

An Assay for the Detection of Xenoestrogens Based on a Promoter Containing Overlapping EREs

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Xenoestrogens could be implicated in the decrease of male fertility and in the increased incidence of testicular and breast cancers in humans. To predict their deleterious effects, various *in vivo* or *in vitro* tests have been proposed to assay the xenoestrogenic activity. We have designed an assay for the detection of xenoestrogens based on a novel estrogen responsive unit formed by two overlapping estrogen response elements (overEREs). This construct is able to mediate a synergistic activation of transcription by 17 β -estradiol. We have used the overERE unit to assay the estrogenic activity of synthetic compounds, mostly organochlorine compounds. By using the overERE construct, we were able to detect the estrogenic activity of compounds at concentrations 10- to 100-fold lower than a single ERE (i.e., we detected the estrogenic effect of endosulfan at a concentration of 10⁻⁵ M with ERE, whereas the overERE unit allowed us to detect a significant estrogenic activity of endosulfan at a lower concentration (10⁻⁶ M). Some compounds did not exhibit any estrogenic activity when tested with a classical ERE, whereas they were potent xenoestrogens when the overERE was used (i.e., Betanal). The assays we have developed are very sensitive and can be performed quickly. Moreover, because the promoter that we used contains only an overlapping ERE as a regulatory unit, the interference of the tested molecules with other regulatory pathways can be avoided. **Key words:** detection assay, ERE, estrogen receptor, overlapping ERE, transcription, xenoestrogens. *Environ Health Perspect* 107:563-566 (1999). [Online 3 June 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p563-566massaad/abstract.html>

Xenoestrogens are chemical compounds released in the environment that mimic the action of natural estrogens. Although their chemical structure is different from that of estradiol, these compounds can bind the estrogen receptor and activate the transcription of its target genes. Environmental estrogens were suspected to play a role in decreasing the quantity and quality of human semen during the last 50 years (1,2) and in increasing the incidences of cryptorchidism (3-5) and testicular cancer (6-8) in men and breast cancer in women (9) in industrialized countries, at least in some studies. The deleterious action of endocrine disruptors was observed in wildlife; for example, the decreased fertility of alligators and turtles in Lake Apopka, Florida, could be due to a spill of the pesticide Kelthane (10). Thus, it is important to develop appropriate tests to assess the estrogenic activity of synthetic compounds.

The target receptor of xenoestrogens is the estrogen receptor (ER), a member of the nuclear receptors superfamily. Nuclear receptors mediate the cellular actions of several hormones and effectors such as steroid hormones, triiodothyronine (T₃), retinoic acid, and vitamin D (11). There are two large families of ligand-activated nuclear receptors: the glucocorticoid receptor family and the estrogen/thyroid hormone receptor family (12,13). Most of the receptors of the latter family bind DNA as heterodimers with the retinoid X receptor. Their responsive elements generally consist of direct repeats of the AGGTCA half-site (14). Interestingly,

the estrogen responsive element (ERE) is different in that it is usually palindromic and consists of inverted half-sites separated by three base pairs (15). In the case of the ER, following hormonal stimulation, the ER binds to an ERE as a homodimer, thus modulating gene expression (16-19).

We have previously described the interaction of two dimers of the estrogen receptor with an overlapping ERE (overERE), which consists of two EREs separated by five base pairs (center to center) (20). We have shown that such an overERE sequence is functional and cooperatively binds a tetramer of ER. Furthermore, the two overEREs display a synergistic transcriptional activation. Thus, binding of two ER dimers on opposite sides of the DNA double helix enhances their transcriptional efficacy.

In this report we have used this construct to perform a detection assay of the estrogenic activity of chemical compounds. In transfection experiments, estradiol activated transcription from an overERE containing promoter four to six times better than from a single ERE containing promoter. This synergistic effect was observed using either the natural hormone (17 β -estradiol) or organochlorine xenoestrogens, and improved the detection of these compounds by at least one order of magnitude.

Materials and Methods

Cell culture. The human hepatoma cell line HepG2 (21) was cultured in Dulbecco's Modified Eagle Medium (DMEM) without

phenol red and supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μ /ml streptomycin (Diamant, Puteaux, France), and 0.5 μ g/ml fungizone (Squibb, Princeton, NJ).

Chemicals. 17 β -Estradiol, DDT, and isophorone were purchased from Sigma Aldrich (Saint Quentin, France), and Gesaprim, Madit, Illoxan, and Betanal were from AgreVo (Paris, France). Toxaphene, dieldrin, and endosulfan were purchased from Supelco (Bellofonte, PA).

Plasmids. The plasmid Δ MTV-CAT was derived from the plasmid MMTV-CAT by deletion of the sequence from position -190 to -88 of the mouse mammary tumor virus long terminal repeat (22). A *Hind*III site, created at the deletion site, was used as a cloning site for all oligonucleotides used in this study. These oligonucleotides were also subcloned into the *Hind*III site of the Tk-CAT plasmid. The double stranded oligomers (ERE and overERE) have 5' extensions that are compatible with a *Hind*III site. However, the restriction site is lost in the recombinant plasmid. The sequence of ERE is as follows, with ERE sites underlined: strand A, 5'AGCTGCTCAGCT AGGTCA CTG CGACCT CTA₃' ; strand B, 5'AGCTAGTAG AGGGC GAG TGACCT AGTGAGC3'. The sequence of overERE is as follows: strand A, 5'AGCTGCTCAGCT AGGTCAGGTCGACCTGACCT CTA₃' ; strand B, 5'AGCTAGTAG AGGTCAGGTCGACCTGACCT AGTGAGC3' (ERE sites are underlined). The luciferase plasmid (RSV-Luc) was purchased from Promega (Madison, WI).

Transfection experiments. Transfection experiments were performed as described by Massaad et al. (23). One day before the transfection, HepG2 cells (10⁶ cells/10-cm dish)

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were seeded into phenol red-free DMEM containing 10% fetal calf serum. Ten milliliters of fresh medium with 10% charcoal-treated serum was added to the cells 2–3 hr before the transfection. The serum was treated as follows: 100 ml of fetal calf serum was incubated with 1 g of Norit A activated charcoal (Sigma Aldrich) and 0.1 g of dextran T70 (Pharmacia Biotech, Uppsala, Sweden) and heated at 56°C for 30 min. This procedure was repeated twice and the mixture was filtered through a 0.22- μ m membrane. The chloramphenicol acetyltransferase (CAT) plasmids (5 μ g of DNA), the human ER (hER) expression vector (5 ng and 5 μ g), and the luciferase expression vector (1 μ g) were introduced into the cells by the calcium phosphate coprecipitation technique (23) followed by a glycerol shock. Following the glycerol shock, 10 ml of fresh medium containing 5% charcoal-treated serum was added to the cells. Sixteen hours later, serum-free medium was added and cells were treated with reference hormone or pesticides. After an additional 24-hr incubation, cells were homogenized for CAT and luciferase assays.

Luciferase assay. We used luciferase activity to normalize the transfection efficiency in all culture dishes (24). The luciferase assay was performed using a luciferase assay system kit from Promega and the manufacturer's instructions. Briefly, the transfected cells were washed twice with 5 ml calcium- and magnesium-free phosphate-buffered saline (PBS) and lysed in 500 μ l of Reporter Lysis Buffer 1X (Promega) for 15 min. After a 5-min centrifugation, 20 μ l of the supernatant was mixed with 100 μ l of luciferase assay reagent (Promega) at room temperature. The luciferase activity was measured using a luminometer 30 sec after addition of the assay reagent.

CAT assay. The CAT activity was determined using the two-phase assay developed by Neumann et al. (25). Briefly, 60 μ l of cellular extract, heated at 65°C for 10 min, was incubated with 1 mM chloramphenicol, 30 μ l acetyl CoA, and 0.5 μ Ci [³H]-acetyl CoA (NET-290 L, NEN) at 37°C for 30 min. The solution was then transferred to a minivial and layered with 4 ml of Econofluor (NEF 969, NEN). After vigorous mixing, the two phases were allowed to separate for at least 15 min, and the radioactivity was then counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not unreacted acetyl-CoA, can diffuse into the Econofluor phase. For these experiments, blanks were obtained by assaying CAT activity in cells that had undergone the same treatment in the absence of a CAT plasmid.

Statistics. Statistical analyses of the data were performed using the Mann Whitney *U* test.

Results

We designed a novel sequence (overERE) formed of two overlapping ERE sequences. This was possible because a classical ERE consists of two half-sites separated by three base pairs. We selected these nucleotides and adjacent nucleotides in order to design two overlapping identical EREs. This sequence binds cooperatively two dimers of ER on the opposite sides of the DNA double helix and could elicit transcriptional activation by estrogens (20).

To compare the efficiency of transcriptional activation of a classical consensus ERE and the overERE unit, we subcloned these constructs in a Δ MTV-CAT plasmid and made transient transfection in HepG2 cells using these constructs and an ER expression vector. As shown in Figure 1, 17 β -estradiol activated both ERE and overERE constructs at 0.01 nM, but the transcriptional activation elicited by overERE was 3- to 4-fold higher than ERE. These results show that two overlapping EREs elicit a synergistic activation of transcription. The median effective concentration (EC₅₀) elicited by estradiol on these two constructs were slightly different (EC₅₀ of ERE = 0.1 nM; EC₅₀ of overERE = 0.03 nM).

We compared the responsiveness of ERE- and overERE-containing promoters to various classes of xenoestrogens.

α -Endosulfan (1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite), a well known insecticide that mimics estrogen activity (26), was added at different concentrations (Figure 2). The transcriptional activation elicited by the overERE sequence was 3- to 4-fold higher than that elicited by the ERE sequence at the same concentration of endosulfan. As a consequence, when using the ERE construct, we were able to detect the estrogenic effect of endosulfan at a concentration of 10⁻⁵ M, whereas the overERE sequence allowed us to detect a significant estrogenic activity of endosulfan at a lower concentration (10⁻⁶ M).

We also assayed the estrogenic activity of toxaphene, another insecticide (26). Toxaphene is a mixture containing polychloro bicyclic terpenes, with chlorinated camphene predominating. The transcriptional activation of the overERE construct by toxaphene was more potent than that of the ERE construct (Figure 3).

In the case of dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo,exo-1,4:5,8-dimethanonaphthalene) (26), an insecticide used for

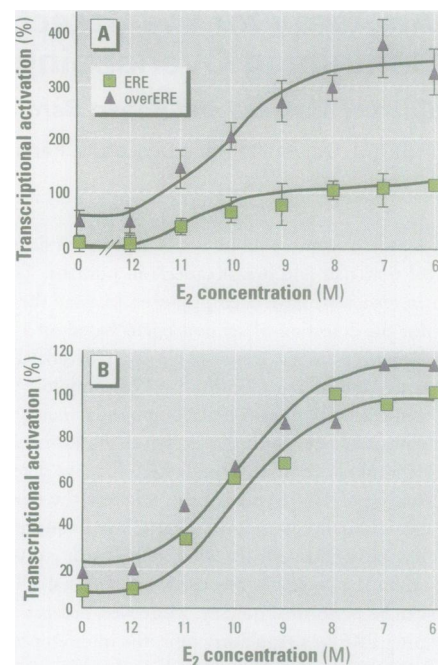


Figure 1. Functionality of the overERE construct. Abbreviations: CAT, chloramphenicol acetyltransferase; E₂, 17 β -estradiol; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element; SE, standard error. (A) Human hepatoma HepG2 cells were transiently transfected with either the Δ MTV-ERE-CAT or the Δ MTV-overERE-CAT plasmid and the ER expression vector. Increasing concentrations of E₂ were added and CAT activity was assayed. Results were calculated as a percent of the maximal activity of the ERE construct (10⁻⁶ M E₂) and are expressed as the mean \pm SE of four independent experiments. (B) For each curve, results are expressed as the percent of the maximal activity of the ERE or overERE constructs, respectively, at 10⁻⁶ M E₂. Results shown are the mean \pm SE of four independent experiments.

lice and louse flies on sheep, overERE was 5- to 6-fold more potent in activating transcription than ERE at a 10⁻⁶ M concentration and was approximately 3-fold more potent at 10⁻⁵ M (Figure 4).

These results indicate that synergistic activation of transcription elicited by the overERE sequence was observed for both the natural hormone and for organochlorine chemicals with estrogenic activity. Moreover, the overERE was able to reveal the estrogenic activity of compounds at concentrations 10-fold lower than ERE.

We then screened the estrogenic activity of commonly used compounds by using the assay described above. We incubated the HepG2 cells, transfected by either ERE-CAT or overERE-CAT plasmids, with increasing concentrations of Illoxan (diclofop methyl, 360 g/L, a herbicide used to kill weeds in wheat fields (Figure 5). Illoxan weakly activated the transcription mediated by overERE at a concentration of 1 μ M;

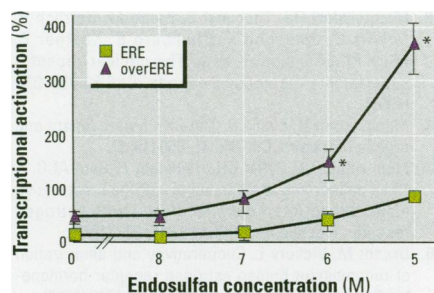


Figure 2. Effect of endosulfan on human hepatoma HepG2 cells. Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with either the Δ MTV-ERE-CAT or Δ MTV-overERE-CAT(s) plasmids and ER expression vector. Increasing concentrations of endosulfan were added to the culture medium. Results are expressed as the percent of the maximal activity of the ERE construct (10^{-5} M endosulfan) and are means \pm standard errors of five independent experiments.

*Significantly different ($p < 0.05$) from both the value of the untreated overERE-transfected cells and from the value of the ERE-transfected cells treated with the same amount of compound.

this induction was clear at 10 μ M Illoxan (3-fold induction as compared to the activity in untreated cells). The transactivation elicited by ERE was weak even at 100 μ M. These results show that Illoxan mimics the estrogenic activity and can be classified as an endocrine disruptor.

We also tested the estrogenic activity of Betanal (phenmedipham, 157 g/L), a herbicide used to kill weeds in beet fields (Figure 6). Betanal did not elicit a transactivation mediated by ERE. In contrast, Betanal clearly activated the overERE-containing plasmid at 10^{-5} M. The estrogenic activity of Betanal was revealed only by the use of the overERE.

To determine if some commonly used commercial products elicit estrogenic activity, we used the detection assay described above to test the following compounds: Gesaprime, Madit (herbicide), Productif (insecticide; AgreVo), Isophorone, and Decis (insecticide). DDT (insecticide), a well-known xenoestrogen, was also tested as a positive control. Increasing concentrations of each compound were added to HepG2 cells transiently transfected with the overERE-CAT plasmid and ER expression vector. In Table 1, we have reported only the lowest concentration of the tested compound at which a detectable estrogenic activity was observed. Only Gesaprime and, as expected, DDT displayed estrogenic activity at a concentration of 10^{-6} M. The other tested compounds (Medit, Isophorone, Decis, and Productif) did not elicit any detectable estrogenic activity even at the high concentrations tested (not shown).

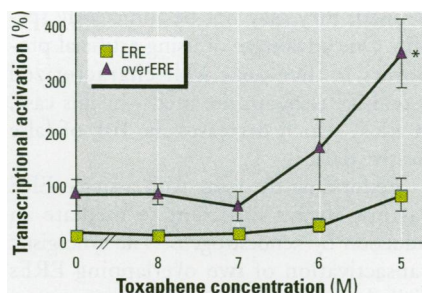


Figure 3. Effect of toxaphene on human hepatoma HepG2 cells. Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with either Δ MTV-ERE-CAT or Δ MTV-overERE-CAT plasmids and ER expression vector. Increasing concentrations of toxaphene were added to the culture medium. Results are expressed as the percent of the maximal activity of the ERE construct (10^{-5} M toxaphene) and are the means \pm standard errors of four independent experiments.

*Significantly different ($p < 0.05$) from both the value of the untreated overERE-transfected cells and from the value of the ERE-transfected cells treated with the same amount of compound.

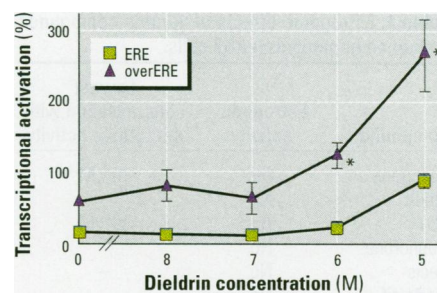


Figure 4. Effect of dieldrin on human hepatoma HepG2 cells. Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with either Δ MTV-ERE-CAT or Δ MTV-overERE-CAT (s) plasmids and ER expression vector. Increasing concentrations of dieldrin were added to the culture medium. Results are expressed as the percent of the maximal activity of the ERE construct (10^{-5} M dieldrin) and are the means \pm standard errors of four independent experiments.

*Significantly different ($p < 0.05$) from both the value of the untreated overERE-transfected cells and from the value of the ERE-transfected cells treated with the same amount of compound.

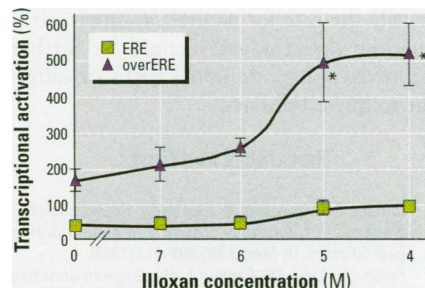


Figure 5. Effect of Illoxan on human hepatoma HepG2 cells. Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with either Δ MTV-ERE-CAT or Δ MTV-overERE-CAT (s) plasmids and ER expression vector. Increasing concentrations of Illoxan were added to the culture medium. Results are expressed as the percent of the maximal activity of the ERE construct (10 M Illoxan) and are the means \pm standard errors of four independent experiments.

*Significantly different ($p < 0.05$) from both the value of the untreated overERE-transfected cells and from the value of the ERE-transfected cells treated with the same amount of compound.

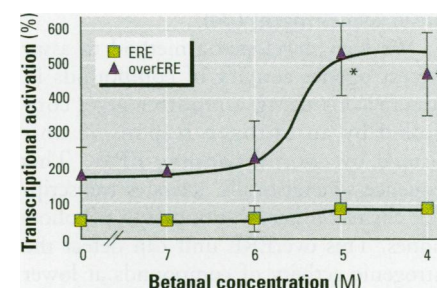


Figure 6. Effect of Betanal on human hepatoma HepG2 cells. Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with either Δ MTV-ERE-CAT or Δ MTV-overERE-CAT plasmids and ER expression vector. Increasing concentrations of Betanal were added to the culture medium. Results are expressed as the percent of the maximal activity of the ERE construct (10^{-4} M Betanal) and are the means \pm standard errors of four independent experiments.

*Significantly different ($p < 0.05$) from both the value of the untreated overERE-transfected cells and from the value of the ERE-transfected cells treated with the same amount of compound.

Discussion

Humans and animals are exposed to endogenous estrogens, phytoestrogens, and xenoestrogens. The manufactured estrogens have deleterious effects on wildlife (27). Xenoestrogens have been implicated in the decrease of male fertility and in the increase of the incidence of testicular and breast cancers in humans (28,29). To predict their deleterious effects, various *in vivo* and *in vitro* tests have been developed to assay xenoestrogenic activity. *In vivo* tests include

the uterotrophic bioassay, the evaluation of the weight of the rat uterus in response to estrogenic activity. Another test measures the induction of the vitellogenin gene that is induced severalfold by estrogens in female fish. Male fish are put in a medium containing the test compound and vitellogenesis is then measured (30). An example of an *in vitro* assay is the E-SCREEN assay, which measures the proliferative effect of estrogens on their target cells (e.g., MCF-7 cells and ZK-75 cells) (31). Other bioassays

Table 1. Estrogenic effects of various compounds on human hepatoma HepG2 cells.

Compound	Estrogenic activity	Lowest concentration with detectable activity
Gesaprine	Yes	10 ⁻⁶ M
Madit	No	—
DDT	Yes	10 ⁻⁶ M
Isophorone	No	—
Decis	No	—
Productif	No	—

Abbreviations: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; MTV, mammary tumor virus; overERE, overlapping estrogen responsive element. HepG2 cells were transiently transfected with Δ MTV-overERE-CAT plasmid and ER expression vector; increasing concentrations (10⁻⁸ M–10⁻⁵ M) of the test compounds were added to the culture medium. The CAT activity was assayed to assess the estrogenic activity. Only the lowest concentration at which detectable estrogenic activity was observed is shown. Each compound was tested in four independent experiments.

measure the induction of specific gene products by estrogens [e.g. progesterone receptor (32) and pS2 (33)]. Finally, the transfection of recombinant yeast cell lines (YES) by both an ER and an ERE-containing promoter is a sensitive technique to screen xenoestrogens (34).

We have developed a method to assay the estrogenic activity of compounds in human cells using a reporter gene controlled by an estrogen responsive unit formed by two overlapping EREs. This sequence synergistically activates transcription elicited either by estradiol or xenoestrogens. This overERE unit can detect the estrogenic activity of compounds at lower concentrations than can a single ERE, indicating its higher sensitivity. For example, with the overERE we were able to detect the estrogenic activity of Illoxan at 10 μ M, whereas we could hardly detect an increase of transactivation with ERE at 100 μ M. Moreover, some substances that failed to display estrogenic activity when tested with the ERE construct elicited clear xenoestrogenic activity with overERE (e.g., Betanal).

The overERE assay described here presents several advantages in terms of specificity and sensitivity. As previously described, the vitellogenin, progesterone receptor, and pS2 genes are biomarkers used to assay estrogenic activity of endocrine disruptors (32,33). The promoters of these genes do not only contain EREs but they also include other transcription factor binding sites that could be affected by the tested compounds. Our assay is based on the induction of an estrogen responsive unit formed of EREs only. Thus, the possible effect of the tested molecules on other transcription factors is avoided. More generally, *in vitro* assays involving transfection methods are usually very sensitive, but when natural promoters

are used, they may not be sufficiently specific. One advantage of using artificial promoters is that only well-characterized responsive elements are used—in this case, an ERE, which decreases the risk of false positive data.

Performing the assay with a single ERE was not always sufficient to mediate an induction by xenoestrogens. The synergistic transactivation of two overlapping EREs elicited a potent induction, allowing us to detect smaller amounts of xenoestrogens. Moreover, in some cases, a single ERE failed to be activated by some compounds that have a clear estrogenic activity (e.g., Betanal). Another advantage of the overERE assay is that it is performed in human cells.

In conclusion, the xenoestrogen detection assay described here consists of the transfection of human cells with a reporter gene driven by an artificial promoter. The sensitivity of the assay stems from the synergistic properties of two overlapping EREs. Its improved specificity is due to the fact that the promoter does not contain additional responsive elements, which is usually the case for natural promoters. It is possible that the assay could be further improved using additional EREs or different recipient cell lines.

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