5020	1000	0
Dexamethasone	1000	0
o,p'-DDT	1000	110 ± 3
o,p'-DDE	1000	101 ± 10
o,p'-DDD	1000	105 ± 3
p,p'-DDT	1000	97.7 ± 6.9
p,p'-DDE	1000	0
p,p'-DDA	1000	0

The strain expressing LexA-hER was grown with the indicated ligands at concentrations sufficient to induce transcriptional activation. In addition, agonists for other steroid hormone receptors, such as dexamethasone and R5020, were tested. ICI 164,384 (ICI), an ER antagonist, was also used. After 12 hr of incubation, the cells were lysed, and transactivation was measured by the β-galactosidase assay.

* Percent activity was obtained by dividing the mean ± SEM values for 3–6 samples, expressed in Miller units, by the maximum value obtained with estradiol (100 nM).

scriptionally activate the hER, while *p,p'*-DDE and *p,p'*-DDA metabolites were not. The compounds that activated transcription gave a maximal response at concentrations just 100-fold higher than the estradiol concentration used to induce maximal response. These yeast data corroborate results of the competitive binding assays, since the same metabolites of DDT that bind the hER are able to transactivate the receptor.

Additive Transcriptional Activation Response of DDT Isomers and Metabolites

Since organisms are not exposed to environmental estrogens alone, but in mixtures with other estrogens, we were interested in learning whether DDT isomers and metabolites plus estradiol, or plus other DDT isomers and metabolites, could elicit an additive transactivation response. Yeast cells expressing LexA-hER were incubated with 100 nM estradiol, 0.3 nM estradiol, or 40 nM *o,p'*-DDT. In addition, 0.3 nM estradiol and increasing concentrations, i.e. 40 nM, 100 nM, or 1 μM, of the specified DDT isomers and metabolites were given to this transformed yeast strain (Fig. 4). The combination of 0.3 nM estradiol plus 40 nM *o,p'*-DDT yielded a response that was not statistically different from the sum of the separate responses observed with 0.3 nM estradiol or 40 nM *o,p'*-DDT alone. The responses for 100 nM estradiol, 0.3 nM estradiol, and 40 nM *o,p'*-DDT were 100, 21.5, and 23.4%, respectively. With 0.3 nM estradiol plus 40 nM, 100 nM or 1 μM *o,p'*-DDT, the transcriptional response also increased in increments. Similar responses were observed for estradiol plus increasing concentrations of *o,p'*-DDE or *p,p'*-DDT. Thus, the transcriptional response of the yeast incubated with DDT isomers and metabolites that activated the hER, e.g. *o,p'*-DDT, *o,p'*-DDE, and *p,p'*-DDT, was additive with that of estradiol. Furthermore, these data are consistent with the interpretation that DDT isomers and metabolites act as agonists, even in the presence of estradiol, and transactivate the hER by the same mechanism as estradiol.

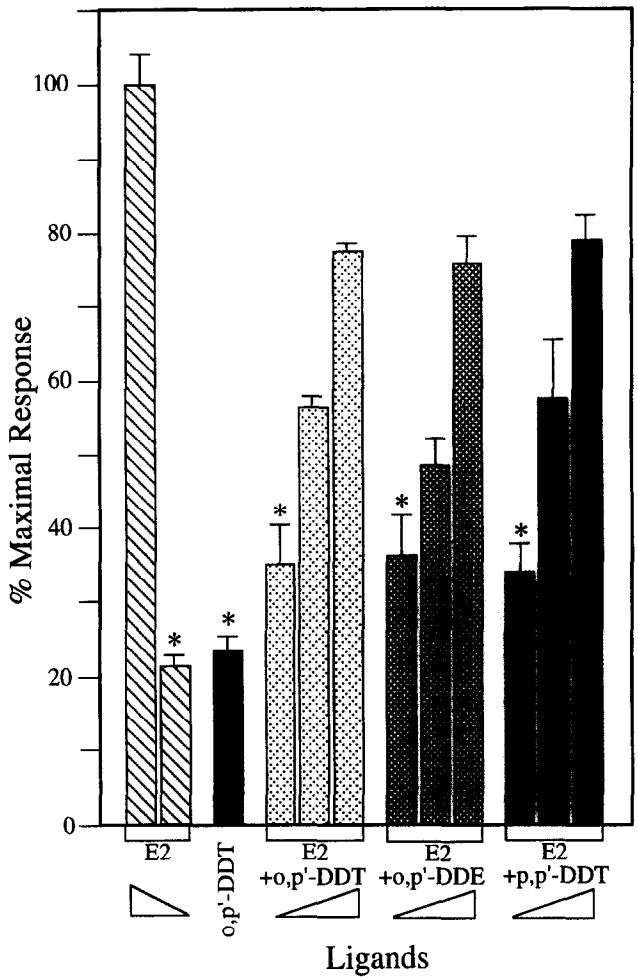

FIG. 4. Transactivation of the LexA-hER in yeast by the combination of estradiol plus a DDT isomer or one of its metabolites. The strain expressing LexA-hER was incubated with 100 or 0.3 nM estradiol (E2), or 40 nM *o,p'*-DDT (concentrations for the first three responses, respectively). In addition, a constant concentration of 0.3 nM estradiol was incubated plus increasing concentrations, i.e. 40 nM, 100 nM, and 1 μM, of the specified DDT isomers and metabolites. After 12 hr of growth, the yeast cells were lysed, and transactivation was measured by the β-galactosidase assay. One hundred nanomolar estradiol gave a maximal (100%) response. The percent maximal response was obtained by dividing the responses for specific concentrations of ligand by the maximal response obtained with estradiol. Data (mean SEM, N = 3) are shown from a representative experiment. Key: (*) the percent maximal responses were not significantly different ($P > 0.05$) from the sum of percent maximal response for 0.3 nM estradiol alone plus 40 nM *o,p'*-DDT alone.

An analogous experiment was conducted with 40 nM

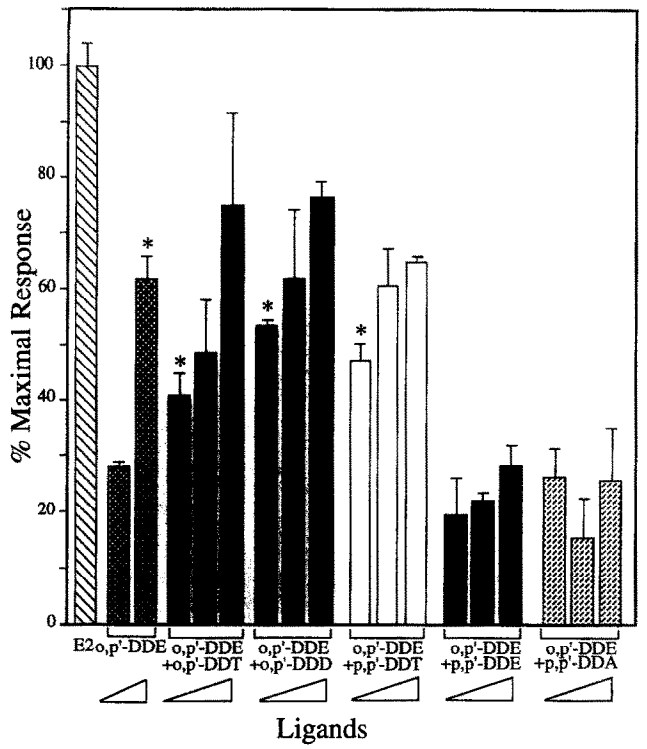

FIG. 5. Transactivation of the LexA-hER in yeast by the combination of one DDT metabolite plus another. One hundred nanomolar estradiol or 40 or 80 nM *o,p'*-DDE was incubated with the yeast (concentrations for the first three responses, respectively). In addition, a constant concentrations, 40 nM, of *o,p'*-DDE was given to cells with increasing concentrations, 40 nM, 100 nM, or 1 μM, of a select DDT isomer or metabolite. After 12 hr of growth, the yeast cells were lysed, and transactivation was measured by the β-galactosidase assay. The concentration of 100 nM estradiol (E2) elicited a 100% response. A representative experiment is shown, where three samples were tested for each pair of DDT isomers or metabolites. The percent maximal response was obtained by dividing the response for a specific concentration of ligand by the maximal response obtained with estradiol. Data are shown for a representative experiment, by mean ± SEM, where N = 3. Key: (*) the % maximal responses were not significantly different ($P > 0.05$) from that for 80 nM *o,p'*-DDE alone.

o,p'-DDE plus 40 nM, 100 nM, or 1 μM concentrations of other DDT isomers and metabolites (Fig. 5). Yeast cells expressing LexA-hER were given 40 and 80 nM *o,p'*-DDE alone to serve as controls, which yielded responses of 27.9 and 61.9%, respectively. The DDT isomers and metabolites that activate the receptor, i.e. *o,p'*-DDE, *o,p'*-DDT, *o,p'*-DDD, and *p,p'*-DDT, all transcriptionally activated the receptor in an additive, concentration-dependent manner, when incubated in combination with 40 nM *o,p'*-DDE. The treatments of 40 nM *o,p'*-DDE plus 40 nM *o,p'*-DDT, *o,p'*-DDD, or *p,p'*-DDT gave responses that were not significantly different from the control of 80 nM *o,p'*-DDE alone. The addition of *p,p'*-DDE or *p,p'*-DDA, compounds that do not activate or bind to the hER, with the *o,p'*-DDE did not induce a greater signal than that of *o,p'*-DDE alone.

Progesterone Receptor Induction by DDT Isomers and Metabolites in MCF-7 Cells

To determine whether the DDT isomers and metabolites were estrogenic in human cells, progesterone receptor induction and estrogen receptor down-regulation were measured in MCF-7 cells. The progesterone receptor level was measured by binding to [^{3}H]R5020, a progesterone receptor specific ligand. In Fig. 6, it is apparent that progesterone receptor was not detectable when vehicle alone was given to the cells. However, the cells demonstrated an increase in progesterone receptor up to 87 fmol/mg when treated with 10 nM estradiol. Progesterone receptor induction in response to 10 μM DDT isomers or metabolites was expressed relative to that of estradiol (100% induction). The following levels were observed: 43.7% for *p,p'*-DDT, 109% for *o,p'*-DDT, 73.6% for *o,p'*-DDD, 74.7% for *o,p'*-DDE, and 0% for *p,p'*-DDA. With respect to estrogenicity, the relative order of potency of the DDT isomers and metabolites was: *o,p'*-DDT > *o,p'*-DDD ≥ *o,p'*-DDE > *p,p'*-DDT, with *o,p'*-DDT having a potency similar to that of 10 nM estradiol, and *p,p'*-DDA not eliciting any response.

MCF-7 cells were treated with different concentrations of *o,p'*-DDT to ascertain whether there was a concentration-dependent increase in the progesterone receptor (Fig.

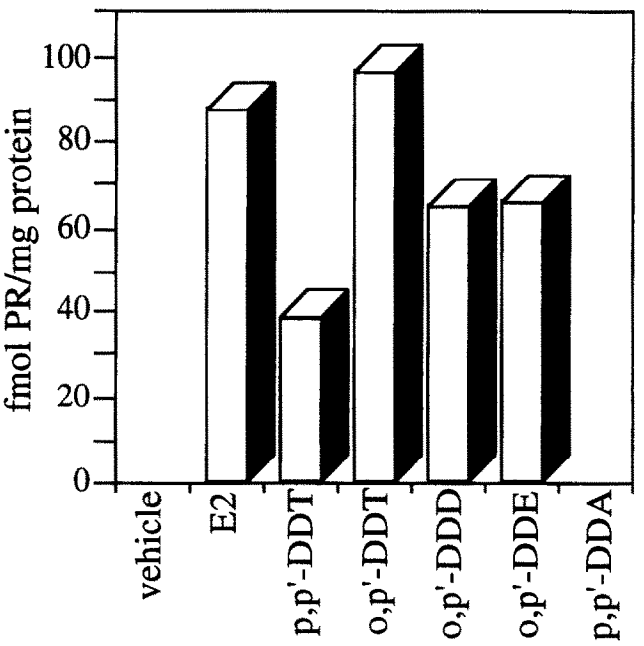

FIG. 6. Progesterone receptor (PR) induction by DDT isomers and metabolites in MCF-7 cells. The MCF-7 cells were grown to near confluence in MEM plus 10% bovine calf serum treated with charcoal–dextran. The medium was then brought to a concentration of 0.1% ethanol (vehicle), 10 μM DDT isomers and metabolites, or 10 nM estradiol (E2). After 3 days of exposure to the ligands, MCF-7 cell protein extract was obtained, and a receptor binding assay was conducted. Data are shown from a representative experiment, where N = 2.

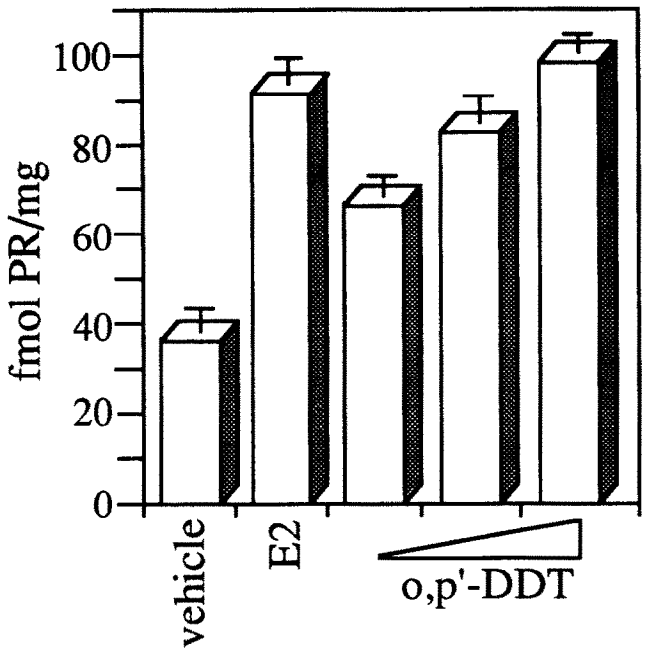

FIG. 7. Concentration-dependent progesterone receptor (PR) induction by *o,p'*-DDT in MCF-7 cells. The MCF-7 cells were grown for 3 days in the same conditions stated above in the legend of Fig. 6. In the medium, a final concentration of 0.1% ethanol (vehicle), 10 nM estradiol (E2), or 10 nM, 100 nM, or 1 μM *o,p'*-DDT was then given to the cells. Following 4 days of exposure to the ligands, whole cell extracts were prepared, and a receptor binding assay was conducted. Values are means ± SEM, N = 4.

7). With exposure to 10 nM, 100 nM, or 1 μM *o,p'*-DDT, there was a concomitant increase of 69.0, 79.9, and 102.0 fmol/mg in the level of progesterone receptor. Cells treated with 10 nM estradiol produced 95.2 fmol/mg progesterone receptor, an amount similar to that of cells exposed to 1 μM *o,p'*-DDT. This level of induction was 3-fold higher than the level for vehicle-treated cells.

Down-Regulation of the ER by DDT Isomers and Metabolites in MCF-7 Cells

In agreement with the results for progesterone receptor induction, ER down-regulation measured by western blot was observed after treatment of MCF-7 cells with DDT isomers and metabolites for 3 days (Fig. 8). Measurement of estrogen down-regulation is an endpoint for exposure to estrogens. The down-regulation of hER corresponded to the potencies of DDT isomers and metabolites for progesterone receptor induction, that is estradiol-treated cells exhibited a 71.2% reduction in hER level as compared with the control (100% response), whereas *p,p'*-DDT, *o,p'*-DDT, *o,p'*-DDD, and *o,p'*-DDE gave reductions of 24.6, 46.1, 37.8, and 32.4%, respectively. As expected, the signal for *o,p'*-DDT was closest to that of estradiol, and the signal for *p,p'*-DDA was similar to that of vehicle alone, indicating that the *o,p'*-DDT was the most potent estrogen, as opposed to *p,p'*-DDA, which was not an estrogen.

DISCUSSION

Although DDT was banned in the U.S. and many other countries more than 20 years ago, significant levels are still found today in the blood and adipose tissue of people in these countries [4, 26]. The prevalence of DDT is probably due to its properties, i.e. chemical stability, excellent lipid solubility, and resistance to metabolism. As a result of the continued risk of exposure to this organochlorine, it is important to determine possible deleterious effects of DDT, such as its estrogenicity. Potentially, the molecular mechanism of action of DDT may serve as a model for other important organochlorines that are putative environmental estrogens, such as certain insecticides, polychlorinated biphenyls, and plastics [27–29].

Our present studies agree with previous studies in which *o,p'*-DDT, but not *p,p'*-DDT, was shown to bind to the rat ER *in vitro* [30]. However, we found that *o,p'*-DDT, its

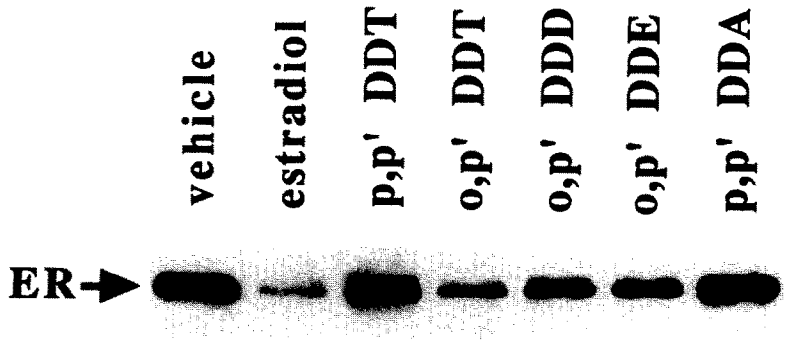

FIG. 8. Down-regulation of the ER by DDT isomers and metabolites. MCF-7 cells were grown to near confluence in MEM and 10% charcoal-dextran treated bovine calf serum. Medium with a 10 μm concentration of the DDT isomers and metabolites, or 10 nM estradiol (E2) was given to cells for 3 days. Subsequently, whole cell extracts were prepared, and samples were resolved by SDS–PAGE. Proteins were transferred to a PVDF membrane, which was probed with an anti-hER antibody. The ECL system visualization method was used to locate anti-hER antibody binding.

metabolites, and *p,p'*-DDT were able to bind to the hER in an *in vitro* competitive binding assay. In addition, all of the *o,p'*-DDT isomers and metabolites appeared to have similar affinities for the hER. Past studies done *in vitro* with rat ER show that even though other *o,p'*-DDT isomers and metabolites such as *o,p'*-DDE and *o,p'*-DDD could compete for binding to the ER, the relative affinity for binding was much lower for *o,p'*-DDE and -DDD than -DDT [8]. Past *in vivo* studies done in rats indicate that *o,p'*-DDD, DDE, and *p,p'*-DDT have little or no estrogenic action, as measured by uterotropic response [31]. The finding that there is a difference in the DDT isomers and metabolites that bind the hER versus the rat ER suggests that humans may be sensitive to more of the DDT metabolites. Since most of the studies with DDT isomers and metabolites have been conducted in rodent or avian [7, 9, 32] models, the studies may not represent the true potency of DDT isomers and metabolites in humans. The difference in binding of DDT isomers and metabolites to the rat versus the human ER might be explained by the eight amino acid difference in the ligand binding domain of the two receptors.

From our yeast transcriptional activation assays, it is evident that DDT isomers and metabolites may serve as transcriptional activators of an hER expression-reporter system. This transactivation was shown to be effective in a concentration-dependent manner at concentrations found in human tissues, and the DDT isomers and metabolites that activated the system were the same as those that could bind to the hER *in vitro*. In addition, DDT isomers and metabolites could activate with just 140- to 300-fold lower potency than estradiol in a yeast cellular system, while in the *in vitro* competitive binding assays, there was a 1000-fold lower potency than estradiol. The increased potency of the DDT isomers and metabolites in the yeast system could indicate that the biological activity of these compounds is greater than that indicated by the affinity of these compounds for the hER. Alternatively, maybe the hydrophobic DDT isomers and metabolites are concentrated within the yeast cells and are more likely to form complexes with the hER than they are at the same concentration in a cell-free system.

One recent study [33] suggests that certain DDT isomers and metabolites may bind to and inhibit transcriptional activation of the androgen receptor. While the authors show that the *p,p'*-DDE metabolite competes for binding with a radiolabeled androgen to the rat androgen receptor, they did not demonstrate competition for binding to the human androgen receptor. Additionally, it is rather surprising that *p,p'*-DDE is the only metabolite to bind to the rat androgen receptor with a high affinity and block transactivation. However, this evidence may provide a further explanation for the "estrogenic" phenotype seen in animals treated with DDT isomers and metabolites. Possibly the *p,p'*-DDE is acting as an androgen antagonist, while the *o,p'*-DDT isomer, its metabolites, and *p,p'*-DDT are functioning as estrogen agonists.

Since transactivation is measured apart from the DNA binding function of the ER in the LexA-hER system, the data show that DDT isomers and metabolites can activate the transactivation functions of the receptor independent of the DNA-binding function of the hER. It is evident that both our hER and LexA-hER expression-reporter systems produce a signal that is specific for the activation of the hER. Specificity for transactivation of the hER is demonstrated by a high response for estradiol, which can be partially inhibited by estrogen antagonists, little or no signal for the estrogen antagonists given alone, and virtually no signal for steroids that are potent agonists for other receptors. While the concentration, i.e. 100 nM, that elicits a maximal response in the two yeast expression-reporter systems is rather high when compared with ligand concentrations used in mammalian cellular reporter systems [34, 35], other researchers utilized similar maximal response concentrations in experiments with yeast reporter systems [36–38]. A factor that may interfere in ligand-activation of the hER expressed in yeast is an estrogen-binding protein [39]. This protein binds to estradiol with a high affinity, and may serve as a cellular sink for estradiol. In this case, the total amount of ligand given to the yeast would not be available to bind the hER.

Although we were able to show some transactivation inhibition by estrogen antagonists, this inhibition was not as high as that described for cellular or animal models [40, 41]. Furthermore, other groups [38, 42, 43] have reported that estrogen-stimulated transcriptional response cannot be blocked at all by estrogen antagonists. This may be due to different strain-specific properties of the used, e.g., the lack of uptake of estrogen antagonists through the yeast cell wall, or the effective elimination of these compounds by the P-glycoproteins. These proteins are homologous to mammalian MDR proteins, known to export hydrophobic compounds, i.e. the yeast a-factor mating pheromone [42].

Nonetheless, our novel transactivation evidence shows that DDT isomers and metabolites are able to function as estrogen agonists in combination with either estradiol or other DDT isomers and metabolites. Since the responses of DDT isomers and metabolites with estradiol are additive, they most likely stimulate the hER by the same activating mechanism as estradiol. These findings imply that even low concentrations of DDT isomers and metabolites may have additive effects in humans. Thus, mixtures of DDT isomers and metabolites may be able to elicit a more potent estrogenic response than initially anticipated.

A recent paper also using the expression-reporter system with the hER and *lacZ* reporter gene shows that a DDT isomer, *o,p'*-DDT, can transcriptionally activate the hER in a concentration-dependent manner at low concentrations that may be found in the human body [36]. However, there are several distinctions that can be made between the study mentioned above and this present study. One major difference is that in the past study, the researchers used a differ-

ent version of the hER expression-reporter system. Their system appears to have a signal much closer to the background than the present yeast strain used. In addition, the past study is concerned with demonstrating that certain environmental estrogens, namely diethylstilbestrol (DES), *o,p'*-DDT, and octyl phenol, may be more potent estrogens than estradiol *in vivo* because of their bioavailability. Exogenous estrogens, such as DES, have a significantly lower binding affinity to specific serum proteins, like the sex hormone binding globulin. This serum protein binds endogenous estrogens, i.e. estradiol, with a high affinity. So these researchers used the yeast hER expression-reporter system to show that with estradiol or the other exogenous estrogens listed above, increasing concentrations of either sex hormone binding globulin or two different types of sera could elicit a concentration-dependent decrease in the transcriptional activation of the hER. The estradiol-induced signal seemed to be inhibited much more than that for the exogenous estrogens, like *o,p'*-DDT. The Arnold *et al.* study [36] does not address the effect of DDT isomers in combination with the other endogenous or exogenous estrogens. In contrast, this present paper uses a similar yeast strain transformed with hER and *lacZ* reporter gene to demonstrate that certain DDT isomers and metabolites can have an additive effect with either estradiol or another DDT isomer or metabolite on transcriptional activation by the hER.

When MCF-7 human mammary carcinoma cells were used to examine the estrogenic effect of DDT isomers and metabolites, there was induction of the progesterone receptor in response to some of the DDT metabolites. In addition, when the cells were incubated with the *o,p'*-DDT isomer, there was a concentration-dependent increase in the progesterone receptor. The MCF-7 datum suggests that as in the yeast system, DDT isomers and metabolites activate the hER with a greater potency than is predicted by their *in vitro* binding datum. Possibly these hydrophobic compounds are being concentrated inside cells to levels higher than those initially given. These DDT isomers and metabolites were the same compounds that bound to the hER and could transcriptionally activate the receptor. The data for the ER down-regulation were similar to that of progesterone receptor induction, in that those DDT isomers and metabolites that bound and activated the ER were able to decrease the level of ER in the MCF-7 cells with the same order of potency.

In conclusion, some DDT metabolites are estrogenic and may activate the hER. These organochlorines and other estrogenic organochlorines may be more harmful to humans than previous studies using rodent and avian models have indicated. The interaction of the DDT isomers and metabolites with the hER appears to be rather unique since more of the DDT metabolites can bind and activate the human receptor than the rat ER. The relative estrogenicity of these compounds appears to depend specifically on both the chemical structure of the metabolites and the position of chlorines on different structural isomers. For example, *p,p'*-DDT was shown to bind the hER, whereas it did not show any binding to the rat ER. The DDT isomers and metabolites transcriptionally activate the hER in yeast at the concentrations found in humans in a concentration-dependent and additive manner. Thus, the yeast hER reporter systems function as specific, sensitive, and reliable screens for potential estrogens. Furthermore, the advantages of working with the yeast reporter systems over mammalian cellular reporter systems are numerous. Yeast cells grow faster, are less expensive, and are better genetically characterized. With the increasing concern over the plethora of environmental estrogens, this system seems ideal for testing a broad range of compounds for estrogenic activity.

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