

## Assaying Estrogenicity by Quantitating the Expression Levels of Endogenous Estrogen-Regulated Genes

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Scientific evidence suggests that humans and wildlife species may experience adverse health consequences from exposure to environmental chemicals that interact with the endocrine system. Reliable short-term assays are needed to identify hormone-disrupting chemicals. In this study we demonstrate that the estrogenic activity of a chemical can be evaluated by assaying induction or repression of endogenous estrogen-regulated "marker genes" in human breast cancer MCF-7 cells. We included four marker genes in the assay—*pS2*, transforming growth factor  $\beta$ 3 (*TGF $\beta$ 3*), monoamine oxidase A, and  $\alpha$ 1-antichymotrypsin—and we evaluated estrogenic activity for 17 $\beta$ -estradiol ( $E_2$ ), diethylstilbestrol,  $\alpha$ -zearalanol, nonylphenol, genistein, methoxychlor, endosulphan, *o,p*-DDE, bisphenol A, dibutylphthalate, 4-hydroxy tamoxifen, and ICI 182.780. All four marker genes responded strongly to the three high-potency estrogens ( $E_2$ , diethylstilbestrol, and  $\alpha$ -zearalanol), whereas the potency of the other chemicals was  $10^3$ - to  $10^6$ -fold lower than that of  $E_2$ . There were some marker gene-dependent differences in the relative potencies of the tested chemicals. *TGF $\beta$ 3* was equally sensitive to the three high-potency estrogens, whereas the sensitivity to  $\alpha$ -zearalanol was approximately 10-fold lower than the sensitivity to  $E_2$  and diethylstilbestrol when assayed with the other three marker genes. The potency of nonylphenol was equal to that of genistein when assayed with *pS2* and *TGF $\beta$ 3*, but 10- to 100-fold higher/lower with monoamine oxidase A and  $\alpha$ 1-antichymotrypsin, respectively. The results are in agreement with results obtained by other methods and suggest that an assay based on endogenous gene expression may offer an attractive alternative to other E-SCREEN methods. **Key words:**  $\alpha$ 1-antichymotrypsin, competitive PCR, differential display, endocrine disruptors, estrogen, estrogenicity assay, gene expression, monoamine oxidase A, *pS2*, *TGF $\beta$ 3*. *Environ Health Perspect* 108:403–412 (2000). [Online 17 March 2000] <http://ehpnet1.niehs.nih.gov/docs/2000/108p403-412jorgensen/abstract.html>

The presence of endocrine disruptors in our environment has caused an increasing concern of their possible impact on wildlife and human health (1,2). Investigators have focused on a possible decrease in human semen quality and an undisputed increased incidence of testicular cancer over the past few decades (3,4). Although hypothetical, these changes may be caused by intrauterine exposure of the male fetus to estrogens or antiandrogens (5). In addition, significant increases in the incidences of prostate, endometrium, and breast cancer, and malformations of the external and internal genitals have been observed over the past 40–50 years, and may also be associated with increased exposure to estrogens (6–8).

Environmental estrogens include a variety of very different chemicals such as polychlorinated biphenyls (PCBs), organochlorine pesticides, alkylphenols, phthalates, and food antioxidants (2,9–11). In addition, many plants and fungi contain compounds with estrogenic activity—the phytoestrogens and mycoestrogens (2). The chemical structures of these chemicals vary substantially, which makes it difficult to predict their estrogenicity solely on a structural basis.

Hence, there is a strong need for reliable short-term methods that can rapidly detect chemicals with estrogenic properties. This is

reflected in the ambitious Endocrine Disruptor Screening and Testing Program (EDSTP), which was recently proposed by the U.S. Environmental Protection Agency (EPA), for which the EPA is considering more than 87,000 substances as potential candidates for testing. These compounds include pesticides, commercial chemicals, ingredients in cosmetics, food additives, nutritional supplements, and certain mixtures. The EDSTP is available online from the EPA website (12).

According to the EDSTP, the compounds will be tested for effects on the estrogen, androgen, and thyroid systems in humans, fish, and wildlife by a combination of *in vitro* and *in vivo* screening assays. The assays proposed by the EPA to detect estrogenic chemicals include estrogen receptor (ER) binding assays (13,14), transcriptional activation assays (15), rodent 3-day uterotrophic assays (16), rodent 20-day pubertal female assays (17), and the fish gonadal recrudescence assay (18). For chemicals that are positive in the screening, this will be followed by longer-term studies to determine whether the chemicals cause adverse effects in humans, fish, and wildlife and to establish a quantitative relationship between the dose and the adverse effect.

**Short-term estrogenicity assays in the EDSTP.** The validity and usefulness of some of the assays included in the EDSTP and

other short-term estrogenicity assays was recently evaluated in a comparison study by Andersen et al. (19).

The *in vitro* assays are based either on measurement of direct binding to isolated receptors or on the induction of a reporter gene regulated through the ER. ER binding assays involve the competition of the test compound with radiolabeled estradiol for specific binding to the ER in whole cells or in cell homogenates (13,14). Alternatively, specific binding to isolated recombinant receptors could be measured (19,20). The direct binding assay can easily be automated and thus scaled to accommodate testing of a large number of compounds. However, the ER binding assay only shows how well the tested compound binds to the ER, but it does not define the ligand as an agonist or antagonist. Furthermore, chemicals that are metabolized to estrogenic compounds in mammalian cells are not detected in the cell free binding assays.

In reporter gene-based assays, yeast or mammalian cells are transfected with the human ER and a reporter gene, such as  $\beta$ -galactosidase or luciferase, under the control of an estrogen responsive promoter. The activity of the reporter gene is directly related to the transcriptional activation activity of the test compound (15). The yeast-based reporter gene assays can easily be automated, but they do not discriminate between estrogenic and antiestrogenic chemicals (19,20). Mammalian reporter gene assays are under development, and they may eventually replace the current yeast-based assays (21–23).

In the rodent 3-day uterotrophic assay, estrogenicity is estimated as an increase in uterine tissue mass in ovariectomized or immature rodents after 3 days of treatment (16); in the rodent 20-day pubertal female assay, estrogenicity is indicated by accelerated vaginal opening in weanling rats after daily treatment beginning at 21 days of age (17). Another commonly used *in vivo* estrogenicity assay measures the level of the yolk protein

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vitellogenin in male fish, which is very low in male fish but increases in a dose-dependent manner after exposure to estrogenic compounds (18); this is one of the primary end points in the fish gonadal recrudescence assay. The *in vivo* assays have several advantages, especially the following: they take into consideration the effects of metabolism, plasma-protein binding, and pharmacokinetics; and they typically cover a broader range of mechanisms of actions than *in vitro* assays. Because of their cost, complexity, and ethical concerns, however, animal models are not suited for large-scale screening of chemicals.

**Currently used alternative assays.** There are several attractive short-term assays that are not included in the EDSTP, including the cell proliferation assay (E-SCREEN) described by Soto et al. (9). The E-SCREEN assay is based on the dose-response relationship between the proliferation of human estrogen-dependent breast cancer cells and the concentration of estrogen to which the cells are exposed during 6 days of incubation (9). The sensitivity of the E-SCREEN assay is relatively high, and it can discriminate between estrogen agonists and antagonists. However, the proliferative response is an indirect effect, and the assay is complicated by the toxicity of some compounds. Also, it is unclear to what degree proestrogens may, or may not, be activated to their estrogenic form in cultured cells.

In a clinical setting, the radioimmunoassay (RIA) is commonly used to measure serum estradiol concentrations. Because it is antibody-based, the RIA can only measure 17 $\beta$ -estradiol (E<sub>2</sub>), although similar assays probably could be developed for other compounds. However, RIA assays have a detection limit of 10–20 pmol/L and cannot be used to measure, for example, the concentration of estradiol in the serum of prepubertal children (24).

Recently, Nishikawa et al. (25) proposed an assay based on yeast two-hybrid measurements of protein-protein interactions between ER and coactivators. Because estrogen agonist binding leads to the dissociation of corepressors and recruitment of coactivators, this assay measures both the ability of a compound to bind to the receptor and whether it recruits coactivators; therefore, it yields more information than a simple binding assay. Furthermore, other protein-protein interactions could be included: for example, interactions with corepressors could discriminate between agonists and antagonists because antagonists generally do not displace corepressors.

The sensitivity of the currently available assays, measured as the lowest detectable concentration of estradiol, is very different. The most sensitive is the MCF-7 cell proliferation

assay, in which concentrations as low as 0.1 pM can be measured (9); this assay may actually be able to measure concentrations in the femtomolar range (26). The yeast-based reporter gene assays are generally several orders of magnitude less sensitive (27), although measurements of very low estradiol concentrations have been reported (15). The fish vitellogenin assay can detect estradiol at approximately 4 pM (18), whereas direct binding assays generally require concentrations in the nanomolar range (19,28).

Thus, there is a need for additional very sensitive assays that can be used to verify results obtained by, for example, MCF-7 cell proliferation assays.

**Assaying the expression levels of endogenous genes.** An alternative assay could be based on quantitation of estrogen-induced changes in the expression levels of endogenous genes, either in cultured cells or in selected tissues from exposed animals. For example, Petit et al. (29) measured the induction of vitellogenin in trout hepatocyte cultures to identify estrogenic compounds. With an endogenous gene expression assay, it is possible to assay for induction of genes that are known to be regulated by different signaling pathways as well as for genes that are regulated directly by the ER. Compared to this, a reporter gene assay will only reveal how a single gene or promoter is regulated; for example, effects caused by cross-talk between different signaling pathways may not be detected. In addition, assaying gene expression in several tissues from exposed animals ensures that effects derived both from the test compound and from its metabolites are detected, including tissue-specific effects.

In the end, all the end points currently used to determine estrogenicity in animals (and cells) are derived from changes in gene

expression, and these changes in the expression of endogenous genes could be as good an end point as, for example, increased uterine weight, provided the responsible genes have been identified.

In this study we show that a cell-based endogenous gene expression assay is very sensitive and that it can be used to assay the estrogenicity of different putative estrogenic chemicals (Figure 1). Estrogenicity is evaluated as induction or repression of four endogenous estrogen-regulated "marker genes" selected from a collection of previously identified estrogen-regulated genes. The assay is performed in human estrogen-dependent breast cancer MCF-7 cells, and changes in gene expression are assayed by a competitive polymerase chain reaction (PCR) method and displayed on PAGE gels as for differential display of reverse transcribed mRNAs technology (DDRT-PCR) (30,31). The expression levels are subsequently quantitated by phosphor imaging.

## Materials and Methods

Detailed step-by-step manuals for all procedures related to DDRT-PCR can be obtained from the DD Base web site (32).

**Cell culturing and hormone exposure.** To avoid any sample-to-sample contamination, we cultured all cells used in this study in 25 cm<sup>2</sup> flasks. Human estradiol-dependent MCF-7 breast cancer cells (a gift from P. Briand; The Danish Cancer Society, Copenhagen, Denmark) were grown in DMEM medium (Gibco BRL, Rockville, MD) containing 5% fetal bovine serum (FBS; Gibco BRL), 1 nM insulin (Roche, Basel, Switzerland), 2 mM L-glutamine (Gibco BRL), 1  $\times$  nonessential amino acids (Gibco BRL), and 25 IU/mL penicillin-streptomycin (Gibco BRL). The medium was changed every 2–3 days. Six days before the addition

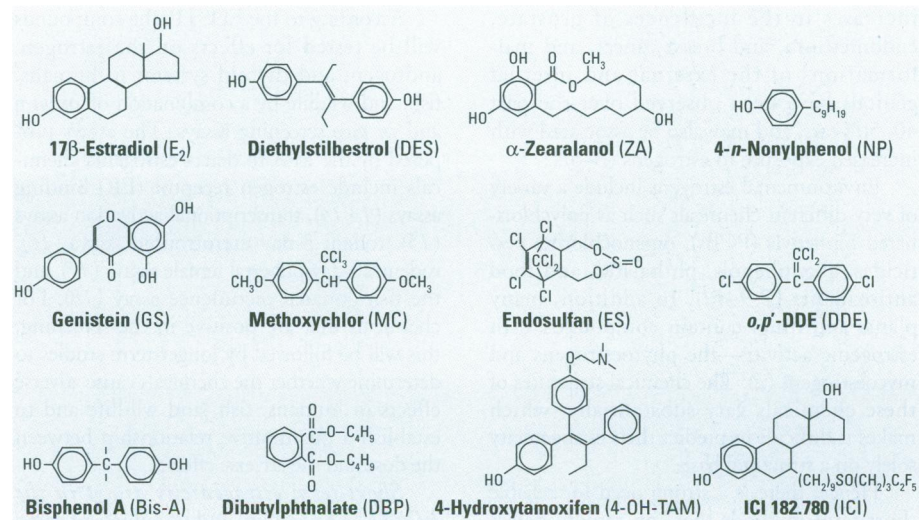


Figure 1. Chemical structures of the compounds used in the study.



of the test compounds, the cells were washed in phosphate-buffered saline (PBS; Gibco BRL), and the medium was substituted with a phenol red-free DMEM medium (Gibco BRL) containing 5% dextran charcoal-stripped FBS and the standard additives described above. Steroids were removed from FBS essentially as described by Darbre et al. (33). Briefly, FBS was incubated with 0.5% activated charcoal (Sigma-Aldrich, St. Louis, MO) and 0.05% dextran T-70 (Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min at 55°C; the charcoal particles were removed by centrifugation at 4°C for 20 min at 4,500g. This step was repeated, and the stripped serum was sterile filtered and stored in aliquots at -20°C. Forty to 50% confluent cells were rinsed in PBS, and fresh medium containing the respective test compound was added. The control cells received only the vehicle (ethanol).

**Test chemicals.** The test compounds were bisphenol A (BisA; I-0635; Sigma-Aldrich); 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*o,p*-DDE; 12,389-7; Sigma-Aldrich); dibutylphthalate (DBP; D-2270; Sigma-Aldrich); diethylstilbestrol (DES; D-4628; Sigma-Aldrich); endosulphan (ES; C131200; Ehrenstorfer GmbH, Augsburg, Germany); E<sub>2</sub> (E-2758; Sigma-Aldrich); genistein (GS; G-6649; Sigma-Aldrich); methoxychlor (MC; M-1501; Sigma-Aldrich); nonylphenol technical grade (NP; 29,085-8; Sigma-Aldrich); 4-hydroxy tamoxifen (4-OH-TAM; H-6278; Sigma-Aldrich); ICI 182,780 (AstraZeneca Pharmaceuticals, Westborough, MA); and  $\alpha$ -zearalanol (ZA; Z-0292; Sigma-Aldrich).

**Isolation of RNA.** Cells were harvested by adding 1 × trypsin-EDTA (Gibco BRL) and collected by centrifugation (1,000g for 5 min.). Total RNA was prepared using the RNeasy total RNA kit (Qiagen, Hilden, Germany) as described by the manufacturer, and stored in diethylpyrocarbonate-treated H<sub>2</sub>O (DEPC-H<sub>2</sub>O) at -80°C. Contaminating DNA was removed from the total RNA by treating with 5 U DNase-1 (Amersham Pharmacia Biotech) in 20 mM Tris-HCl, pH 7.5, and 7.5 mM MgCl<sub>2</sub> at 37°C for 30 min. The DNase was removed by incubation with 25 µg/mL proteinase K in 5 mM Tris-HCl, pH 7.5, and 10 mM EDTA, pH 8.0, at 37°C for 15 min. The RNA was then extracted with phenol/chloroform and collected by ethanol precipitation. The total RNA was dissolved in DEPC-H<sub>2</sub>O at a concentration of 1–5 µg/µL and stored at -80°C.

**cDNA synthesis.** One microgram total RNA and 0.5 µg HT11V primer (Table 1) in 10 µL DEPC-H<sub>2</sub>O were mixed and heated to 65°C for 1 min. The samples were quickly transferred to 42°C, and 10 µL cDNA Synthesis Mix and 7–8 U AMV Reverse Transcriptase (Stratagene, La Jolla, CA) were

added. The final composition of the reaction buffer was 130 mM Tris-HCl, pH 8.3; 5 mM MgCl<sub>2</sub>, 20 mM KCl, and 0.625 mM each of deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). The samples were incubated at 42°C for 1 hr, and then 80 µL of 0.1% Triton X-100 in H<sub>2</sub>O was added. The samples were denatured at 95°C for 1 min and stored in aliquots at 80°C.

**Competitive PCR.** One microliter cDNA was used in competitive PCR reaction mixtures performed in total volumes of 12 µL (final concentrations, including contributions from the cDNA): 12 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.9 mM MgCl<sub>2</sub>; 0.1% Triton X-100; 0.005% gelatin; 14 µM each dATP, dCTP, dGTP, and dTTP; 1 µCi [<sup>35</sup>S]αdATP (Amersham Pharmacia Biotech); 10 pmol each upstream and downstream primers (Table 1, Table 2); and 1 U AmpliTaq (Perkin-Elmer, Norwalk, CT). PCR was performed in a Perkin-Elmer 9600 PCR machine (Perkin-Elmer) and the cycle conditions were as follows: 1 cycle of 2 min at 95°C and 40 cycles of 30 sec at 95°C, 1 min at 40°C, 1 min at 72°C, and 1 cycle of 5 min at 72°C. After PCR, 10 µL loading

buffer (8% ficoll 400, 10 mM EDTA, 10 mM NaOH, 0.1225% bromophenol blue, and 0.1225% xylene cyanol in formamide) was added and the samples were denatured for 2 min at 96°C. Samples were then loaded onto a 5% polyacrylamide “sequencing type” gel run on the ALF-Express sequencer (Amersham Pharmacia Biotech). After electrophoresis at 25 W for 3 hr, gels were transferred to Whatmann 3 MM paper (Whatmann, Maidstone, UK), dried, and analyzed by autoradiography and phosphor imaging.

**Amplification of cDNA fragments from differential display gels.** We excised differentially expressed bands from the dried gels and recovered the DNA content by shaking the sample in 50 µL Te buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA) at 95°C for 15 min. We used 5 µL for PCR amplification in a total volume of 27 µL (final concentrations) 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.8 mM MgCl<sub>2</sub>; 0.1% Triton X-100; 0.005% gelatin; 70 µM each dATP, dCTP, dGTP, and dTTP; 2.5 U AmpliTaq (Perkin-Elmer); 10 pmol upstream primer; and 10 pmol “extended” downstream primer (T7HT11V; Table 1). Cycle conditions were as described

**Table 1.** Downstream primers.

Primer	Sequence
cDNA synthesis and differential display (HT11V and HT11VN)	
HT11A	AAGCTTTTTTTTTTA
HT11C	AAGCTTTTTTTTTTC
HT11G	AAGCTTTTTTTTTTG
HT11AC	AAGCTTTTTTTTTTAC
HT11AG	AAGCTTTTTTTTTTAG
HT11GA	AAGCTTTTTTTTTTGA
HT11GG	AAGCTTTTTTTTTTGG
HT11CC	AAGCTTTTTTTTTTCC
HT11CT	AAGCTTTTTTTTTTCT
Amplification (T7HT11V)	
T7HT11A	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTTA
T7HT11C	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTTC
T7HT11G	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTTG
Sequencing (T7)	
SEQ-17	<b>CY5-TAATACGACTCACTATAGGGA</b>

**Table 2.** Selected estrogen-regulated genes in human breast cancer cells.

Identity	Accession no.	Upstream primer	Downstream primer	Size (bp)	Regulation
$\alpha$ 1-Antichymotrypsin	J05176	CCCTCCTTTCTGCATTAG <sup>a</sup>	HT11C	380	↑
EBP50	AF015926	AGCACTGATCCCAGTTA	HT11GG	600	↑
p-Liv	U41060	GTGCTCAGTGCT <sup>a</sup>	HT11GA	150	↑
pS2	X00474	TTCCTGGGTGTCAC <sup>a</sup>	HT11G	350	↑
EST	AC005384	CAAAGGTACTCCTTTAT	HT11GG	250	↑
EST	AA677552	AGCACTGATCCCAGTTA	HT11CT	180	↑
EST	AA291280	CACGCATAGACTG	HT11AC	170	↑
Monoamine oxidase A	X60819	CTGTCTGTCCAGTTAA <sup>a</sup>	HT11GG	250	↓
Ribosomal protein L9	U09953	ACTACCTCAGTCTCAA	HT11CC	300	↓
TGFβ3	X14149	CACGCATAGACTG <sup>a</sup>	HT11AG	140	↓
EST	AA731207	AGCACTGATCCCAGTTA	HT11CC	280	↓
EST	AA772139	AGCACTGATCCCAGTTA	HT11CC	200	↓
EST	H81588	AGCACTGATCCCAGTTA	HT11GG	300	↓

Abbreviations: EBP50, ezrin-radixin-moesin binding phospho protein; EST, expressed sequence tag; TGFβ3, transforming growth factor β3. Arrows indicate regulation by 17β-estradiol: upregulated (↑), downregulated (↓).

<sup>a</sup>Indicates that the upstream primer is a targeted primer; downstream primer sequences are presented in Table 1.

for differential display above, except that the annealing temperature was 42°C. Reamplified DDRT-PCR fragments were purified from 2% agarose gels as follows: the fragments were carefully excised from the agarose gel and transferred to tubes containing small siliconized glass wool plugs. A hole was pinched in the bottom of each tube, and the DNA/buffer spun out of the agarose piece and into another tube.

We performed sequencing reactions as cycle sequencing (34) using the Thermo-Sequenase enzyme (Amersham Pharmacia Biotech) and a "T7 promotor complementary" primer (Seq-T7; Table 1) that matched all fragments amplified with the extended T7HT11V primers (31). We used ALFexpress sequencers (Amersham Pharmacia Biotech) for all sequencing and DDRT-PCR gel electrophoresis.

**Quantitation of expression levels.** DDRT-PCR gels and Northern blots were analyzed on a Fujifilm Bas-2500 phosphor imager (Fuji, Tokyo, Japan). We adjusted exposure time to the level of radioactivity on the gels/blots (between 3 and 24 hr). We normalized lane-to-lane variation in intensity either by counting a constant band or by correcting according to the background immediately above or below the quantitated band. The marker band in one lane was framed, using the software supplied with the phosphor imager, and the same frame was then copied to the corresponding band in the other lanes to ensure that identical areas were used in all lanes. To normalize the intensity of the different lanes, the marker band frames were subsequently copied together and moved to another position, covering either a constant band or a blank area (background) close to the marker band. All frames were then counted by the phosphor imager software, and the data was transferred to MS Excel (Microsoft, Redmond, WA). We selected one lane and calculated the ratios between a constant band or background in that lane and the values counted at the corresponding position in each of the other lanes. We used these ratios to normalize the intensities of the marker bands by multiplying the counts in each marker band, after subtracting the background value determined in that lane, with the corresponding ratio.

We used the following formula for normalization:

$$NMB_{\text{lane } 1} = \frac{(MB_{\text{lane } 1} - BG_{\text{lane } 1})}{(CB_{\text{lane } 1} / CB_{\text{lane } 1})}, \text{ and}$$

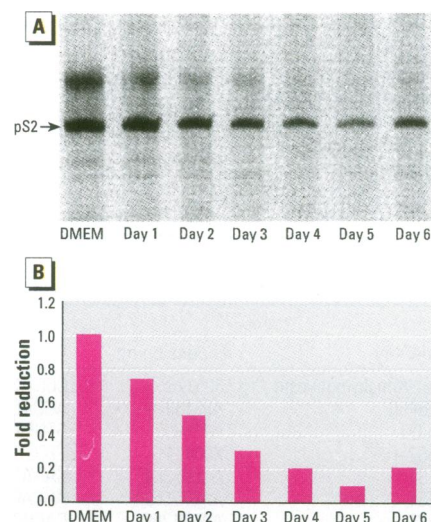
$$NMB_{\text{lane } 2} = \frac{(MB_{\text{lane } 2} - BG_{\text{lane } 2})}{(CB_{\text{lane } 1} / CB_{\text{lane } 2})}; \text{ etc.,}$$

where  $N$  = normalized,  $MB$  = marker band,  $BG$  = background, and  $CB$  = constant band.

## Results

We developed the endogenous gene-based assay because in a screening for estrogen-regulated genes, we observed a strong correlation between the expression of some genes and the concentration of estrogen in the cell media. This suggested that expression of endogenous genes could be a useful method for assaying the estrogenicity of compounds and that individual genes could respond differently to different estrogens. To evaluate whether endogenous gene expression could be used to assay compounds for potential estrogen activity, we first optimized the different steps in the procedure and then tested the estrogenicity of a range of compounds.

**Adjustment to "estrogen-free" cell culture conditions.** To determine the time needed for the cells to adjust their gene expression to "estrogen-free" conditions, we cultured MCF-7 cells in a standard DMEM medium and transferred them to an estrogen-free medium; RNA was harvested on 6 consecutive days. The expression level of the estrogen-inducible pS2 mRNA, a widely used indicator of estrogenicity, was evaluated by DDRT-PCR using a targeted upstream primer. This demonstrated that the level of pS2 mRNA expression was relatively high when the cells were cultured in the standard DMEM medium and that the level decreased in a time-dependent manner after incubation in the estrogen-free medium (Figure 2). After 3–4 days, the expression level of pS2 was reduced sufficiently to perform the experiments. However, to



**Figure 2.** Adjustment of gene expression to estrogen-free cell culture conditions in MCF-7 cells cultured in a standard DMEM medium, transferred to an estrogen-free medium, and harvested on 6 consecutive days. (A) pS2 (indicated by arrow) was detected using DDRT-PCR. (B) Quantitative estimate of the relative change in gene expression was obtained using phosphor imaging and pS2 and HT11G primers (Tables 1 and 2).

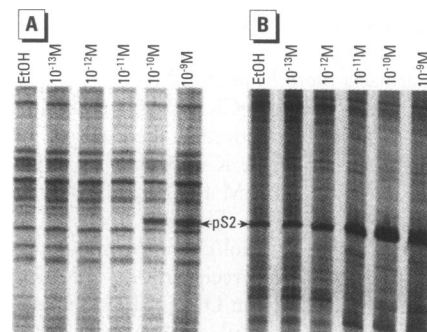
ensure that the cells adapted completely to estrogen-free conditions, all experiments were performed on cells that were incubated for at least 6 days in estrogen-free medium.

**Screening for estrogen-regulated genes in MCF-7 cells.** The use of endogenous genes and the DDRT-PCR technology to assay for estrogenicity implies the prior identification of estrogen-regulated bands, corresponding to potential marker genes, on DDRT-PCR gels. Thus, we screened MCF-7 cells that were either unexposed or exposed to  $10^{-8}$ M  $E_2$  for 24 and 48 hr, respectively, for estrogen-regulated genes, using random 13-mer upstream primers and anchored poly-dT downstream primers. Application of more than 400 different primer combinations resulted in detection of almost 100 estrogen-responsive genes (35). Some of these are listed in Table 2 together with their corresponding accession numbers, upstream primer sequences, and the size and regulation of the bands.

Because most primer combinations result in the display of 100–150 bands, the expression levels of 40,000–60,000 bands have been investigated. Comparing this with the 20,000–30,000 genes which are expressed in a human cell at a given time (36) suggests that the majority of the estrogen-regulated genes in MCF-7 cells may have been detected.

**Optimization of the endogenous gene expression assay.** The most important parameter in selecting a marker gene is that its expression is highly sensitive to the treatment, that is, the intensity of the corresponding band must be very different in estrogen-treated cells and untreated cells. Moreover, the gene must be directly regulated by estrogens and not be dependent on prior induction of another gene, and its induction must be reversed by the pure antiestrogen ICI 182,780.

To test the different candidate marker genes, we exposed MCF-7 cells to either



**Figure 3.** Application of targeted upstream primers increased sensitivity in cells exposed to ethanol (control) or increasing concentrations of  $E_2$ . The band corresponding to the pS2 mRNA (indicated by arrow) was detected using DDRT-PCR with either a nontargeted upstream primer (p-Liv) and HT11G (A) or a targeted upstream primer (pS2) and HT11G (B) (Tables 1 and 2).

increasing concentrations of test compound for 24 hr or a fixed concentration for different time periods. The candidate genes were then assayed for dose- and time-dependent effects on mRNA expression as reflected in the intensity of the corresponding bands. The initial selection reduced the number of candidate marker genes to approximately 20 that all responded strongly to estradiol.

In this paper we present estrogen-dependent expression profiles for four representative marker genes: *pS2*, monoamine oxidase A (*MAO-A*), transforming growth factor  $\beta 3$  (*TGF $\beta 3$* ) and  $\alpha 1$ -antichymotrypsin ( *$\alpha 1$ -ACT*).

**Optimization of PCR.** For the 20 originally selected genes, we improved the PCR by optimizing the matches between primers and mRNA because there were several mismatches in the alignment of the random upstream primer sequences used in the screening and the mRNA sequences. We designed the targeted upstream primers by correcting the mismatches and extending the primer with four to five extra nucleotides at the 5' end. Thus, combining the targeted upstream primer with the appropriate downstream primer results in a band of the same size as in the screening (31). Because the primer position is important for the efficiency of PCR amplification, several targeted primers were tested for each gene, and the primer that resulted in the strongest band was selected for further testing.

In most cases, the use of targeted primers significantly increased the sensitivity of the

assay. For example, with a random upstream primer that contained three mismatches within the 3'-six nucleotides of the primer, a concentration of  $10^{-10}$  M  $E_2$  was required to induce a detectable increase in the expression of *pS2* (Figure 3A), whereas application of a targeted upstream primer reduced the required concentration to only  $10^{-12}$  M (Figure 3B).

To further enhance the assay, it can be an advantage to reduce the number of competing bands, which can be done by replacing the one-base-anchored downstream primer (HT11V; Table 1) with a two-base-anchored primer (HT11VN; Table 1). In this study, we used two-base-anchored downstream primers for *TGF $\beta 3$*  and *MAO-A*.

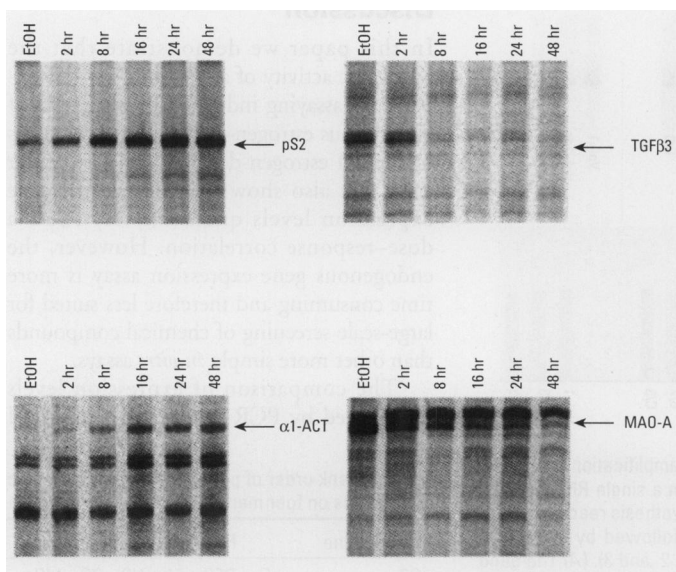
**Rapid induction of marker genes by  $E_2$ .** The observed changes in gene expression could, in principle, be caused by the activation or repression of other genes, for example, a transcription factor that is directly regulated by estrogen. The expression profiles of genes directly regulated by estrogens suggest that primary effects of estrogen are induced within a few hours, whereas secondary effects are not detectable before 16–72 hr incubation (20). We therefore performed a time-course study in which MCF-7 cells were exposed to  $E_2$  for increasing periods of time (0, 2, 8, 16, 24, and 48 hr). For almost all estrogen-responsive genes detected in the screening, including the four marker genes presented in this paper, the changes in gene expression were detectable within 2–8

hr, suggesting that they are primary effects of  $E_2$  (Figure 4).

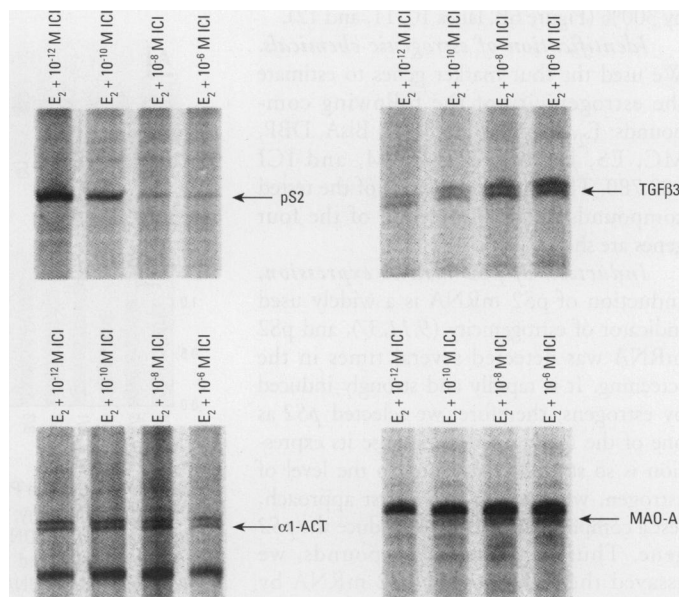
**Estrogen-induced changes in marker gene expression are ER mediated.** To verify that the regulation was mediated by the ERs, we tested the ability of the pure antiestrogen ICI 182,780 to inhibit the  $E_2$ -induced changes in gene expression. For all four marker genes, ICI 182,780 abolished the effect of  $E_2$  in a dose-dependent manner (Figure 5), demonstrating that the observed effects are mediated by the ERs. In fact, ICI 182,780 further reduced or induced the expression levels as compared to ethanol-only samples, which suggests that there are trace amounts of estrogens in the stripped serum, whose effects are reversed by ICI 182,780. The antiestrogen-mediated repression of  $\alpha 1$ -ACT is difficult to detect because the concentration of  $E_2$  used in the competition experiment was relatively low ( $10^{-11}$  M) and a 10-fold higher concentration is required to significantly induce  $\alpha 1$ -ACT expression.

The results presented in this paper are most likely derived only from activation of ER $\alpha$  because we have not been able to detect the ER $\beta$  mRNA in MCF-7 cells by RT-PCR, not even with nested primers and  $2 \times 40$  PCR cycles.

**Consistency of the technology.** Although MCF-7 cells respond strongly to estrogens, the technology used to assay gene expression should be sufficiently robust and results should be reproducible. Because the expression levels are determined by competitive



**Figure 4.** Time-dependent effects of  $17\beta$ -estradiol on marker gene expression. MCF-7 cells were exposed to  $10^{-8}$  M  $E_2$  for 2, 8, 16, 24, or 48 hr and control cells were exposed to ethanol (EtOH) for 24 hr. Bands corresponding to the four marker genes (*pS2*,  *$\alpha 1$ -ACT*, *TGF $\beta 3$* , and *MAO-A*); indicated by arrows) were detected by DDRT-PCR using the following primers: *pS2*/HT11G (*pS2*),  $\alpha 1$ -antichymotrypsin/HT11C ( *$\alpha 1$ -ACT*), *TGF $\beta 3$* /HT11AG (*TGF $\beta 3$* ), and monoamine oxidase A/HT11GG (*MAO-A*) (Tables 1 and 2).



**Figure 5.** Dose-dependent inhibition of  $E_2$ -mediated changes in gene expression by ICI 182,780. MCF-7 cells were exposed to a fixed concentration of  $E_2$  ( $10^{-8}$  M) and increasing concentrations of ICI 182,780 for 24 hr. Bands corresponding to the marker genes (indicated by arrows) were detected by DDRT-PCR using the following primers: *pS2*/HT11G (*pS2*),  $\alpha 1$ -antichymotrypsin/HT11C ( *$\alpha 1$ -ACT*), *TGF $\beta 3$* /HT11AG (*TGF $\beta 3$* ), and monoamine oxidase A/HT11GG (*MAO-A*) (Tables 1 and 2).



PCR amplification of cDNA synthesized from different RNA samples, it is important that the protocols do not introduce significant variation. In addition, because it is impossible to obtain completely identical loading on DDRT-PCR gels, it must be possible to normalize the lanes, according to either the background or to a constant band.

To test the consistency of the method, we performed three cDNA synthesis reactions from a single RNA preparation, followed by three PCR runs of each cDNA; the results for the *pS2* primer set are shown in Figure 6A. The DDRT-PCR gel was scanned by phosphor imaging and the results normalized according to the background just below the *pS2* band (Figure 6B). This showed that the variation introduced by the cDNA synthesis and PCR was < 24 and 16%, respectively, and that the maximal variation between two samples was < 34%. These differences are representative of the variation we have observed among the hundreds of samples that have been analyzed; this variation is small as compared to the severalfold differences among samples treated with different concentrations of estrogens.

To test the power of the normalization, two-thirds and one-third of the volume of the sample in lane 9 (sample C/3) were loaded separately (Figure 6A, lanes 10, 11, and 12). After normalization, the *pS2* values in these lanes were essentially identical, showing that normalization according to the background or a constant band can be used to normalize samples, even when the loaded amounts differ by 300% (Figure 6B, lanes 10, 11, and 12).

#### Identification of estrogenic chemicals.

We used the four marker genes to estimate the estrogenicity of the following compounds: E<sub>2</sub>, ZA, DES, GS, NP, BisA, DBP, MC, ES, DDE, 4-OH-TAM, and ICI 182,780. The relative potencies of the tested compounds on the expression of the four genes are shown in Table 3.

#### Induction of *pS2* mRNA expression.

Induction of *pS2* mRNA is a widely used indicator of estrogenicity (9,11,37), and *pS2* mRNA was detected several times in the screening. It is rapidly and strongly induced by estrogens; therefore, we selected *pS2* as one of the marker genes. Because its expression is so strongly correlated to the level of estrogen, we routinely, as a first approach, test a compound for ability to induce the *pS2* gene. Thus, for all test compounds, we assayed the induction of *pS2* mRNA by DDRT-PCR (Figure 7) and quantitated the expression profiles by phosphor imaging (Figure 8).

The expression level of *pS2* mRNA was increased in a dose-dependent manner by all the tested estrogens; however, their potencies were very different. Based on their relative

potency (Table 3), the chemicals can be divided into three groups. Group 1 includes E<sub>2</sub>, ZA, and DES, which cause detectable increases in *pS2* at 10<sup>-12</sup> M–10<sup>-11</sup> M; Group 2 includes NP and GS, which induce *pS2* at 10<sup>3</sup>- to 10<sup>4</sup>-fold higher concentrations (> 10<sup>-8</sup> M); and Group 3 includes BisA, DDE, MC, ES, and DBP, which require 10<sup>5</sup>- to 10<sup>6</sup>-fold higher concentrations, relative to E<sub>2</sub>, to induce *pS2* (Figure 7, Figure 8). The high-potency estrogens in Group 1 (E<sub>2</sub>, ZA, and DES) cause a significantly greater quantitative change in the expression level of *pS2* as compared to the environmental estrogens in Groups 2 and 3. For example, the expression level of *pS2* is increased almost 25-fold after exposure to E<sub>2</sub> (10<sup>-10</sup> M), but less than 10-fold after exposure to DBP (10<sup>-4</sup> M) (Figure 7, Figure 8).

A slight increase in the expression level of *pS2* mRNA was also observed after exposure to high concentrations (> 10<sup>-7</sup> M) of 4-OH-TAM (Figure 7, Figure 8).

#### Dose-dependent effects of estrogens and antiestrogens on three other marker genes.

To increase the strength of the estrogenicity assay and to analyze whether other genes responded in the same manner as *pS2*, we assayed dose-dependent effects of the test compounds on the three other marker genes.

Exposure to increasing concentrations of E<sub>2</sub> caused an induction of  $\alpha$ 1-ACT and a reduction in the expression levels of TGF $\beta$ 3 and MAO-A (Figure 9). Detectable reductions in the expression levels of TGF $\beta$ 3 and MAO-A could be detected at an E<sub>2</sub> concentration of

10<sup>-13</sup> M (Figure 9), equivalent to, or even below, the dose required to induce *pS2* mRNA expression (Figure 7, Figure 8), whereas detectable induction of  $\alpha$ 1-ACT required a 100-fold higher concentration (10<sup>-11</sup> M) (Figure 9). The other test compounds caused detectable changes in the expression levels of TGF $\beta$ 3 and MAO-A at doses similar to those required for *pS2* induction, but for most chemicals, the concentrations required to induce  $\alpha$ 1-ACT were 10- to 100-fold higher (Figure 9).

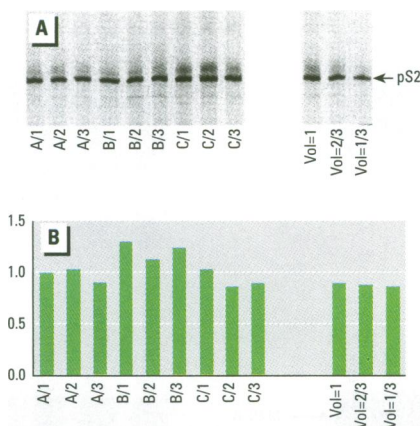
There are significant marker gene-dependent differences in the rank order of potencies of the test compounds (Table 3). For example, the potencies of E<sub>2</sub>, DES, and ZA were essentially identical on TGF $\beta$ 3, whereas the potency of ZA was almost 100-fold lower than the potency of E<sub>2</sub> and DES on *pS2*,  $\alpha$ 1-ACT, and MAO-A. Furthermore, NP and GS affected the expression levels of *pS2* and TGF $\beta$ 3 with similar potencies, whereas the potency of NP was almost 10-fold higher than the potency of GS on MAO-A; the potency of NP on  $\alpha$ 1-ACT was lower than that of GS (Table 3).

The partial estrogen agonist 4-OH-TAM slightly increased expression levels of  $\alpha$ 1-ACT and *pS2* and reduced the expression level of MAO-A, suggesting that 4-OH-TAM acts as a partial agonist on these genes. In contrast, a high concentration of 4-OH-TAM slightly increased the expression of TGF $\beta$ 3 (Figure 9), suggesting that it acts as an antagonist or a selective estrogen receptor modulator (SERM) on TGF $\beta$ 3.

## Discussion

In this paper we demonstrate that the estrogenic activity of a chemical can be evaluated by assaying induction or repression of endogenous estrogen-regulated marker genes in human estrogen-dependent breast cancer cells. We also show that changes in gene expression levels quantitatively show a dose-response correlation. However, the endogenous gene expression assay is more time consuming and therefore less suited for large-scale screening of chemical compounds than other more simple *in vitro* assays.

The comparison of expression levels determined by PCR is a well-characterized



**Figure 6.** Competitive PCR amplification is a highly consistent technology; from a single RNA sample, three independent cDNA synthesis reactions were performed (A, B, and C), followed by three PCR reactions of each cDNA (1, 2, and 3). (A) The band corresponding to *pS2* (indicated by arrow) was detected by DDRT-PCR using the *pS2* and HT11G primers. An equal volume of the sample in lane 9 (C/3) was loaded in lane 10 (vol = 1), and 2/3 and 1/3 the volume were loaded in lanes 11 and 12, respectively. (B) After quantitation by phosphor imaging, the values were normalized according to the background in each lane.

**Table 3.** Rank order of potencies of the estrogenic compounds on four marker genes.

Marker gene	Rank order of potency
<i>pS2</i>	E <sub>2</sub> = DES > ZA > NP = GS > MC > ES = BisA > DDE > DBP
$\alpha$ 1-ACT	E <sub>2</sub> = DES > ZA > GS > NP > ES > BisA = MC > DDE > DBP
MAO-A	E <sub>2</sub> > DES > ZA > NP > GS > DBP > BisA > ES = MC > DDE
TGF $\beta$ 3	E <sub>2</sub> = DES = ZA > NP = GS > BisA > MC > DDE > ES = DBP

method used in a variety of protocols. PCR amplification of cDNA fragments conserves the relative levels as they were in the original mRNA preparations, provided proper procedures are used (38,39). Thus, the level of an amplified radioactively labeled PCR fragment is directly proportional to the level of the corresponding mRNA in the original sample. The low-stringency PCR method we describe is, in principle, similar to multiplex PCR, in which a few DNA fragments are amplified in the same reaction, although we obtain multiplexing using a single primer pair and low-stringency annealing. The result is that we amplify on average approximately 125 PCR fragments in each reaction. Because the competing fragments are identical in all samples prepared with a given primer pair, only the level of the estrogen-sensitive mRNA/cDNA fragment will change, and the intensity of the corresponding band accurately reflects the level of the mRNA in the RNA samples. However, in contrast to Northern blotting, for example, the precise amount of an mRNA is not reflected in the intensity of the band. The DDRT-PCR technology only allows comparison of the expression level of a given mRNA among different samples, where the ratio between any two samples will show the difference in the expression level of the mRNA in the two samples.

The expression levels were quantitated by phosphor imaging scanning and normalized according to either a constant band or the background; this resulted in reproducible results and thus accurate estimates of estrogenic activity. This is shown by the small variation introduced in the cDNA synthesis and PCR (24 and 16%, respectively), as compared to the many fold induction or repression resulting from exposure to the estrogenic compounds.

### The Marker Genes

In this study, estrogenicity was determined by analyzing the expression levels of four marker genes: *pS2*, *MAO-A*, *TGF $\beta$ 3*, and  *$\alpha$ 1-ACT*. Because the assay is based on endogenous gene expression, an arbitrary number of marker genes could, in principle, be included in the assay; the marker genes used in this study could be replaced by other estrogen-responsive genes such as the progesterone receptor.

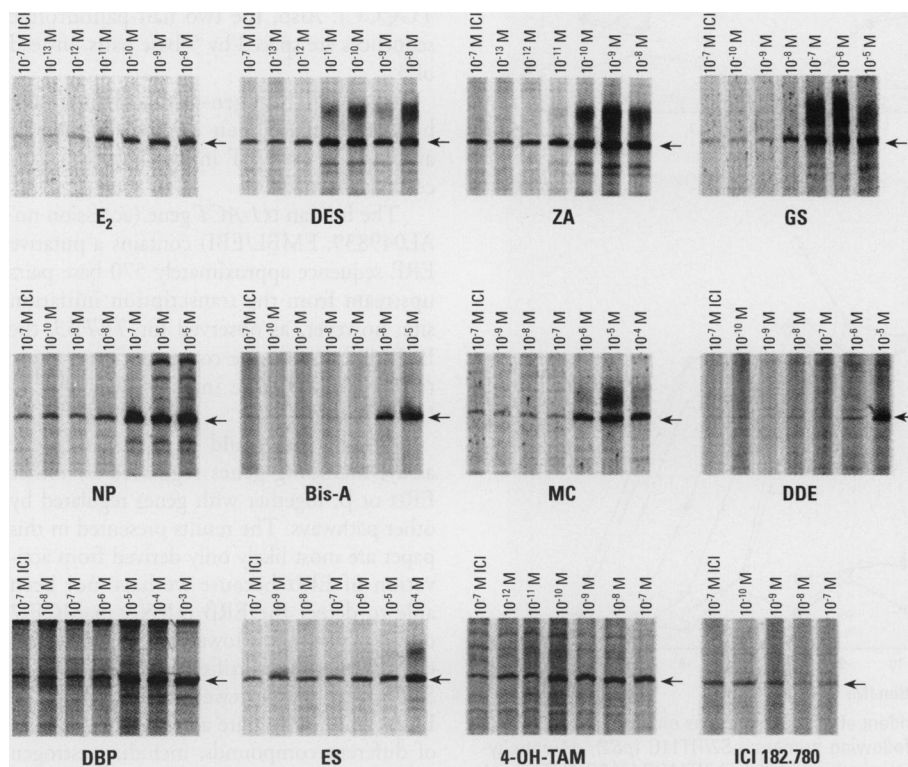
**PS2.** The expression level of pS2 mRNA is a widely used indicator of estrogenicity, and the translation product of pS2 mRNA is also induced in MCF-7 cells in response to estrogen (37). The 9 kDa encoded pS2 protein belongs to the trefoil family of peptides (40), whose members are probably involved in regulation of proliferation; it has been

shown that trefoil proteins activate the Ras/MEK/MAP-kinase signal transduction pathway by direct interaction with epidermal growth factor receptors (41). There are currently no reports in the literature of non-estrogenic compounds that induce pS2 expression in MCF-7 cells.

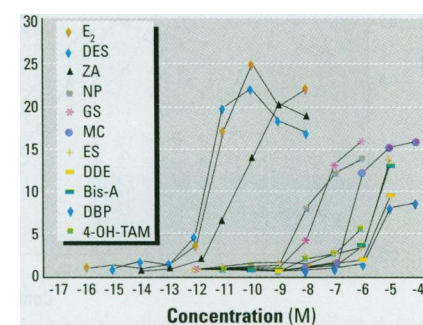
The human *pS2* gene (accession no. X05030; EMBL/EBI, Cambridge, UK) (42) contains an imperfect estrogen response element (ERE) that varies from the consensus palindromic ERE (GGTCANNNTGACC) by one base pair in its right arm (GGT-CACGGTGGCC) (43). Functional imperfect EREs have been demonstrated in a number of estrogen responsive genes, including human *TGF $\alpha$*  (44), human cathepsin D (45), rat progesterone receptor (46), and *Xenopus laevis* vitellogenin genes (47), and are most likely responsible for the observed estrogen-dependent regulation.

**MAO-A.** The *MAO-A* gene encodes one of the two monoamine oxidase proteins (A). The expression and activity of *MAO* genes (A and B) have been investigated both *in vivo* and *in vitro* (48–50). *In vivo* assays have been conducted because there seems to be a correlation between mood changes and estradiol levels that may be mediated through the activity of monoamine oxidases (51–53). The activity and expression of *MAO-A* is inversely correlated both *in vivo* and *in vitro* to the estradiol concentration (49,50), which is similar to the regulation we have observed in MCF-7 cells. However, *MAO* genes may be induced by  $E_2$  in some tissues; Sarabia and Liehr (54) showed that expression of the *MAO-B* gene actually is induced by  $E_2$  in hamster kidneys.

The human *MAO-A* gene (accession no. AL020990; EMBL/EBI) contains several putative ERE sequences that could be responsible for the observed estrogen-dependent regulation of the *MAO* gene. One is located 200 base pairs upstream from the transcription

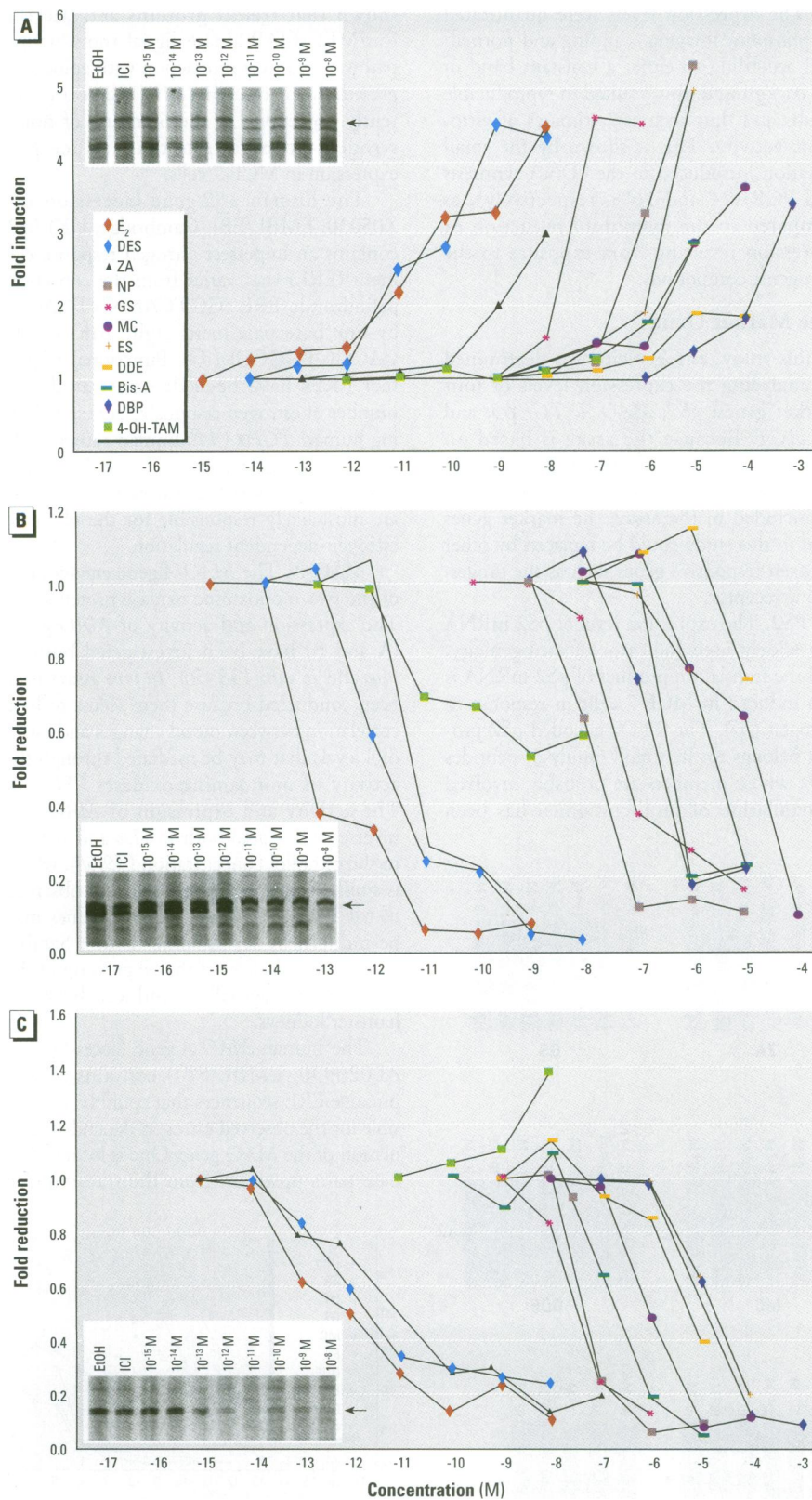


**Figure 7.** Dose-dependent induction of pS2 in MCF-7 cells by different estrogens. MCF-7 cells were exposed for 24 hr to  $E_2$ , DES, ZA, GS, NP, Bis-A, MC, DDE, DBP, ES, 4-OH-TAM, and ICI 182.780. Control cells were exposed to ethanol (EtOH) or ICI 182.780 ( $10^{-7}$  M) for 24 hr. The band corresponding to pS2 (indicated by arrows) was detected by DDRT-PCR using the pS2 and HT11G primers. Quantitation of the expression levels is shown in Figure 8.



**Figure 8.** Quantitative representation of DDRT-PCR expression profiles. The fold induction is relative to EtOH, and the molar concentrations are indicated. To eliminate lane-to-lane variations, we normalized the quantitations according to the background in each lane. The results represent at least two to three independent experiments.





**Figure 9.** Phosphor imaging estimates of the dose-dependent effects of estrogens on the expression level of (A)  $\alpha 1$ -ACT, (B) MAO-A, and (C) TGF $\beta 3$  using the following primers: pS2/HT11G (*pS2*),  $\alpha 1$ -antichymotrypsin/HT11C ( *$\alpha 1$ -ACT*), TGF $\beta 3$ /HT11AG (*TGF $\beta 3$* ), and monoamine oxidase A/HT11GG (*MAO-A*) (Tables 1 and 2). MCF-7 cells were exposed to increasing concentrations of the test compounds and harvested after 24 hr. Fold induction/repression is relative to ethanol (EtOH). Bands corresponding to each of the three marker genes were detected by DDRT-PCR, and the expression profiles were quantitated by phosphor imaging. An example of a DDRT-PCR expression profile (corresponding to  $E_2$ -exposed cells) appears as an insert in each chart. The results represent at least two to three independent experiments.

initiation site and differs from the consensus palindromic ERE by 2 base pairs in its right arm (GGTCACCTTCCC). Moreover, the two half-palindromic sequences are separated by only 2 base pairs. Another putative imperfect ERE is located approximately 1,500 base pairs upstream from the transcription initiation site and contains a single mismatch in the left arm (GGACAAATGTGACC), with 4 base pairs between the two half-palindromic sequences.

**TGF $\beta 3$ .** Repression of TGF $\beta 3$  expression by estradiol in MCF-7 cells has previously been described (55), and cell- and tissue-type-specific expression has also been shown (56). TGF $\beta 3$  expression is regulated by non-estrogenic compounds (57), and it is probably necessary to include additional marker genes to be sure that only estrogens are detected. Knabbe et al. (58) reported that TGF $\beta 3$  inhibits the growth of MCF-7 cells and that secretion of TGF $\beta 3$  is induced by antiestrogens. In our study, however, TGF $\beta 3$  was only slightly increased after exposure to a high concentration of 4-OH-TAM (Figure 9).

The human TGF $\beta 3$  sequence (accession no. X14885; EMBL/EBI) (59) contains a putative imperfect ERE 340 base pairs upstream from the transcription initiation site, which varies from the consensus ERE sequence at two positions, one in each half-palindromic sequence (GGCCAGCAAC-TGCC). Also, the two half-palindromic sequences are spaced by 5 base pairs, instead of 3.

**$\alpha 1$ -ACT.** Estrogen-mediated induction has previously been described for the antiprotease  $\alpha 1$ -ACT in human breast cancer cells (60,61).

The human  $\alpha 1$ -ACT gene (accession no. AL049839; EMBL/EBI) contains a putative ERE sequence approximately 570 base pairs upstream from the transcription initiation site; however, as observed for TGF $\beta 3$ , the ERE differs from the consensus sequence at two positions, one in each arm (AGT-CACTGTGCC).

Other genes could be included in the assay, including genes regulated by either ER $\alpha$  or  $\beta$ , together with genes regulated by other pathways. The results presented in this paper are most likely only derived from activation of ER $\alpha$  because we have not been able to detect the ER $\beta$  mRNA in MCF-7 cells by RT-PCR. However, we cannot rule out that newly identified receptors such as SXR and PXR [reviewed by Blumberg and Evans (62)], which are activated by a variety of different compounds, including estrogen antagonists and agonists, could be involved in the activation/repression of some of the estrogen-sensitive genes we have identified. The reversal of estrogen-induced changes by ICI 182,780 observed for the four marker



genes, however, makes it unlikely that these genes are regulated through the new receptors (i.e., SXR and PXR).

Marker genes are often highly cell-type specific. For example, MAO-A and TGF $\beta$ 3 were expressed in the estrogen-dependent breast cancer cell line T47D, and both were repressed by estrogens; pS2 was undetectable; and the expression level of  $\alpha$ 1-ACT was very low, much below that in MCF-7 cells, although it probably is also induced by estrogen in T47D cells (results not shown). Thus, the diverse action of estrogens on different tissues and cell types is reflected in the genes that are estrogen regulated in the different cells; this suggests that cell-specific differences in gene expression might be exploited for the detection of new SERMs.

**Compounds have different potencies on different genes.** We demonstrated estrogenic activity for all the putative estrogenic test compounds, and the results correlate well with previous reports (9,11,19). Furthermore, because of its conversion to estrogens (63),  $10^{-7}$  M testosterone had an estrogen-like effect on the expression of the marker genes, whereas  $10^{-7}$  M progesterone had no effect on the expression levels (results not shown).

The four marker genes all responded strongly to the high-potency compounds ( $E_2$ , DES, and ZA), but there were some differences in their potency, defined as the minimum concentration required to affect the expression of a marker gene. The potencies of  $E_2$  and DES were similar when assayed with pS2, TGF $\beta$ 3, and  $\alpha$ 1-ACT, whereas MAO-A seems to be more sensitive to  $E_2$  than to DES. The potency of ZA was equal to that of  $E_2$  and DES if assayed with TGF $\beta$ 3, but it was more than 10-fold lower than  $E_2$  and DES for the other three marker genes.

The potencies of the environmental estrogens were much lower than those of  $E_2$ , DES, and ZA. As for the high-potency compounds, there were some differences in the relative potencies of the low-potency compounds on the four genes (Table 3). For example, the potency of NP was equal to the potency of GS on two marker genes (pS2 and TGF $\beta$ 3), but 10- to 100-fold higher and lower, respectively, when assayed with MAO-A and  $\alpha$ 1-ACT.

The relative potency of the compounds generally correlated with the relative change in expression level of the four genes, although this was most evident for pS2. For example, the expression level of pS2 was induced almost 25-fold after exposure to  $10^{-10}$  M  $E_2$ , approximately 16-fold after exposure to  $10^{-6}$  M GS, and < 10-fold after exposure to  $10^{-4}$  M DBP. For the other marker genes, this trend was less consistent. Fold induction/repression is very sensitive to the initial levels, and a slightly elevated level in the

noninduced cells leads to significant variation in the fold change. Thus, the precise fold change varies between experiments, whereas the relative differences between compounds are maintained.

High concentrations ( $10^{-8}$ – $10^{-6}$  M) of TAM-OH seem to have small but detectable  $E_2$ -like effects on the expression of MAO-A, pS2, and  $\alpha$ 1-ACT, whereas similar concentrations may have a small stimulatory effect on TGF $\beta$ 3. However, the required concentrations are so high that the effects may not be ER mediated; this is similar to what has been observed in SK-N-BE/SK-ER3 cells, in which the observed effects of tamoxifen apparently are independent of the presence of ER $\alpha$ . Unfortunately, the possible expression of ER $\beta$  has not been investigated in the SK-N-BE cells, although the lack of  $E_2$  response indicates that neither receptor subtype is present (48,49).

During the screening for estrogen-regulated genes, we detected several related genes that were consistently more sensitive to DES and ZA than to  $E_2$ . These genes, however, show a time-dependent expression profile that is different from the majority of the identified  $E_2$ -regulated genes; their expression was not affected before 8–16 hr exposure to the hormones (35), whereas almost all other  $E_2$ -regulated genes, including the four marker genes, were affected within 2–8 hr.

**Future development.** There are many aspects of the endogenous gene expression assay that could be improved. For example, we are assaying expression levels by radioactive incorporation during PCR, but fluorescent labels could be used instead, with the results analyzed on a DNA sequencer. In our hands, however, fluorescent labels reduce the sensitivity by 10- to 100-fold, although it may be possible to optimize both the PCR and the detection thereby obtaining a sensitivity that is similar to that of radioactivity-based assays. The Taq-Man technology (Perkin-Elmer) may be an attractive alternative. Another possibility is DNA chip technology (64,65). For example, construction of a chip with approximately 100 estrogen-regulated genes and a similar number of unaffected genes would allow almost complete automation of the assay. However, at present, the sensitivity of the chip technology limits its practical use; this will probably change, so DNA chip technology should be considered an alternative to PCR-based methods.

Endogenous gene expression assays could, in principle, be used to evaluate cellular responses to any compound, provided genes that respond to that class of compounds have been identified. Thus, compounds acting through the androgen, thyroid, aromatic hydrocarbon, and other receptors could be identified by determining the expression levels

of genes that are activated through these receptors. Assays to detect effects caused by activation of ER $\beta$  and the androgen receptor could be developed relatively easily from cell lines derived from the prostate.

Furthermore, endogenous gene expression assays are not limited to cell cultures because changes in expression levels of selected genes could be determined in different tissues from exposed animals. The number of animals required to assay thousands of compounds for their estrogenicity could be significantly reduced if gene expression-related end points could be established. For example, when the specific genes responsible for estrogen-induced uterine or breast growth have been identified and a relationship established between their expression and a disease, these genes could be assayed. An end point could then be fold induction of genes X and Y in tissue A and fold repression of gene Z in tissue B.

Finally, an ultimate goal would be to develop an assay to directly analyze human samples, for example, derived from blood. This requires identification of genes expressed in blood cells that are affected by exposure to putative endocrine-disruptor compounds. We are currently analyzing blood samples for the expression of the  $E_2$ -regulated genes that we identified in the screening; preliminary results suggest that many of the genes which are regulated by  $E_2$  in MCF-7 cells are also expressed in various cells present in blood. However, we currently do not have information concerning possible  $E_2$  regulation in the blood, although reports in the literature suggested that there are  $E_2$ -regulated genes in blood and that, for example, MAO-A may be a candidate for an  $E_2$ -regulated gene which could be assayed in human blood samples. (48).

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