Development of a Stably Transfected Estrogen Receptor-Mediated Luciferase Reporter Gene Assay in the Human T47D Breast Cancer Cell Line

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Development of an estrogen receptor-mediated, chemical-activated luciferase reporter gene-expression (ER-CALUX) assay was attempted by stable transfection of luciferase reporter genes in a number of cell lines. Stable transfection of the chimeric Gal4 estrogen receptor and luciferase gene constructs in MCF-7 breast cancer and Hepa.1c1c7 mouse hepatoma cell lines, as well as transfection of a newly constructed luciferase reporter gene pEREtata-Luc in the ECC-1 human endometrial cell line, resulted in constitutive, non-estradiol-inducible clones. Stable transfection of pEREtata-Luc in the T47D breast cancer cell line, however, resulted in an extremely sensitive, highly responsive cell line. Following a 24-h exposure to estradiol (E2), stably transfected T47D.Luc cells demonstrated a detection limit of 0.5 pM, an EC50 of 6 pM, and a maximum induction of 100-fold relative to solvent controls. No clear reduction in responsiveness has been found over extended culture periods (50 passages). Anti-estrogens ICI 182,780, TCDD, and tamoxifen inhibited the estradiol-mediated luciferase induction. Genistein, nonylphenol, and o,p'DDT were the most potent (pseudo-)estrogens tested in this system (EC50 100, 260, and 660 nM, respectively). Determination of interactive effects of the (pseudo-)estrogens nonylphenol, o,p'DDT, chlordane, endosulfan, dieldrin, and methoxychlor revealed that, in combination with 3 pM E2, (pseudo-)estrogens were additive. Slightly more than additive effects (less than 2-fold) were found for combinations of dieldrin and endosulfan tested in the range of 3 to 6 μM. At these concentrations, the combination of endosulfan and chlordane demonstrated additive interaction. The ER-CALUX assay with T47D cells can provide a sensitive, responsive, and rapid *in vitro* system to detect and measure substances with potential (anti-)estrogenic activity.

Key Words: reporter gene assay; *in vitro*; T47D; luciferase; stable transfectant; anti-estrogens.

An increasing number of reports that chemicals in the environment may mimic the natural hormone estrogen, thereby disrupting normal endocrine function, have raised public concern and resulted in a rapidly growing research effort. It has been hypothesized that increased incidence of hormone-sensitive cancers, decreased sperm counts in humans, and compromised gonadal functionality in wildlife may be due to exposure to substances possessing estrogenic activity (reviewed by Colborn *et al.*, 1993). An ever-growing list of environmental, industrial, natural, and pharmaceutical chemicals have been identified as potentially estrogenic. Many of these chemicals, defined as "estrogenic", have structures that deviate considerably from the natural steroid hormone 17β-estradiol, but can nevertheless evoke effects via the estrogen receptor signal transduction pathway. The effects of estrogens are mediated by binding, with high affinity, to a specific estrogen receptor (ER), a member of the nuclear receptor superfamily (Evans, 1988). In addition to the "classical" ERα form, a second ER form (ERβ) has recently been identified that has high homology with ERα but shows a different tissue distribution, ligand binding potential, and trancriptional activity (Kuiper *et al.*, 1997; 1996; 1998; Mosselman *et al.*, 1996;). Estrogen freely enters the cell and binds to the ER, inducing heat-shock protein 90 dissociation and homodimerization (Fig. 1). The homodimer complex binds to specific DNA sequences called estrogen-response elements (ERE) within regulatory regions of estrogen-responsive genes. Transcription is induced and messenger RNA is translated into protein. The synthesis of these new proteins can alter cellular functions and result in profound effects on physiology (Ing and O'Malley, 1995). In females, estrogen is essential for the development of secondary sex characteristics, the reproductive cycle, fertility, and maintenance of pregnancy. Recently, estrogens have been shown to be essential for male fertility as well (Hess *et al.*, 1997).

A number of *in vitro* assays have been developed to screen substances for (anti-)estrogenicity (see review by Zacharewski, 1997). These assays include competitive ligand-binding assays (Berthois *et al.*, 1986; Korach *et al.*; 1978; Migliaccio *et al.*,

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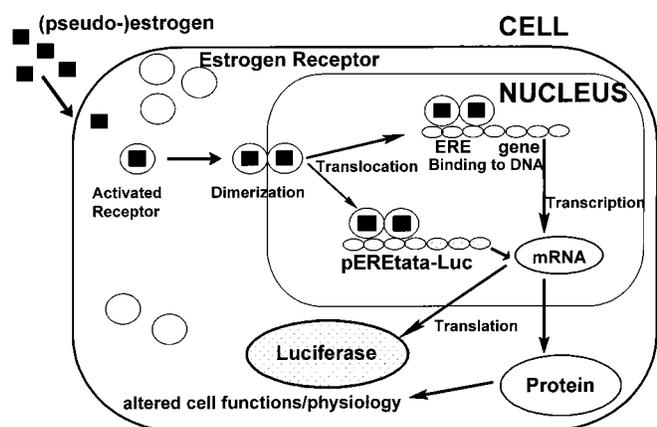
FIG. 1. Mechanism of estrogen action in stably transfected T47D.Luc cells. Pseudo-estrogens enter the cell and bind to and activate the estrogen receptor (ER). Following dimerization, the ER-ligand dimer translocates to the cell nucleus and binds to estrogen response elements (ERE) upstream of genes or present on pEREtata-Luc construct. Transcription and translation of proteins (including luciferase) is induced.

1992), cell proliferation assays (Soto *et al.*, 1995), recombinant receptor/reporter gene assays (Jausons-Loffreda *et al.*, 1994; Pons *et al.*, 1990) and yeast-based screens for estrogens (Connor *et al.*, 1995; Arnold *et al.*, 1996; Routledge and Sumpter, 1997). However, most of these assays suffer from a number of drawbacks that limit their usefulness as a screen for (anti-)estrogenicity. Competitive ligand binding assays for the estrogen receptor are limited because they cannot distinguish between estrogenic and anti-estrogenic substances and do not provide insight into a substance's ability to initiate the molecular cascade of events leading to altered gene expression. Cell proliferation assays are limited by a lack of specificity as mitogens other than estrogens are able to influence the proliferation of human breast cancer cells (van der Burg *et al.,* 1988; Dickson and Lippman, 1995). Yeast-based assays are simple and sensitive, however drawbacks include lack of responsiveness to anti-estrogens (Lyttle *et al.*, 1992; Kohno *et al.*, 1994) as well as possible differences in permeability of compounds through the yeast cell wall, relative to mammalian cell membranes.

In comparison to other existing *in vitro* assays, we believe recombinant receptor and reporter gene assays based on stably transfected cell lines can provide one of the most specific, responsive, and biologically relevant means to screen substances for potential anti-estrogenic, as well as estrogenic, effects with a high throughput potential. This type of assay is based on the receptor-mediated mechanism of action of estrogens, and reporter gene expression is a culmination of a molecular cascade of events involved in receptor transactivation. To our knowledge, only two estrogen-responsive cell lines stably transfected with recombinant receptor and/or reporter genes have previously been developed. Pons and coworkers developed an MCF-7 cell line stably transfected with the

pVit-tk-Luc construct, which consists of the *Xenopus laevis* vitellogenin A2 promoter region containing one estrogen-response element upstream of the *Herpes simplex* virus thymidine kinase promoter that controls expression of the firefly luciferase gene (Pons *et al*, 1990). A HeLa cell line stably transfected with a Gal4-regulated chimeric estrogen receptor and luciferase reporter gene constructs has also been developed (Jausons-Loffreda *et al.*, 1994). In both stably-transfected cell lines, however, only modest sensitivity and responsiveness to estradiol has been reported.

Recently in our laboratories, stably transfected CALUX (chemical-activated luciferase gene expression) cell-line assays have been developed, as a sensitive and rapid method to determine the potency of individual congeners and mixtures of dioxin-like compounds (Aarts *et al.*, 1995; Garrison *et al.*, 1996; Murk *et al.*, 1996). These cell lines express endogenous aryl hydrocarbon (Ah) receptor and are stably transfected with a dioxin-responsive luciferase reporter gene. In the present study we attempted to develop new, highly responsive stably transfected estrogen receptor-mediated ER-CALUX cell lines for the assessment of (anti-)estrogenic substances. ECC-1 human endometrial carcinoma and T47D human breast adenocarcinoma cells expressing endogenous estrogen receptor were stably transfected with a newly constructed estrogen-responsive luciferase reporter gene, pEREtata-Luc. This construct consists of three estrogen-response elements upstream from a TATA box regulating expression of an enhanced luciferase reporter gene construct. Hepa.1c1c7 mouse hepatoma and MCF-7 human breast adenocarcinoma cells were stably transfected with the chimeric receptor and reporter gene constructs Gal4-HEGO and 17m5-G-Luc. Stable transfection of pEREtata-Luc in T47D cells resulted in a sensitive, highly responsive clone, which was further characterized in dose-response studies with estradiol as well as some known estrogenic and anti-estrogenic substances.

MATERIALS AND METHODS

Chemicals. 17β-Estradiol (E2, 99%), methoxychlor (95%), tamoxifen (99%), genistein (99%), al-trans retinoic acid, and ethanol (100%, pa) were purchased from Sigma Chemical Co. 4-Nonylphenol (92.7%) was from Fluka. Bisphenol A (99%) was from Aldrich. Chlordane (97.7%), endosulfan (99%), and dieldrin (98.5%) were from Riedel-de Haan, The Netherlands. ICI 182, 780 was a kind gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, U.K. o,p'DDT and tetrachlorodibenzo-p-dioxin (TCDD) were kindly provided by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). The synthetic progestin Org 2058 was a gift from Organon B. V., Oss, The Netherlands. The synthetic androgen R1881 (methyltrienolone) was purchased from NEN (Boston, MA) and kindly provided by Dr. A. Brinkman, Erasmus University, Rotterdam. For exposure of cells, all chemicals were dissolved in ethanol or dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade, Acros). Antibiotics used for selection of stable clones (puromycin, hygromycin, and G418) were from Duchefa, The Netherlands.

Cell culture. The T47D human breast adenocarcinoma cell line was kindly provided by Dr. R. L. Sutherland (Garvan Institute of Medical Research, Sydney, Australia). The T47D cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supple-

mented with sodium bicarbonate, non-essential amino acids, sodium pyruvate, and 7.5% fetal calf serum (FCS, Integro, Austria). T47D cells were cultured at 37°C, 7.5% CO_2. The ECC-1 human endometrial carcinoma cell line was a kind gift from Dr. P.G. Stayaswaroop (Pennsylvania State University, State College, PA). ECC-1 cells were cultured in RPMI (Gibco) supplemented with 1 ng/ml insulin and 5% FCS (Sigma). The MCF-7 human breast cancer cell line was acquired from the American Type Culture Collection (ATCC, USA). The Hepa 1c1c7 mouse hepatoma cell line was kindly provided by Dr. J. P. Whitlock, Jr. (Stanford University, Palo Alto, CA) The MCF-7 and Hepa cell lines were cultured according to Zacharewski et al. (1995), in phenol red-free Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 10% FCS. ECC-1, MCF-7, and Hepa cells were cultured at 37°C, 5% CO_2.

For ER-CALUX assays, T47D, MCF-7 and Hepa cells were maintained in assay medium without phenol red, supplemented with 5% dextran-coated charcoal treated FCS (DCC-FCS). DCC-FCS was prepared by heat inactivation (30 min at 56°C) of FCS, followed by two 45-min DCC treatments at 45°C as described by Horwitz and McGuire, 1978. ECC-1 cells were assayed in RPMI with phenol red supplemented with 5% DCC-FCS.

Receptor and reporter gene constructs. The reporter gene pEREtata-Luc was constructed using the enhanced luciferase reporter gene pGL3-basic (Promega, U.S.A.). Three tandem repeats of the consensus ERE oligo (GAGCTTAGGTCACTGTGACCT) upstream of the minimal human E_1B TATA promoter sequence (GGGTATATAAT) were inserted in the Sma1-Bgl II site of the multiple cloning site of pGL3-basic (Fig. 2).

The Gal4-HEGO chimeric receptor and Gal4-regulated luciferase reporter gene 17m5-G-Luc were kindly provided by Prof. P. Chambon, INSERM U184, Strasbourg, France. Gal4-HEGO consists of the ligand binding domain of the ER linked to the DNA binding domain (1–147) of the yeast transcription factor Gal4 (Green et al., 1988). The plasmid 17m5-G-Luc consists of luciferase cDNA regulated by the rabbit β-globin basal promoter and five tandem consensus Gal4 17-mer response elements (Jausons-Loffreda et al., 1994). For stable transfection, a second 17m5 luciferase reporter gene (p17m5-G-neo-Luc) was constructed by cloning the Pvu1-EcoRV fragment of pMAMneo-Luc (ClonTech, U.S..) containing the sequence for neomycin (G418) resistance in the Pvu1-EcoRV sites of p17m5-G-Luc.

For selection of stable transfected clones, the following selection plasmids were used: for puromycin selection, pPur (ClonTech, U.S.); for hygromycin selection, pGK-Hyg (Te Riele et al., 1990); and for G418 selection, pSV2-Neo (Hoglund et al., 1992).

Stable transfection: ECC-1, MCF-7 and Hepa.1c1c7 cell lines. Six–16 h prior to transfection, ECC-1, MCF-7, and Hepa.1c1c7 cells were seeded at 50% confluency in three wells of a 6-well plate in 4 ml culture medium per well. Transfection was carried out according to the calcium phosphate precipitation method (Sambrook et al., 1989). In ECC-1 cells, 4.65 mg pEREtata-Luc and 4.65 mg pPur was transfected per well, using 250 μl HEPES-buffered solution (HBS, consisting of 42 mM HEPES, 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, pH 7.05) and 250 μl 250 mM $CaCl_2$ per well. For MCF-7 and Hepa cells, cells were transfected with either 0.6 mg pGal4HEGO, 3 mg p17m5-G-Luc and 1.5 mg pSV2Neo or 0.6 mg pGal4 HEGO and 3 mg p17m5-G-neo-Luc. After 24 h, the cells were trypsinized and per well distributed over five 100-mm petri dishes. Medium was replaced every three days with culture medium containing 1 mg/ml puromycin (ECC-1 cells) or 1000 mg/ml G418 (MCF-7 and Hepa cells) until clones formed which were large enough to isolate (about two to three weeks).

Luciferase-positive clones were screened, using a single-photon detecting camera (Argus 50, Hatamatsu) according to the method described by Pons et al., 1990. Briefly, clones in 100-mm dishes were exposed for 24 h to 10 nM E2. Luciferin (0.3 mM) was added to the culture medium, and dishes were immediately introduced into the dark room of the single-photon counting camera for luminescence measurement. Light intensity was monitored for 5 to 10 min per dish. The image of the luminescent cells was superimposed on to the light field image of the dishes, enabling identification of positive clones. Positive clones were isolated using cloning rings and further cultured in 24-well plates.

To test for luciferase induction, clones were trypsinized and seeded over 3 wells in a white 96-well viewplate (Packard) in assay medium. Following a 24-h incubation, cells were exposed for 24 h to control (0.5% DMSO), 10 nM E2, and 10 nM ICI 182,780. Cells were rinsed twice with 50 ml 0.5× phosphate buffered saline (PBS). Low salt (LS) buffer containing 10 mM Tris pH 7.8, 2 mM DTT, and 2 mM 1,2 diaminocyclohexane-N,N,N′,N′-tetraacetic acid was added at a volume of 20 μl per well. Following a 10-min incubation on ice, 96-well plates were frozen at −80°C for a minimum of 30 min to lyse cells. The plates were thawed on ice and shaken for 5 min at room temperature. Luciferase activity was measured in 96-well viewplates in a luminometer (Labsystems Luminoscan RS), with automatic injection of 100 μl luciferin "flashmix" containing 470 μM luciferin, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)2.5H_2O$, 2.67 $MgSO_4$, 0.1 mM EDTA, 5 mM ATP, and 2 mM DTT, pH 7.8 per well.

Stable transfection: pEREtata-Luc in T47D cell line. Two days prior to transfection, T47D cells were plated at a density of 250,000 cells per 5-cm petri dish on 5 ml of culture medium. Per dish, 18 mg pEREtata-Luc and 2 mg pGK-Hyg were transfected using the calcium phosphate precipitation method described above. Cells were incubated with transfection mixture for 8 h, after which the medium was renewed. The following day, the cells were trypsinized and plated over four 5-cm dishes in culture medium supplemented with 100 mg/ml hygromycin (selection medium). Clones were allowed to grow for 10 days, during which time the selection medium was renewed every two to three days. Dishes containing 10–15 large colonies were trypsinized for 1–3 min at room temperature. Individual clones were resuspended in 2 μl medium and added to 24-well plates containing 2 ml selection medium. Smaller clones were left an extra 2 weeks to grow and were then isolated in the same manner. To test for luciferase induction, confluent 24 wells were trypsinized and one well per clone was seeded in culture medium and incubated for 24 h. Non-transfected T47D cells were used as control. Medium was removed and cells were lysed in 55 μl triton-lysis buffer containing 1% triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA (pH 7.8), and 1 mM DTT. A 50-μl portion of cell lysate was transferred to a black 96-well plate (Packard) to which 50 μl luciferine substrate (LucLite reporter gene assay kit, Packard) was added. Luciferase activity was measured in a scintillation counter (Packard Top Count) for 0.1 min per well.

ER-CALUX assay procedure. T47D cells, stably transfected with pEREtata-Luc from the most responsive clone, were plated in 24-well plates at a density of 50,000 cells in 0.8 ml DF without phenol red + 5% DCC-FCS (assay medium) per well, or in black 96-well viewplates (Packard) at a density of 5000 cells per well in 0.1 ml of assay medium. Following a 24-h incubation, cells were approximately 50% confluent. Assay medium was renewed, and the cells were incubated another 24 h. The medium was again renewed, and the cells were dosed in triplicate in 24-well plates by direct addition of the chemical to be tested, dissolved in ethanol or DMSO to the medium above cells. For 96-well plates, the assay medium containing chemicals was first prepared in 0.8 ml assay medium in 48-well plates, mixed well, and transferred to the microtiter plate at a volume of 0.1 ml per well. In addition to one E2 standard curve in triplicate per experiment, control wells, solvent control wells, and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. The maximum solvent concentration used was 0.2%. Cells were dosed for 24 h prior to luciferase measurement. For 24-well plates, medium was removed, and the cells were lysed in 100 μl triton-lysis buffer. Lysis was carried out by gentle shaking at 4°C for a minimum of one h. A 50-μl sample of the cell lysate was then transferred to a black 96-well plate, 50 μl luciferin substrate was added and the luciferase activity was assayed in a scintillation counter for 0.1 min per well. For 96-well viewplates, 50 μl LucLite was added directly to the medium above cells and the plates were shaken gently for 10 min at room temperature to stimulate cell lysis. The transparent bottom of the viewplates was covered by a black sticker prior to luciferase measurement in a scintillation counter (Packard Top Count).

Potential cytotoxicity of pseudo-estrogens was controlled by microscopic visualization of the cells. In addition, a "CytoLite" (Packard) luminescent, non-separation assay kit for the determination of viable cell numbers was used,

according to manufacturer's specifications, in black 96-well viewplates. T47D.Luc cells were seeded and exposed to pseudo-estrogens in the same manner as outlined in the ER-CALUX assay procedure.

Data and statistical analysis. To determine the EC50 and detection limit of E2 in the ER-CALUX assay, a complete standard curve was included in each assay. The standard curve was fitted (sigmoidal fit, function: $y = a_0 + a_1 / (1 + \exp(-(x-a_2) / a_3))$) using SlideWrite 3.0 for Windows, which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). The EC50 for E2 and pseudo-estrogens was calculated by determining the concentration at which 50% of the maximum luciferase activity was reached using the sigmoidal fit equation. The detection limit was calculated as the luciferase activity elicited by the solvent control plus three times the standard deviation.

To determine estradiol equivalents (EEQs), the luciferase response by (combinations of) pseudo-estrogens was interpolated in the linear range of the corresponding E2 standard curve for the same assay. Data shown are representative of a minimum of 2 independent assays. Statistical analysis was performed on luciferase activity by combinations of pseudo-estrogens, as compared to the arithmetic sum of luciferase activity by individual compounds using ANOVA ($\alpha = 0.01$) in SPSS Version 6.0 for Windows. Standard deviation around the arithmetic sum was calculated by taking the square root of the pooled variance of the luciferase activity by the two individual compounds.

RESULTS

Stable Transfection of Reporter Genes in ECC-1, MCF-7, and Hepa Cell Lines

Following transfection of the pEREtata-Luc reporter gene plasmid in ECC-1 cells and selection with puromycin, 23 luminescent clones were identified using the single photon-counting camera. However, exposure of these clones to 10 nM E2 resulted in no inducible luciferase activity. Luciferase expression in the clones appeared to be constitutive. Similar results were obtained with Hepa.1c1c7 cells transfected with the chimeric receptor and reporter gene constructs pGal4-HEGO and p17m5-G-Luc and selected with G418. Fifteen luminescent clones were identified with the photon-counting camera, of which only one demonstrated estradiol-inducible luciferase expression. This clone showed a 5-fold increase in luciferase activity relative to background following 24-h exposure to 10 nM E2. In subsequent assays, however, luciferase activity decreased to three times control levels, suggesting that integration was unstable (data not shown). In Hepa cells transfected with pGal-HEGO and p17m5-G-neo-Luc, all 12 luminescent clones showed constitutive, estradiol-independent luciferase expression. Three separate attempts to stably transfect the chimeric constructs in MCF-7 cells with different concentrations of G418 selection resulted in the formation of unstable clones that ultimately died.

Stable Transfection of pEREtata-Luc in T47D Cells

A total of 72 T47D clones were isolated and tested for luciferase expression by comparison of activity (without estradiol stimulation) relative to non-transfected cells. Using this method, 28 clones were identified with luciferase expression of
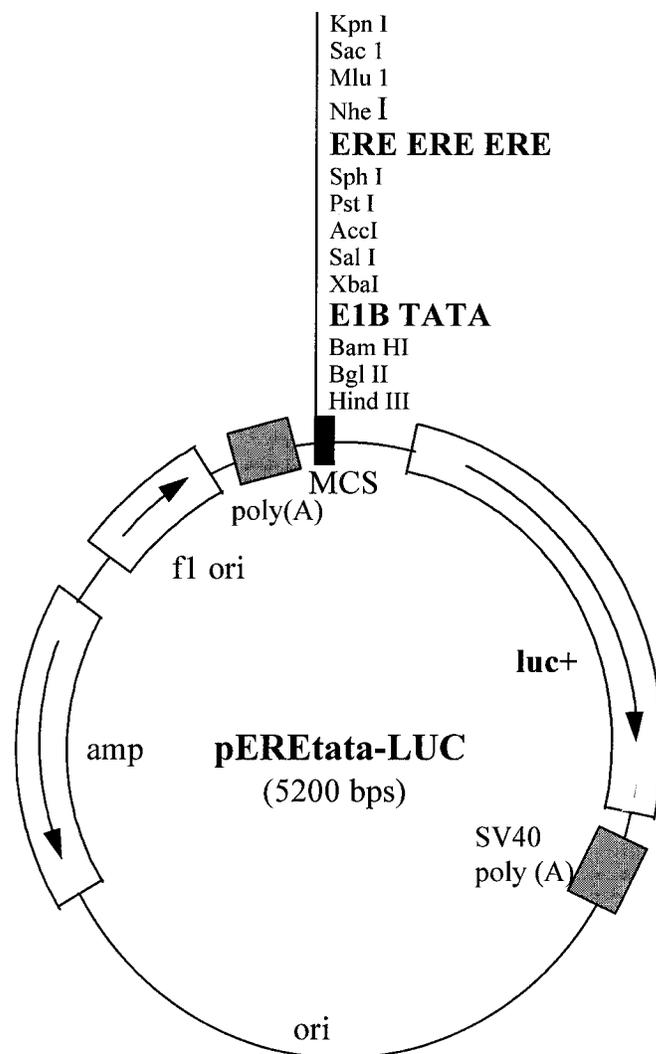

FIG. 2. Schematic representation of the pEREtata-Luc construct. Three estrogen response elements (ERE) upstream of a minimal E_1B TATA promoter were inserted in the multiple cloning site (MCS) of the enhanced luciferase (luc+) vector pGL3-basic.

more than 10-fold induction relative to non-transfected cells. Of these 28 clones, 22 were further characterized, according to the ER-CALUX assay method, in 24-well plates with exposure to 1 nM estradiol. Nine clones were found with greater than 10-fold induction relative to control. Clone ERE.Luc number 42 showed the highest induction and was used for further experiments with the ER-CALUX method.

ER-CALUX Assay with Stably Transfected T47D Cells

Stable transfection of the pEREtata-Luc construct in T47D cells resulted in a sensitive, highly responsive cell line for the assessment of compounds with (anti-)estrogenic activity. As illustrated in Figure 2, by using the ER-CALUX assay with
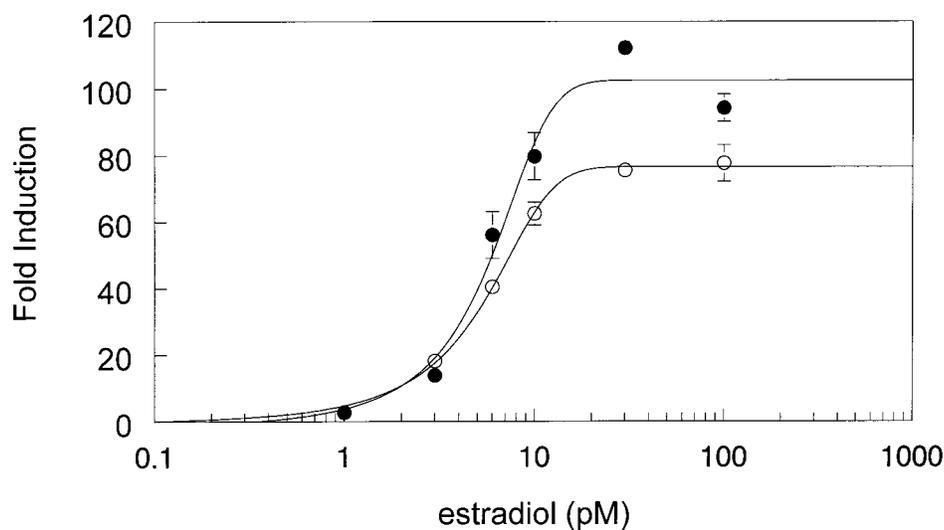
T47D.Luc cells, substances can be tested for (anti-)estrogenicity based on their specific estrogen receptor-mediated mechanism of action. Upon entering the T47D.Luc cell, substances with estrogenic activity will bind to the endogenous estrogen receptor and activate the receptor. The activated receptor dimer subsequently binds to estrogen-response elements (EREs) upstream of the luciferase gene, leading to recruitment of transcription factors to the promoter and ultimately to luciferase gene expression (Fig. 1).

Response to estradiol. The sensitivity and responsiveness of the ER-CALUX assay with the stably transfected T47D ERE.Luc 42 clone (T47D.Luc cells) was assessed by measuring the luciferase activity induced by E2 compared to solvent control, over a period of 9 months, in cells ranging from passages 9 to 48. Exposure of T47D.Luc cells to estradiol for 24 h resulted in dose-response related luciferase induction as measured in both 24-well plates and 96-well plates (Fig. 3). In 24-well plates averaged over 15 assays, 102 ($\pm$76)-fold maximum induction relative to control was reached at 30 pM E2. A detection limit of 0.5 ($\pm$0.1) pM and an EC50 of 5.5 ($\pm$1.8) pM was calculated. Using 96-well plates, a similar EC50 and detection limit were measured in 6 experiments, though maximum induction at 30 pM E2 relative to control was less than in 24-well plates (average 76 ($\pm$ 31)-fold). No clear reduction in luciferase activity over prolonged periods of cell culture was found, demonstrating stable integration of the luciferase gene.

Response to other steroids and retinoic acid. Because the T47D cell line expresses other hormone receptors, such as progesterone, androgen and retinoic acid receptors, we determined the potential effects of other endogenous ligands on the estrogen receptor-mediated luciferase response. Luciferase activity in T47D.Luc cells was determined following 24-h treatment with a synthetic progestin (Org 2058), synthetic androgen (R1881), and retinoic acid. No luciferase activity was found by

these ligands when tested in concentrations ranging from 1 pM to 10 nM (data not shown).

Response to anti-estrogens. Exposure of T47D.Luc cells to the pure anti-estrogen ICI 182,780 and the Ah receptor agonist TCDD at concentrations ranging from 0.01 to 1000 nM resulted in luciferase activity equal to or slightly less than control values (Fig. 4). When tested in combination with the EC50 value (6 pM E2), ICI 182,780 completely inhibited luciferase activity at concentrations at or above 1 nM. TCDD was a potent antagonist when co-administered with 6 pM E2, as a concentration as low as 10 pM inhibited luciferase activity by 50% (Fig. 4). Exposure of cells to tamoxifen alone at lower concentrations (<100 nM) resulted in no luciferase activity, though tamoxifen was
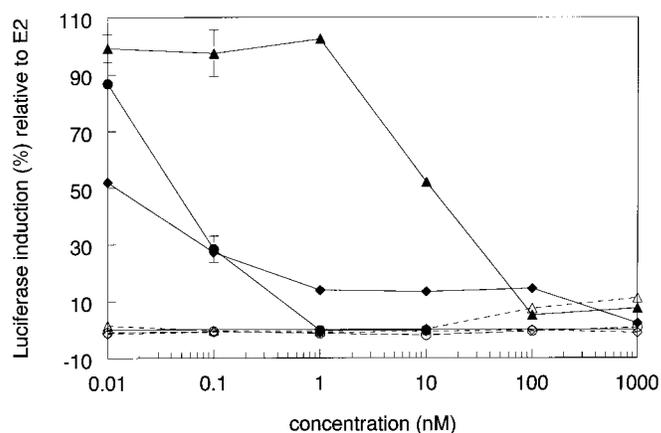
TABLE 1

Potency of Pseudo-Estrogens Relative to Estradiol, Following 24-h Treatment in the ER-CALUX Assay with T47D Luc Cells

	LOEC[a] (nM)	Relative potency[b] (LOEC)	EC50[c] (nM)	Relative potency[d] (EC50)	Maximum effect conc. (nM)[e]	% Effect rel. to estradiol[f]
Natural hormone						
Estradiol	0.0005		0.006	1	0.030	100
Pesticides						
o,p'-DDT	100	5E-6	660	9.1E-6	10000	168 (2.0)
Methoxychlor	600	8E-7	5720	1.0E-6	30000	167 (12.9)
Dieldrin	1000	5E-7	24490	2.4E-7	60000	104 (11.8)
Endosulfan	1000	5E-7	5920	1.0E-6	10000	59 (2.6)
Chlordane	1000	5E-7	6240	9.6E-7	10000	52 (4.5)
Industrial chemicals						
4-Nonylphenol	100	5E-6	260	2.3E-5	1000	135 (1.3)
Bisphenol A	100	5E-6	770	7.8E-6	10000	153 (2.3)
Phytoestrogen						
Genistein	100	5E-6	100	6.0E-5	1000	127 (2.4)

[a] Low effect concentration; test concentration at which estrogenic effect is detected.

[b] Ratio between dose of compound and that of estradiol needed to achieve an estrogenic effect.

[c] Concentration at which 50% of maximum luciferase activity is reached.

[d] Ratio between EC50 of compound and EC50 of estradiol.

[e] Concentration at which maximum estrogenic effect is detected.

[f] Per cent luciferase activity (standard deviation) at concentration eliciting maximum estrogenic effect relative to 30 pM estradiol.

slightly estrogenic at 100 and 1000 nM (about 10% activity relative to 6 pM E2) (Fig. 4). Tamoxifen was far less anti-estrogenic when tested in combination with E2 than ICI 182, 780, as concentrations above 100 nM were necessary to inhibit the E2-induced luciferase response by about 90%.

Response to pseudo-estrogenic compounds. The specificity of the ER-CALUX assay was determined by testing the luciferase activity induced by a number of compounds previously shown in other *in vitro* assays to be estrogenic. These compounds were also estrogenic when tested in T47D.Luc cells (Table 1). Genistein, nonylphenol, and o,p'DDT were the most potent pseudo-estrogens tested (EC50 100, 260 and 660 nM, respectively) (Table 1). With the exception of dieldrin and methoxychlor, all compounds tested at concentrations above 30 μM demonstrated cytotoxic effects (data not shown). The organochlorine pesticides tested induced luciferase in a dose-response related manner (Fig. 5). A number of compounds, including nonylphenol, genistein, o,p'DDT, methoxychlor, and bisphenol A showed maximum luciferase activity exceeding that of estradiol at 30 pM, though at concentrations 3000–300,000 times that of estradiol (Table 1).

Combination effects of pseudo-estrogens. Pseudo-estrogenic compounds were tested in combination with a low concentration of E2 (3 pM) in order to determine potential interactive effects when tested in mixtures (Fig. 6). All combinations tested showed additive luciferase activity, i.e.,

not significantly different from the arithmetic sum of the compounds tested individually. To determine interactive effects, the pseudo-estrogens endosulfan, dieldrin and chlordane were also tested together at concentrations eliciting luciferase activity within the lower linear range of the estradiol standard curve (between 1 and 6 pM E2 equivalents). The combination of endosulfan with dieldrin at concentrations between 3 and 6 μM resulted in luciferase activity showing more than additive in-
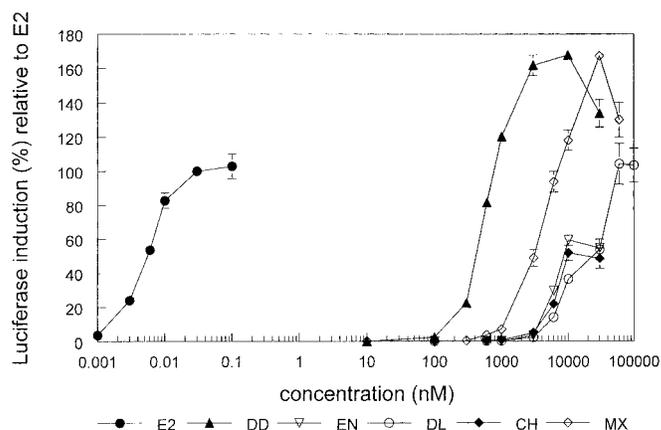

FIG. 5. Luciferase induction (%) of pesticides relative to 30 pM estradiol (E2) in the ER-CALUX assay. T47D.Luc cells were treated for 24 h with o,p'DDT (DD), chlordane (CH), endosulfan (EN), dieldrin (DL), and methoxychlor (MX) ($n = 3$, avg $\pm$ std).
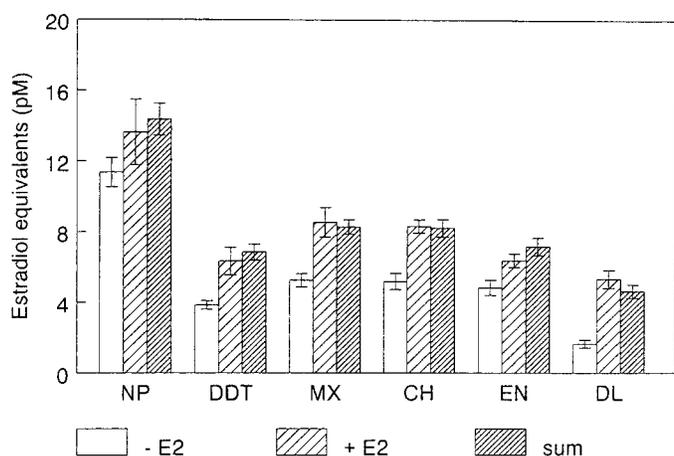
FIG. 6. Interactive estrogenic activity of environmental chemicals with estradiol (E2) in the ER-CALUX assay. T47D.Luc cells were exposed for 24 h to environmental chemicals with (striped bars) or without (open bars) co-administration of 3 pM E2. Double-striped bars show the arithmetic sum of luciferase activity obtained by adding the induction by the individual compound with induction elicited by 3 pM E2. o,p'DDT (DDT) and 4-nonylphenol (NP) were tested at a concentration of 200 nM. Methoxychlor (MX) was tested at a concentration of 2 μM. Chlordane (CH), dieldrin(DL), and endosulfan (EN) were tested at a concentration of 4 μM ($n = 6$, ave ± std).

FIG. 8. Interactive effects of mixtures of the pseudo-estrogens endosulfan (END) and chlordane (CHL) in the ER-CALUX assay. T47D.Luc cells were treated with each pseudo-estrogen and with the two combined (END + CHL). Double-striped bars on the right show the arithmetic sum (SUM) of luciferase activity obtained by adding the induction elicited by the individual compounds ($n = 6$, avg ± std; EC50 endosulfan: nM; EC50 chlordane: 6240 nM).

teraction. The luciferase activity elicited by these combinations significantly exceeded the arithmethic sum of their individual luciferase activity, though by a factor of less than 2 (Fig. 7). At 7 and 8 μM, the interaction between endosulfan and dieldrin was additive or slightly less than additive (Figure 7). The combination of endosulfan and chlordane tested at concentrations between 3 and 5 μM resulted in additive luciferase

activity (Fig. 8). When combined at concentrations higher than their individual EC50 values (6 to 8 μM), luciferase activity was less than additive (Fig. 8).

DISCUSSION

Stable Transfection in ECC-1, MCF-7, and Hepa Cell Lines

Attempts to stably transfect the pEREtata-Luc construct in ECC-1 cells, as well as the chimeric Gal4-HEGO/17m5-G-Luc constructs in Hepa.1c1c7 and MCF-7 cells, resulted in constitutive, non-estradiol-inducible luciferase expression. Jausons-Loffreda and co-workers also reported some hormone-independent luminescent clones after stable tranfection of chimeric Gal4 constructs in HeLa cells (Jausons-Loffreda *et al.*, 1994). The reasons for the presence of these constitutive clones are not clear, but may be due to events during integration that resulted in a mutation in the promoter region of the luciferase gene, or to the influence of promoters around the integration site causing constitutive, E2-independent luciferase expression. Only one Hepa clone that was stably transfected with Gal4HEGO/17m5-G-Luc demonstrated estradiol-inducible luciferase activity, though this induction decreased in subsequent assays, indicating instability of the clone. Subculturing of this clone could result in clones with a more homogeneous population of luciferase-expressing cells.

ER-CALUX Assay in Stably Transfected T47D Cell Line

Stable transfection of the pEREtataLuc construct in T47D cells resulted in a sensitive, highly responsive cell line for the screening of compounds with anti-estrogenic activity. Estradiol concentrations as low as 0.5 pM were detected, and an EC50 of
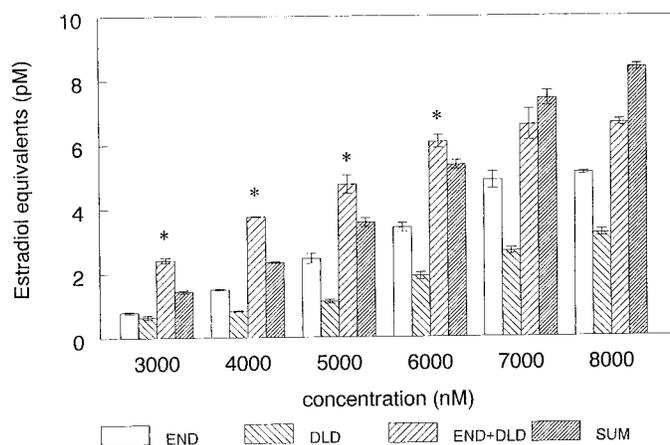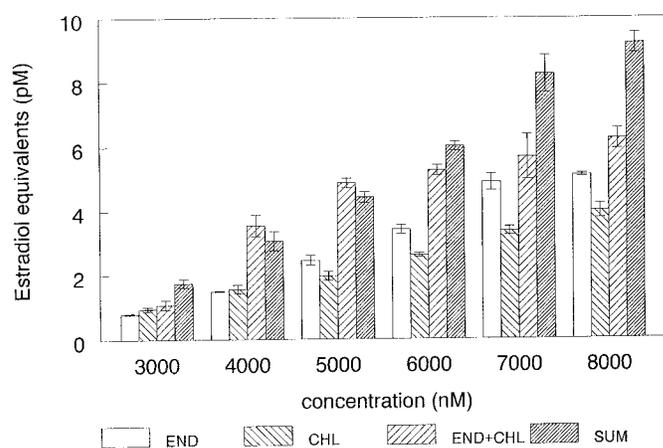

FIG. 7. Interactive effects of mixtures of the pseudo-estrogens endosulfan (END) and dieldrin (DLD) the ER-CALUX assay. T47D.Luc cells were treated with each pseudo-estrogen and with the two combined (END + DLD). Double-striped bars on the right show the arithmetic sum (SUM) of luciferase activity obtained by adding the induction elicited by the individual compounds ($n = 6$, avg ± std, *significantly higher than arithmetic sum, $p < 0.01$).

TABLE 2
Potency Values for Maximum Effect Concentrations of Pseudo-Estrogens Relative to Estradiol
According to *in Vitro* Assays for Estrogenicity

Compound	Assay	Relative potency	Reference
o,p'DDT	ER-CALUX	3 E-6	
	Transient transfection MCF-7	1 E-4	Klotz *et al.,* 1996
	E-SCREEN	1 E-6	Soto *et al.,* 1994
Methoxychlor	ER-CALUX	1 E-6	
	Transient transfection HeLa cells	0	Shelby *et al.,* 1996
Dieldrin	ER-CALUX	5 E-7	
	E-SCREEN	1 E-6	Soto *et al.,* 1994
Endosulfan	ER-CALUX	3 E-6	
	Transient transfection HeLa cells	0	Shelby *et al.,* 1996
	E-SCREEN	1 E-6	Soto *et al.,* 1994
4-Nonylphenol	ER-CALUX	3 E-5	
	Transient transfection HeLa cells	1 E-3	Shelby *et al.,* 1996
	Transient transfection MCF-7	1 E-3	White *et al.,* 1994
	E-SCREEN	3 E-6	Soto *et al.,* 1992
Genistein	ER-CALUX	3 E-5	
	E-SCREEN	1 E-4	Mayr *et al.,* 1992

6 pM was found following a 24-h exposure to estradiol. The assay is also highly responsive, as luciferase induction in 24-well plates by 30 pM E2 was increased by about 100-fold relative to solvent controls. The assay appears also to be specific for estrogens, as other endogenous ligands tested, such as synthetic progestin and androgen, or retinoic acid, did not induce luciferase activity. Anti-estrogenicity of substances such as ICI 182,780 and tamoxifen could be demonstrated by measuring inhibition of luciferase induction with co-administration of estradiol. The prototypical Ah receptor agonist TCDD was also a potent anti-estrogen when measured in the ER-CALUX assay. These results are consistent with previous studies which demonstrate the anti-estrogenicity of TCDD in breast cancer cell lines, likely due to the complex cross talk between the AhR and ER signal transduction pathways (reviewed by Safe, 1995*).

Response of pseudo-estrogens. A number of pseudo-estrogenic compounds were tested individually and in combination in order to determine their potency in the ER-CALUX assay. The phyto-estrogen genistein, nonylphenol, and the pesticide o,p'DDT were the most potent of the pseudo-estrogens tested. The environmental estrogens demonstrating induced luciferase activity in the ER-CALUX assays have also been previously shown to be estrogenic in both *in vivo* and *in vitro* assays: 4-nonylphenol (Jobling and Sumpter, 1993; Soto *et al.*, 1991; White *et al.*, 1994), o.p'DDT (Bitman, 1969, Fry and Toone, 1981, Klotz *et al.*, 1996), methoxychlor (Gray *et al.*, 1989, Schlenk *et al.*, 1997), endosulfan (Arnold *et al.*, 1996), dieldrin (Haake *et al.*, 1987) and chlordane (Cranmer *et al.*, 1984). Comparison of the potency values relative to estradiol shows that in general, lower potency values for environmental estrogens are calculated in the ER-CALUX than demonstrated with

other ER-mediated reporter gene assays, such as transient transfections of ERE-reporter genes in HeLa and MCF-7 cells (Table 2). However, this factor of 10–100 fold difference in potency could be explained by the differences in estradiol concentrations required to produce a maximal response. For the ER-CALUX assay, maximum luciferase induction is reached at 30 pM E2. For other assays, estradiol concentrations ranging from 1 nM (Shelby *et al.*, 1996) to 10 nM (White *et al.*, 1994) are used for calculation of relative potency. ER-CALUX relative potency values correlate well with those reported in MCF-7 cell proliferation assays, in which a maximum E2 effect concentration of 10–30 pM is also found (Soto *et al.*, 1992, 1994, 1995).

Interestingly, when tested at high concentrations (>1000 nM), a number of compounds such as genistein, nonylphenol, bisphenol A, o,p'DDT, and methoxychlor elicited luciferase induction higher than that of the maximum induction by estradiol (Table 1). In a screening of estrogenic compounds using a recombinant yeast strain, Routledge and Sumpter also reported that exposure to both the genistein and the alkylphenol 4-*tert*-octylphenol resulted in higher reporter gene activity than by estradiol (Routledge and Sumpter, 1997). The mechanism of this super-agonism is not clear, but may be due to stimulated receptor and/or co-activation factor renewal by pseudo-estrogens or to effects on luciferase stability.

When tested in combination with 3 pM E2 in the ER-CALUX assay, all test compounds showed additive luciferase activity (Fig. 6). However, when the pseudo-estrogens endosulfan, dieldrin, and chlordane were tested in combination in the ER-CALUX assay, the resulting interaction was not consistently additive. The combination of dieldrin with endosulfan resulted in luciferase activity slightly more than additive,

TABLE 3

Comparison of Detection Limit (dl), EC50, and Responsiveness to Estradiol in Recombinant Receptor/Reporter Gene Assays with Stable or Transient Transfection of Estrogen-Receptor-Mediated Reporter Genes in Mammalian Cell Lines

Reference	Transfection	DNA construct	Cell line	dl (pM)	EC50 (pM)	Induction
This study	Stable	pEREtata-Luc	T47-D	0.5	6	100-fold
Pons *et al.,* 1990	Stable	pVit-tk-Luc	MCF-7	ns	20	5-fold
Kramer *et al.,* 1997	Stable	pVit-tk-Luc	MCF-7	ns	1.5	2.2-fold
Balaguer *et al.,* 1996	Stable	pGal4HEGO/ p17m5-G-Luc	HeLa	18	40	10-fold
Zacharewski *et al.,* 1995	Transient	pGal4HEGO/ p17m5-G-Luc	MCF-7	ns	20	50-fold
White *et al.,* 1994	Transient	pEREBLCAT	MCF-7	ns	ns	6-fold
Jobling *et al.,* 1995	Transient	pERE-tk-Luc	MCF-7	ns	50	10-fold

Note. ns, data not shown.

though significantly higher, than the arithmetic sum of the individual compounds (Fig. 7). The combination of endosulfan and chlordane was additive at the lower concentrations tested (3 to 6 μM). Gaido and coworkers have also reported the additive effects of endosulfan and chlordane when tested in combination in a yeast estrogen screen (Gaido *et al.*, 1997). Less than additive luciferase induction by the combination of endosulfan and chlordane was observed at higher concentrations (7 and 8 μM) (Fig. 8). However, these concentrations were higher than the EC50 level of the individual compounds, so it is not surprising that the arithmetic sum of their individual activities was higher than activity elicited by the combination. These results indicate that weak environmental estrogens may act cumulatively and further research is warranted in the *in vivo* effects of mixtures of pseudo-estrogens.

Comparison of ER-CALUX with other in vitro assays. To our knowledge, in comparison to other recombinant receptor and/or reporter gene assays using stably or transiently transfected mammalian cells, the ER-CALUX assay is the most sensitive and responsive stably-transfected, estrogen-responsive cell line in existence (Table 3). The sensitivity and specificity of the ER-CALUX assay may be explained in part by the use of a minimal E_1B promoter (TATA box only) in the pEREtata-Luc construct. Enhancer and promoter regions are absent in this minimal promoter. Other promoters used in response to element-regulated reporter genes generally consist of several hundred base pairs of 5′ flanking regions, which may cause higher background activation levels (Cavailles *et al.*, 1988, 1989). Also, the vigorous serum-stripping method used may contribute to the low background levels in the ER-CALUX assay. The presence of serum-borne estrogens can result in high constitutive reporter gene activity and can be demonstrated by assessing the ability of anti-estrogens to reduce background levels (Bondy and Zacharewski, 1993). In the ER-CALUX assay, ICI 182,780 at concentrations up to 100 nM did not significantly antagonize background luciferase activity, demonstrating that serum-borne estrogens were very

low. The ER-CALUX-assay procedure was also optimized to ensure the lowest possible background levels. Cells are plated 2 days prior to exposure with a medium renewal step on the second and third days. These medium renewal steps reduced background levels of serum-borne estrogens by a factor of 2 (data not shown).

The high responsiveness of the ER-CALUX assay may be due in part to the choice of the cell line. The T47D cell line appears to provide the cellular environment, such as high estrogen receptor levels and the availability of transcription factors, that are necessary for gene transcription. The presence of both ERα and ERβ subtypes in T47D cells has been confirmed using RT-PCR (Dotzlaw *et al.*, 1996). Estrogen receptor levels in the cytosol of T47D cells have been reported to be 67.6 $\pm$ 6.2 fmol/mg cytosol protein (Watanabe *et al.*, 1990). The use of an enhanced luciferase reporter gene that was modified for optimal transcriptional activity in transfected eukaryotic cells (Sherf and Wood, 1994) may also have contributed to higher induction values. Comparison of EREtata constructs with enhanced vs normal luciferase in transient transfections in the 293 human embryonic kidney cell line showed a factor 5 increase in fold induction (data not shown).

The use of the ER-CALUX assay in stably transfected T47D.Luc cells offers many advantages in comparison to other *in vitro* bioassays, such as transient transfections of reporter gene constructs, yeast based assays, and cell proliferation assays, for assessing estrogenic substances. The ER-CALUX assay is rapid and simple, and detection in microtiter volumes is possible, allowing for qualitative and quantitative assessment of luciferase induction in a high throughput setup. Compared to transient transfections of estrogen-responsive reporter genes, the ER-CALUX assay is less cumbersome and time consuming, as it is not necessary to transfect DNA for each experiment. Problems with differences in quality of DNA and changes in luciferase activity due to cell culture conditions found with transient transfections (Zacharewski, 1997) are therefore avoided. A number of yeast-based assays have been

developed to assess estrogenic substances that are very sensi-
tive and responsive (Arnold *et al*., 1996; Routledge and
Sumpter, 1997). Unlike the ER-CALUX assay, however, lack
of antagonism of ER-mediated reporter gene expression by
anti-estrogens has been demonstrated in yeast-based assays
(Kohno *et al*., 1994; Lyttle *et al*., 1992). Permeability of
compounds through yeast cell wall may also be a problem not
encountered in the ER-CALUX assay (Legler, manuscript in
preparation). In comparison to cell proliferation assays with
estrogen-responsive cell lines like MCF-7, such as the E-
SCREEN (Soto *et al*., 1995), the ER-CALUX assay is more
specific, rapid, sensitive, and responsive.

We believe that the ER-CALUX assay can form a valu-
able contribution to a careful selection of *in vitro* and *in vivo*
assays used to identify and determine the anti-estrogenicity
of compounds. The ER-CALUX assay is also currently
being used to identify anti-estrogenic substances in complex
environmental mixtures, such as sediment and water ex-
tracts (Legler, Ms. in prep.). Of course, the *in vitro* ER-
CALUX assay cannot completely reflect complex *in vivo*
events such as the pharmacokinetics, metabolism, and ac-
cumulation of a compound. T47D.Luc cells possess some
metabolic capabilities such as the cytochrome P450
enyzmes involved in the hydroxylation of estrogens and
xenobiotics. For example, P450IA (data not shown, Kuil *et
al*., 1998; Spink *et al*., 1998); P4501B (Spink *et al*., 1998)
and 17β-hydroxysteroid dehydrogenase (Piao *et al*., 1997)
activity has been reported in T47D cells. In an *in vitro* assay,
cross-talk with other mechanisms not directly involving
interaction with the ER signal transduction pathway may
also be missed. In addition, caution should be exercised
when using a breast cancer cell line expressing both ERα
and ERβ subtypes for the prediction of tissue-specific ef-
fects. Tissues such as prostate and ovary have been found to
contain prominent ERβ expression (Kuiper *et al*., 1997).
Differences in the binding affinities of compounds such as
phytoestrogens, as well as in the transcriptional activity for
both ER subtypes, have been demonstrated (Kuiper *et al*.,
1998). Further research can determine whether estrogenic
potency predicted in the ER-CALUX assay using human
T47D.Luc cells may be extrapolated to other species such as
fish, birds and reptiles. If not, recombinant reporter gene
assays should be developed using species specific estrogen
receptors in estrogen-sensitive cell lines from various spe-
cies. To address many of these questions, biological valida-
tion is currently underway in fish to compare the reproduc-
tive effects of (mixtures of) estrogens *in vivo* to effects
measured by the ER-CALUX assay.

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