A wide range of chemicals with diverse structures derived from plant and environmental origins are reported to have hormonal activity. The potential for appreciable exposure of humans to such substances prompts the need to develop sensitive screening methods to quantitate and evaluate the risk to the public. Yeast cells transformed with plasmids encoding the human estrogen receptor and an estrogen responsive promoter linked to a reporter gene were evaluated for screening compounds for estrogenic activity. Relative sensitivity to estrogens was evaluated by reference to 17β-estradiol (E2) calibration curves derived using the recombinant yeast cells, MCF-7 human breast cancer cells, and a prepubertal mouse uterotrophic bioassay. The recombinant yeast cell bioassay (RCBA) was approximately two and five orders of magnitude more sensitive to E2 than MCF-7 cells and the uterotrophic assay, respectively. The estrogenic potency of 53 chemicals, including steroid hormones, synthetic estrogens, environmental pollutants, and phytoestrogens, was measured using the RCBA. Potency values produced with the RCBA relative to E2 (100) included estrone (9.6), diethylstilbestrol (74.3), tamoxifen (0.0047), α-zearalanol (1.3), equol (0.0085), 4-nonylphenol (0.005), and butylbenzyl phthalate (0.0004), which were similar to literature values but generally higher than those produced by the uterotrophic assay. Exquisite sensitivity, absence of test compound biotransformation, ease of use, and the possibility of measuring antiestrogenic activity are important attributes that argue for the suitability of the RCBA in screening for potential xenoestrogens to evaluate risk to humans, wildlife, and the environment. Key words: estrogenic, environmental contaminants, estrogen, metabolism, phytoestrogens, potency, receptor, recombinant yeast cell bioassay, uterotrophic. Environ Health Perspect 105:734–742 (1997)

There is considerable concern within the scientific community and media that the increasing occurrence of endocrine-related abnormalities in humans and wildlife may be associated with exposure to environmental pollutants capable of mimicking or modulating the action of natural hormones. Appreciation that many ubiquitous compounds of natural and anthropogenic origins are estrogenic has led to the hypothesis that exposure to such compounds in utero may be involved in falling sperm counts and disorders of the male reproductive tract (1). Although such estrogenic compounds are structurally heterogeneous (2), there are similarities including lipophilicity that facilitate accumulation in food-producing animals and potentiate risks associated with entry into the human food chain (3). In the United Kingdom these concerns have been given added impetus following a report by the Institute for Environment and Health (4), which made several recommendations aimed at investigating the risks of hormonal mimics; these included the need to develop robust and reliable assays capable of screening chemicals for estrogenic activity. Given that prediction of estrogenic potency through structural information alone is not yet possible, development of such generic assays is necessary because many existing assay systems are not sufficiently robust for nonexpert operation or may not accurately reflect human potency.

Methods of detecting and assessing estrogenic compounds have been previously described (5) and critically evaluated (6). Biotransformation and consequent alteration of hormonal activity by test systems is an important consideration because pharmacological metabolites produced by the action of cytochrome P450 enzymes can be more potent than the parent compounds (7,8). Clearly, although such metabolic effects are intrinsic to in vivo models, they may not be so readily reflected by in vitro test systems. Estrogen-sensitive human breast cancer cells, such as MCF-7 cells, express the human estrogen receptor and have been used to develop E-screen tests for chemicals (9) and contaminants in animal feeds (10). Uterotropic assays such as the classical mouse uterus weight bioassay (11) have limitations (6), but are still preferred methods for many investigators (12). Uterotropic assays also offer the potential to combine biochemical and histological analysis of estrogen-mediated events and further adaptation to measure antiuterotropic activity (13).

The use of receptors linked to reporter genes in transformed cellular systems to detect biologically active xenobiotics has been proposed as a strategy to define chemicals by their functional properties (2). This approach facilitates detection of not only receptor ligand binding but also response element occupancy and gene activation. The recombinant yeast cells used in the present report have recently been used in an ultrasensitive RCBA for the determination of prepubertal plasma estrogens (14). These cells contain an expression plasmid that includes the CUP1 metallothionein promoter fused to the human estrogen receptor cDNA and a reporter plasmid which contains two copies of the frog vitellogenin estrogen response element upstream of the yeast iso-1-cytochrome c promoter fused to the structural gene for β-galactosidase. Similar recombinant yeast cell systems have been used for the detection of xenoestrogens (15,16) and various estrogen receptor studies (17–19). The particular advantage of the transformed yeast cell line approach is that the cells are robust and substrate auxotrophy may be used to continuously select for estrogen sensitivity. Moreover, expression of human estrogen receptor and ability to automate suggest that these yeast cells have much to offer the analyst requiring an in vitro screening assay that affords some reflection of potential estrogenic activity in humans.

In the present study, bioassays were evaluated by comparison of the potency of test compounds relative to 17β-estradiol (E2) since this natural estrogen is the most commonly accepted positive control used in such in vivo and in vitro assays (20,21). Validation of the recombinant yeast cell bioassay (RCBA) is addressed through comparison of potency values with an alternative established in vivo bioassay and with potency values produced using similar in vitro assays described in the literature. Similar approaches to validation have been commonly used (6,20) and serve to identify some of the merits and limitations of an assay relative to other in vitro and in vivo methods. The metabolic fate of test compounds in the selected bioassay is another
**Table 1. Comparison of RCBA results and estrogenic potencies described in the literature**

| Source* | Compound tested | RP | RIE | RPL |
|---------|-----------------|----|-----|-----|
| 1       | Steroidal estrogens |    |     |     |
| 17β-Estradiol | 100 | 100 | –    |
| 17β-Estradiol-3β-(o-glucuronide) | 0.32 | 89.6 | –    |
| 17β-Estradiol-3-sulfate | 0.01 | 20.4 | –    |
| 17β-Estradiol-3-glucuronide-3- sulfate | 0 | 0 | –    |
| 17α-Estradiol | 5.25 | 100 | 49.9, 86c |
| Estrone | 9.6 | 100 | 5.766, 86c |
| Estradiol | 0.63 | 88 | 16.14c |
| Steroids |    |     |     |     |
| Cholesterol | 1 | 0 | –    |
| Androstenedione | 0 | 0 | –    |
| Testosterone | 0.001 | 0.8 | 0.01c |
| Androstenediol | 0.023 | 31 | –    |
| Dehydroepiandrosterone | 0.0018 | 1.2 | –    |
| Cortisol | 0 | 0 | 0.01c |
| Progesterone | 0 | 0 | 0.01c |
| α-Norgestrel | 0.0004 | 3.1 | –    |
| Synthetic estrogens |    |     |     |     |
| 17α-Ethynylestradiol | 88.8 | 100 | 191b, 95c |
| Mestranol | 7.3 | 80 | 8d |
| Diethylstilbestrol | 74.3 | 100 | 246b, 73c, 76a |
| Hexestrol | 30.6 | 89 | 74b, 55b, 319b, 5f |
| Dienestrol | 25.4 | 84 | 26c, 102a |
| Triphenylethylene antiestrogens |    |     |     |     |
| Tamoxifen | 0.0047 | 46 | <0.01d |
| 4-Hydroxytamoxifen | 0.0073 | 56 | – |
| Nafoxodine | 0 | 0 | 0.01d |
| Fungal resorcyclic acid lactones |    |     |     |     |
| Zearelanone | 0.26 | 91 | 1.4a, 0.85b, 0.5f |
| α-Zearelanol | 8.7 | 67 | 27c |
| β-Zearelanol | 0.068 | 58 | 0.43c, 0.043c, 0.07c |
| α-Zearelanol (zeareanol) | 1.3 | 84 | 17b, 47c |
| β-Zearelanol | 0.46 | 0.5 | 3.4f |
| Physosterogens |    |     |     |     |
| Coumestrol | 0.67 | 75 | 0.11a |
| Equol | 0.085 | 50 | 0.2b |
| Daidzein | 0.0013 | 5.4 | 0.00072, 0.01f |
| Formononetin | 0.0056 | 23 | 0.0004 |
| Isoflavone | 0.0091 | 61 | 0.0048b, 0.0005f |
| Genistein | 0.049 | 61 | 0.08b, 0.011f |
| 4-Alkylphenols |    |     |     |     |
| 4-Nonylphenol (technical grade) | 0.005 | 57 | 0.003d |
| 4-Nonylphenol (straight chain) | 0.0022 | 38.3 | – |
| 4-Octylphenol | 0.003 | 22.3 | 0.03f |
| 4-tert-Octylphenol | 0.00036 | 43 | – |
| Organochlorines |    |     |     |     |
| DDT | 0.00003 | 0.8 | – |
| o,p'-DDT | 0.00011 | 2.4 | – |
| o,p'-DDE | 0.00004 | 1.0 | – |
| 2,3,7,8-Tetrachlorodibenzo-p-dioxin | 0.26 | 5.5 | – |
| Methoxychlor | 0.003 | 55 | – |
| 2-Chloro-4-biphenyl | 0.06 | 73.3 | 0.025b |
| 2'-Chloro-4-biphenyl | 0.0037 | 32 | – |
| 2',5'-dichloro-4-biphenyl | 0.62 | 65 | 0.19b |
| 2,4,8-Trichloro-4-biphenyl | 1.0 | 67.6 | 2.38b |
| 2',3',4',5'-Tetrachloro-4-biphenyl | 0.82 | 77 | 1.05b |
| 3,3',5,5'-Tetrachloro-4,4'-biphenyliod | 0.016 | 83.5 | 0.074b |
| Other contaminants |    |     |     |     |
| Bisphenol A | 0.005 | 51 | 0.003b, 0.05-0.02b |
| Di-n-butylphthalate | 0 | 0 | – |
| Butylbenzylphthalate | 0.0004 | 5.3 | 0.0003b |
| Bis(2-ethylhexyl)phthalate | 0 | 0 | – |
| Butylhydroxytoluene | 0 | 0 | – |

Abbreviations: E2, 17β-estradiol; RP, relative potency compared to E2 (100) by molar mass determined with the recombinant yeast cell bioassay; RIE, relative inductive efficiency indicates the ratio between maximal

(continued, next column)

**Materials and Methods**

*Source of chemicals. [3H]Coumestrol (518 GBq/mmol; custom synthesis), di-n-butyl [carboxyl-14C]phthalate (962 MBq/mmol), [monoethyl-3H]diethlylstilbestrol (3.4 TBq/mmol), 4-n-nonyl[ring (2.6(n)-3H)phenol (1.96 TBq/mmol; custom synthesis), [6,7-3H]17β-estradiol (1.97 TBq/mmol), [3H)testosterone (3.88 TBq mmol), and α-[3H]zearanol (1.92 TBq/mmol; custom synthesis) were obtained from Amersham International (Little Chalfont, U.K.). Radiochemical purity was checked by radio-HPLC using the conditions described below and found to be greater than 96% in all cases. Sources of chemicals tested for estrogenic potency are provided in Table 1.*

**Transformed yeast cells.** Recombinant estrogen sensitive yeast cells (14) were stored in 30% glycerol at -80°C and grown as required at 30°C on selective media agar plates containing 0.17% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% (NH4)2SO4, 2% dextrose, L-leucine (60 μg/ml), L-histidine (20 μg/ml) and 2% Bacto agar (Difco, East Molsey, U.K.).

**RCBAs of estrogens.** Procedures that were followed were similar to those described previously (14). Briefly, a small colony of

(continues, next column)
yeast cells was sampled from an agar plate and incubated for 18 hr at 30°C by continuous shaking in 2 ml selective media broth. The yeast cells were collected by centrifugation and diluted in 1 M mannose to an optical density of 0.1 (A490). Yeast cell suspension was dispensed (20 μl) into wells of 96-well plates (Nunc; Life Technologies, Paisley, U.K.) containing 75 μl of 50 μM copper sulfate in selective media and incubated with 25 μl calibration standard. Calibration standards were prepared in ethanol at a concentration of 10 mM and serially diluted (×10) in selective media broth. After an 18-hr incubation at 30°C, the yeast cell density in the wells was estimated from the optical density at 630 nm using a Dynatech microtiter plate reader (Dynatech Labs, Billingshurst, U.K.). Induction of β-galactosidase activity was measured by addition of 100 μl of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 50 mM mercaptoethanol, 1 mM MgSO4) containing 2 mg/ml α-nitrophenyl-β-D-galactopyranoside (ONPG), 0.1% sodium dodecyl sulfate, and 250 units/ml lyticase (Sigma Chemical Co., Poole, Dorset, U.K.) to each well, and quantified by determination of α-nitrophenol production expressed as (OD410nm/OD630nm) × 1000/min. Wells were read at 6 min and at 4 hr according to the level of induced β-galactosidase activity.

**RCBA of antioestrogens.** β-Galactosidase activity induced by E2 (100 fm–100 nM) in the presence of the potent antioestrogen 4-hydroxytamoxifen (1 nM–10 μM) was investigated with recombinant yeast cells as described above.

**Estrogenic potency of test compounds in RCBA.** A range of chemicals was tested for estrogenic activity in at least two separate experiments using the RCBA. Calibration curves of E2 and test compounds were produced by analysis of standards (100 fm–10 nM for E2 and potent estrogens and 10 pM–10 μM for weak estrogens) in selective media for estrogenic activity. Each calibration standard of a test compound was analyzed in 8 wells (1 column) and assay blanks (vehicle only) in 16 wells (2 columns) of a 96-well microtiter plate. Each test compound was assayed in a separate 96-well microtiter plate and the wells were sealed with film plate sealers. β-Galactosidase activity in blank wells (typically OD410nm 0.080 ± 0.012 and 0.109 ± 0.009 at 6 min and 4 hr, respectively) was subtracted from wells containing standard concentrations of test chemicals. The relative potency of test compounds was determined from the concentration of E2 and test compound that provided 50% induction of β-galactosidase activity (EC50) and calculated by dividing the EC50 for the test compound by the EC50 for E2. The relative potency of weak estrogens that failed to induce β-galactosidase to 50% of the total E2 activity was determined from the concentration of E2 and test compound that provided the same induction of β-galactosidase activity and calculated similarly. The relative inductive efficiency (RIE) was determined as the ratio of maximal β-galactosidase activity induction with test compound to E2 × 100.

**Uterotrophic assay.** Prepubertal 18-day-old C57BL/6J female mice (Harlan U.K. Ltd., Huntingdon, U.K.) were injected scutaneously with 0.1 ml test compound dissolved in corn oil at the doses shown in Table 2, on three consecutive days, essentially as described by Rubin et al. (17). In each assay, five concentrations of E2 (calibrant) and three concentrations of test compound were injected using seven animals per dose. Mice were sacrificed on the fourth day and the weights of the animal and uteri recorded; a sample of vagina was fixed in phosphate buffered saline containing 1% formaldehyde and processed for routine histological evaluation. Results are expressed as the mean ratio of uterine weight to body weight. The relative potency of test compounds was determined by interpolation from calibration curves of the molar dose of E2 and test compounds that provided similar increases in ratios of uterine wt/body wt × 100. Care and treatment of the mice was in accordance with the Animals (Scientific Procedures) Act 1986 of the United Kingdom and supervised by a veterinary surgeon.

**Human breast cancer cell proliferation assay.** MCF-7 human breast cancer cells were maintained in Eagles minimal essential medium (EMEM) supplemented with 5% horse serum and 1% antibiotic-antimycotic solution.

### Table 2. Effect of test compounds on prepubertal mouse uterine weight and comparison with the RCBA

| Compound                    | Dose (ng) | Uterine wt/body wt (mean ± SD) | Relative uterotrophic potency (molar) | Relative potency (RCBA) |
|-----------------------------|-----------|-------------------------------|---------------------------------------|-------------------------|
| Control                     | 0         | 0.097 ± 0.030                 | –                                     | –                       |
| 17β-Estradiol               | 5 ng      | 0.172 ± 0.028**               | 100                                   | 100                     |
|                             | 10 ng     | 0.247 ± 0.030*                | –                                     | –                       |
|                             | 25 ng     | 0.405 ± 0.114f                | –                                     | –                       |
|                             | 50 ng     | 0.420 ± 0.121f                | –                                     | –                       |
|                             | 100 ng    | 0.594 ± 0.126f               | –                                     | –                       |
| Diethylstilbestrol          | 5 ng      | 0.076 ± 0.029                 | 234                                   | 74.3                    |
|                             | 50 ng     | 0.137 ± 0.034**               | –                                     | –                       |
| 4-Nonylphenol (technical grade, Sigma) | 0.086 ± 0.030  | 0.00036                      | 0.005                               | –                       |
|                             | 1 mg      | 0.094 ± 0.021                 | –                                     | –                       |
|                             | 5 mg      | 0.351 ± 0.192*               | –                                     | –                       |
|                             | 20 mg*    | –                              | –                                     | –                       |
| 4-Octylphenol               | 0.5 mg    | 0.074 ± 0.034                 | 0                                     | 0.003                   |
|                             | 1 mg      | 0.097 ± 0.034                 | –                                     | –                       |
|                             | 5 mg      | 0.085 ± 0.036                 | –                                     | –                       |
| Coumestrol                  | 1 μg      | 0.104 ± 0.026                 | 0.024                                 | 0.67                    |
|                             | 10 μg     | 0.132 ± 0.009f               | –                                     | –                       |
|                             | 100 μg    | 0.436 ± 0.182f               | –                                     | –                       |
| Benzybutylphthalate         | 0.05 mg   | 0.086 ± 0.024                 | 0                                     | 0.0004                  |
|                             | 0.5 mg    | 0.093 ± 0.031                 | –                                     | –                       |
|                             | 5 mg      | 0.108 ± 0.027                 | –                                     | –                       |
| Dibutylphthalate            | 0.05 mg   | 0.119 ± 0.036                 | 0                                     | 0                       |
|                             | 0.5 mg    | 0.101 ± 0.028                 | –                                     | –                       |
|                             | 5 mg      | 0.091 ± 0.014                 | –                                     | –                       |
| α-Zearalenol                | 1 μg      | 0.139 ± 0.029**               | 0.028                                 | 1.3                     |
|                             | 10 μg     | 0.190 ± 0.039f               | –                                     | –                       |
|                             | 100 μg    | 0.411 ± 0.27**               | –                                     | –                       |
| Bisphenol A                 | 0.05 mg   | 0.098 ± 0.014                 | 0                                     | 0.005                   |
|                             | 0.5 mg    | 0.092 ± 0.021                 | –                                     | –                       |
|                             | 5 mg*     | –                              | –                                     | –                       |

Abbreviations: RCBA, recombinant yeast cell bioassay; SD, standard deviation. Potency values determined with the murine uterotrophic assay are presented with the RCBA for comparison. Statistical significance is relative to untreated controls.

*Animals from this treatment group were withdrawn from the study due to toxic effects of the dose. *p<0.05; **p<0.01; ***p<0.001.
After was washed with above dilution, the cultures were treated with medium containing phenol red-free and replaced with medium containing phenol red-free EMEM, dextran charcoal stripped FCS, supplemented as above with the exception of insulin. After a further 3-day incubation period to enable the MCF-7 cells to return to basal estrogen non-stimulated status (18) the cultures were treated with medium containing E2 (1-10,000 pM dissolved in ethanol vehicle) and incubated for 5 days. The cell number per flask was determined by counting cell nuclei as described previously (20).

**Biotransformation of steroids and xenoestrogens by yeast cells.** Recombinant yeast cells were suspended at an appropriate dilution in minimal media so as to provide an optical density of 0.1 (A630 nm); these and similar blank cultures (without yeast cells) were incubated at 30°C with 100 nM 37 KBq/ml tritium labeled coumestrol, E2, diethylstilbestrol, 4-nonylphenol, testosterone, and a-zearanol and 10 μM [14C]di-n-butylphthalate for 18 hr. The medium was sampled after incubation periods of 1, 4, and 18 hr; it was then centrifuged and stored at -20°C prior to analysis by radio-HPLC.

**Metabolite analysis.** Samples of medium were analyzed for the presence of metabolites by reverse phase radio-HPLC using either a Hypersil BDS C18 3 μm 100 × 4.6 mm (Life Sciences International, Runcorn, U.K.) or a Spherisorb ODS 5 μm 250 × 4.6 mm (Jones Chromatography, Hengoed, U.K.) column (for di-n-butylphthalate only) and chromatographed at a flow rate of 1 ml/min. Conditions were selected to provide separation of parent compounds from metabolites using conditions similar to those described previously (27). Di-n-butylphthalate, testosterone, and E2 analysis used an isocratic mobile phase consisting of 35% acetonitrile/65% water for 15 min and a-zeralanol, coumestrol, and diethylstilbestrol analysis used a linear gradient mobile phase system consisting of 50:50 water methanol containing 0.1% acetic acid to 100% methanol, 0.1% acetic acid, over 25 min. 4-Nonylphenol analysis employed a linear gradient from 20:80 methanol/water to 100% methanol over 15 min, which was maintained for an additional 5 minutes.

The above studies were all conducted at the Central Veterinary Laboratory, United Kingdom, between July 1995 and February 1997.

**Results**

**Evaluation of bioassay sensitivity to E2.** The sensitivity of the RCBA, MCF-7 human breast cancer cell, and uterotrophic bioassays were compared by evaluation of respective E2 calibration curves. Direct comparison of the two in vitro assays with the in vivo assay was enabled by conversion of the concentration units of E2, used in the RCBA (100 fm–100 nM) and the MCF-7 proliferation (1–10,000 pM) bioassays to total moles added to the in vitro system during the assay because this could be equated with the quantity of...
administered dose (mole per mouse) used in the in vivo mouse uterotrophic assay. The endpoints recorded for each bioassay, namely induction of β-galactosidase activity (RCBA), increase in cell number (MCF-7 cell proliferation assay), or uterine weight (prepubertal mouse uterine weight assay), were normalized to percentage response. E₂ significantly (Students t-test) increased RCBA β-galactosidase activity (p<0.01), MCF-7 cell proliferation (p<0.05), and uterine weight (p<0.01) at total doses of 10<sup>-17</sup>, 5 × 10<sup>-15</sup>, and 1.83 × 10<sup>-11</sup> moles respectively, relative to controls.

E₂ calibration curves generated with these three bioassays are shown in Figure 1. In terms of molar dose, the RCBA was approximately two and five orders of magnitude more sensitive to E₂ than the MCF-7 cells and uterotrophic bioassays, respectively. The dynamic range of percentage response to E₂ [mean ± standard deviation (SD)] was at least an order of magnitude greater for the RCBA (17.37 ± 10.8–7754 ± 3370%) than either the MCF-7 proliferation (30 ± 13–175 ± 25%) or uterotropic (79 ± 28–472 ± 130%) bioassays. E₂ calibration curves for the recombinant yeast cell and uterine weight bioassays without prior data normalization are shown in Figure 2 and Table 2, respectively. Potential interference of estrogenic residues leaching from the plastic microwells in the RCBA was determined by comparing E₂ calibration curves prepared in untreated and washed (5 times with 0.1 ml ethanol) 96-well plates. No significant difference between either blank values (wells without E₂) or E₂ calibration curves prepared in the different plates was found.

**Application of the RCBA to test compounds.** Treatment of recombinant yeast cells with estrogens for 18 hr produced dose-dependent increases in β-galactosidase activity of approximately 2–3 orders in magnitude. Typical dose–response curves (not all from the same experiment) for E₂, coumestrol, equol, bisphenol A, 4-nonylphenol, zearalanol, and dibutylphthalate are shown in Figure 2.

Relative estrogenic potency and inductive efficiency of test compounds in the RCBA. Dose–response curves for 53 chemicals were produced using the RCBA; from these, estrogenic potency and maximal β-galactosidase induction values relative to E₂ were determined (Table 1). The synthetic stilbenes diethylstilbestrol, hexestrol, and dienestrol and the synthetic steroid 17α-ethynylestradiol had the highest relative potencies and were of a similar order to E₂. Environmental pollutants such as the 4-alkylphenols were among the weakest estrogens tested, with relative potencies 4–5 orders of magnitude less than E₂. Steroids including cholesterol, androstenedione, progesterone, and cortisol had either very weak or undetectable estrogenic activity. The triacylhydride antiestrogens nafodixone, tamoxifen, and 4-hydroxytamoxifen [a potent metabolite of tamoxifen (28)] also had weak estrogenic activity. Many of the test compounds produced relative inductive efficiencies of less than 100%; this was taken to indicate that they are partial estrogen receptor agonists. Potency values of some of the compounds tested with the RCBA were also taken from literature sources and are listed for comparative purposes in Table 1.

**Effect of E₂ + 4-hydroxytamoxifen in RCBA.** The effect of 4-hydroxytamoxifen (10<sup>-9</sup>–10<sup>-5</sup> M) and E₂ (10<sup>-13</sup>–10<sup>-7</sup> M), both singly and in combination, on β-galactosidase activity induction is shown in Figure 3. β-Galactosidase activity was significantly stimulated (p<0.05) by E₂ (10<sup>-13</sup>–10<sup>-7</sup> M) and by 4-hydroxytamoxifen (10<sup>-9</sup>–10<sup>-5</sup> M) compared with blank wells. The potential antagonist activity of 4-hydroxytamoxifen was investigated by addition of 4-hydroxytamoxifen (10<sup>-9</sup>–10<sup>-5</sup> M) to E₂ calibration curves (10<sup>-13</sup>–10<sup>-7</sup> M). β-Galactosidase activity stimulated by 1 nM E₂ was significantly reduced (p<0.05) by inclusion of 4-hydroxytamoxifen (10<sup>-9</sup>–10<sup>-5</sup> M) compared with 1 nM E₂ alone; indicating antagonism of E₂ binding; to clarify this point, these data (box, Fig. 3) are replotted as percentage inhibition of E₂-stimulated β-galactosidase activity in Figure 4. Similarly, β-galactosidase activity stimulated by 10<sup>-8</sup> and 10<sup>-7</sup> M 4-hydroxytamoxifen compared with E₂ alone. 4-Hydroxytamoxifen displayed agonist activity when tested in isolation; as a result, β-galactosidase activity was significantly (p<0.05) elevated by relatively high doses of 4-hydroxytamoxifen when tested with a low dose of E₂ (e.g., 10<sup>-13</sup> M E₂ + 10<sup>-9</sup>–10<sup>-5</sup> M 4-hydroxytamoxifen) when compared with E₂ alone (Fig. 3).

**Determination of relative estrogenic potency using the mouse uterine weight assay.** The uterotrophic effect and relative estrogenic potencies of E₂, diethylstilbestrol, 4-nonylphenol, 4-octylphenol, coumestrol, α-zearalanol, bisphenol A, dibutylphthalates, and benzyloxyphthalates are shown in Table 2. 4-Nonylphenol, coumestrol, α-zearalanol, and diethylstilbestrol significantly (p<0.05) increased uterine weight at the doses shown. After the first injection of the highest doses of 4-nonylphenol and bisphenol A, symptoms of acute toxicity were evident and, as a consequence, these mice were immediately withdrawn from the study and euthanized.

**Xenobiotic biotransformation.** Analysis of incubation medium revealed no evidence of xenobiotic metabolism following incubation of yeast cells with E₂, coumestrol, diethylstilbestrol, testosterone, and 4-nonylphenol. In the case of α-zearalanol, a single metabolite (5% of total) was detected in the medium after 18 hr (data not shown).

**Discussion**

High sensitivity is an important requirement for screening assays to enable detection of compounds of low potency that may be of
biological significance through chronic exposure and/or high abundance in the environment. The effect of E₂ on MCF-7 cell proliferation and uterotrophic activity found in the present study was similar to that observed in previous reports (10,11,29).

The recombinant yeast cells were designed and engineered for exquisite sensitivity to estrogens (17,30,31); overexpression of human estrogen receptor, high amplitude frog vitellogenin estrogen response elements, and their tandem arrangement in the reporter plasmid all serve to amplify β-galactosidase production and hence sensitivity to E₂ (14). However, evaluation of the relative merits of these bioassays for estrogens cannot be based on sensitivity criteria alone; summary findings for the different assays used in this report and a similar recombinant yeast screen are presented for comparison in Table 3. Adaptation of reporter gene assays to the 96-well plate format enables automation on standard laboratory instrumentation and a marked reduction in sample size. In contrast, the prepubertal mouse uterine assay requires a large mass of sample, is relatively labor intensive, requires the sacrifice of large numbers of animals, and is sensitive to the potentially diverse toxic effects of test compounds.

Interaction of activated receptor–ligand complex with human estrogen responsive elements and the opportunity to measure diverse estrogen-regulated gene products such as the progesterone receptor is a recognized advantage of cancer cell-based screens (24). Uterotrophic bioassays also offer the simultaneous measurement of other markers of estrogen action including vaginal and uterine cell proliferation, which may be used to provide definitive confirmation of estrogenic activity (32). One potential disadvantage of MCF-7 cells is that under certain conditions they may also express receptors for other classes of steroids (33), and other hormones including progestagens have been shown to increase breast cancer cell proliferation (34). Similarly, under certain circumstances uterotrophic assays are limited in selectivity for estrogens by sensitivity to androgens and progestagens (6). For these reasons, expression of single class of steroid receptor, as in the RCBA system, is highly desirable since this provides a system with appropriate sensitivity and specificity to a single class of hormone. Moreover, the genetic simplicity of yeast cells separates estrogen receptor signaling from the confounding effects of other signaling pathways in the cell.

Evaluation of the relative estrogenic potency of steroids from different hormone classes is necessary to demonstrate and establish assay selectivity for estrogens. This important element of assay validation also provides a reference scale for estrogenic activity found with the RCBA relative to compounds generally accepted to be either estrogens or without estrogenic activity. Many of the compounds tested with the recombinant yeast cell bioassay did not induce β-galactosidase activity to the same extent as E₂. Similar observations have been made with the E-screen breast cancer cell proliferation assay and were interpreted as indicating that the xenobiotics tested were partial agonists (24). Evaluation of estrogenic activity with the RCBA in terms of relative potency alone can be misleading because several androgens (e.g., testosterone) have estrogenic potencies of a similar order to those of, for instance, the alkylphenols. Consideration must also be given to the relative inductive efficiency of β-galactosidase activity by test compounds. In this regard androgens were much weaker estrogens than alkylphenols. Although androgens have uterotrophic (6) and mitogenic activity (33) at pharmacological doses in vivo, they are unlikely to be of physiological significance as estrogens because endogenous concentrations are several orders of magnitude less than used in such assays. A slight response to testosterone has been reported with a similar yeast screen for estrogens (15); indeed, the potency of testosterone found with the RCBA was an order of magnitude lower than that found with a competitive binding radioreceptor assay (22). Synthetic estrogens including 17α-ethynylestradiol and the stilbenes were among the most potent estrogens tested comparable with E₂. This contrasts with the relative estrogenic potency of most of the phytoestrogens and environmental pollutants, which were several orders of magnitude less potent than E₂. Phytoestrogens have been ranked by potency in a HeLa cell

![Figure 4. Percentage inhibition of 17β-estradiol (E₂, 1nM) induced β-galactosidase activity in recombinant yeast cells by 4-hydroxytamoxifen (1 nM–10 μM). Error bars represent 1 standard deviation (n = 8).](image)

**Table 3. Comparison of assays used to determine estrogenic activity**

| Attribute                        | RCBA | Recombinant yeast screen | MCF-7 breast cancer cells | Mouse uterotrophic assay |
|----------------------------------|------|--------------------------|---------------------------|--------------------------|
| Sensitivity to E₂                | 100 fm | 7.3 pM (2 ng/l) | 1 pM | 18 pmol |
| ER                               | Human | Human | Human | Mouse |
| Estrogen response elements       | Frog | Not stated | Human | Mouse |
| Detection of antiestrogenic activity | Poor | Not tested | Good | Good |
| Assay endpoints                  | β-gal | β-gal | Cell proliferation, etc., e.g., PgR | Uterine weight, etc. (e.g., PgR) in other tissues (e.g., vagina) |
| Steroid receptors expressed      | ER | ER | ER, PgR, AR, GR | ER, PgR, AR, GR |
| Detection of proestrogens        | No | Not stated | Limited ? | Yes |
| Assay duration                   | 18 hr | 3–4 days | 7 days | 4 days |
| Automation in 96-well plate format | Yes | Yes | Yes | No |

Abbreviations: RCBA, recombinant yeast cell bioassay; β-gal, β-galactosidase assay; ER, estrogen receptor; PgR, progesterone receptor; AR, androgen receptor; GR, glucocorticoid receptor. *Recombinant yeast screen described by Routledge and Sumpter (19).
cotransfection reporter gene assay (20) and were found to operate as complete agonists in this system. The phthalates and certain organochlorines (DDT, o,p'-DDT, and DDE) had little estrogenic activity in the recombinant yeast cell bioassay in conflict with other reports (9,36). Various factors including protein binding (16) and molecular permeation into the yeast cells (18), which have not been considered in the present study, cannot be excluded and may provide a partial explanation for these discrepancies. The transformed yeast cells used in the RCBA are a stable cell line and, as such, are robust and well suited for use by other laboratories in further studies, which may reveal the nature of these disparities and further identify strengths and limitations of this assay. Stimulation of β-galactosidase activity by 2,3,7,8-tetrachlorodibenz-p-dioxin was an unexpected but reproducible finding and merits further investigation of the mechanism. Competitive binding studies have shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin does not bind to the estrogen receptor, but dioxins can evoke estrogenic and diverse potent antiestrogenic effects through various cellular mechanisms (37,38).

Comparison of the relative estrogenic potency values derived from the RCBA with those using similar in vitro methods described in the literature (Table 1) provides a further basis for validation against current methods. The RCBA produced relative estrogenic potency values that were within an order of magnitude of those described in the literature with very few exceptions (e.g., daidzein). Indeed, variation of this order are apparent between literature values (see Table 1) both within and between laboratories according to the particular assay used (20,39). Both the RCBA and mouse uterotrophic assay indicate that 4-nonylphenol is a more potent estrogen than 4-octylphenol, contrary to other reports (24,40), although the potency of 4-nonylphenol obtained with the RCBA was very similar to the E-screen (24). Variation in the estrogenic potency of some 4-tert-nonylphenol preparations has been found (15), raising the possibility that the purity of chemicals may provide an explanation for the disparity between assays; alternatively, differences in biotransformation or those inherent to the assay system are plausible explanations. However, this comparative study demonstrates that the RCBA provides estimates of relative estrogenic potency that are reproducible and broadly similar with other in vitro methods. In addition to yeast cells, reporter gene assays have also been introduced into breast cancer cells and other cell lines and produced comparable potency values for alkylphenolic compounds (40). Such reporter gene assays (19,20) and breast cancer cell proliferation assays (10,24) are typically sensitive to E2 at concentrations of 1 pM (Table 3).

4-Hydroxytamoxifen is a metabolite of tamoxifen and a potent antiestrogen (28). Although the RCBA demonstrates the ability of 4-hydroxytamoxifen to significantly inhibit E2-induced β-galactosidase activity, the response was only weak. The incomplete suppression of E2-induced β-galactosidase activity observed may be due to the intrinsic estrogenicity of certain triarylethylene antiestrogens like tamoxifen (41). Given the relatively poor response to the potent antiestrogen 4-hydroxytamoxifen, it is anticipated that this system would not have sufficient sensitivity to detect weaker antiestrogens. More significant inhibition of E2-induced activity by triarylethylene antiestrogens has been demonstrated in reporter gene assays in mammalian cells (19,20,40) and breast cancer cell proliferation assays (42). A yeast cell system similar to the RCBA (18) was sensitive to the estrogen but not antiestrogenic activity of tamoxifen. Thus, these data indicate the need for further adjustment of either the RCBA assay conditions or to the plasmids that confer such extreme sensitivity to estrogen to provide more sensitive detection of antiestrogenic activity. Various co-activators and transactivating sequences have been identified that may be required for steroid receptor transcriptional activity and discrimination between agonist- and antiestrogen-activated receptors in specific cell types (43). Antiestrogens may also operate by other cellular mechanisms including antagonism of calmodulin-regulated processes (42) to which steroid receptor reporter gene systems are likely to be insensitive. Assays with the potential to detect antiestrogens are of particular value because they may be important in evaluating certain classes of compounds such as the phytoestrogens and dioxins, which may have antiestrogenic activity and provide chemoprevention of certain cancers (38,44).

In vitro bioassays for estrogenic activity are of particular value because they provide a means of confirming the potential biological significance of in vitro findings (36). The uterotrophic assay used in the present study produced estrogenic potencies that were relatively imprecise because only three doses of test compound were assayed. Nonetheless, with the exception of diethylstilbestrol, lower potency values were derived using the uterotrophic assay compared with the recombinant yeast cell assay. Moreover, several compounds that have estrogenic activity in vitro, including the phthalates, 4-octylphenol, and bisphenol A, had no detectable uterotrophic activity. Histological evaluation of vagina sections enabled further confirmation of estrogenic activity, as in all cases, uterotrophic activity was associated with cornification and thickening of the stratified squamous epithelium. Clearly, the biological significance of very weak estrogens detected with highly sensitive in vitro bioassays requires careful evaluation if activity in vivo is absent or found only at very high doses, which may exceed those giving rise to acute in vivo toxicity. Pharmacokinetic parameters (adsorption, metabolism, tissue distribution, and excretion) may have a significant influence on estrogenic activity in vivo, which cannot be readily accounted for in in vitro assays. These and other inherent differences between the RCBA and the murine uterotrophic assay will produce different potency values as found for diethylstilbestrol and other chemicals shown in Table 2. Chronic exposure in vivo may facilitate the bioaccumulation of pollutants and their metabolites and thereby give rise to estrogenic activity not evident in acute (3 day) uterotrophic studies. The current situation is further complicated by recent conflicting reports (45,46) relating to the potential for substantial synergistic interactions between xenosterogens.

Biotransformation of xenobiotics in vivo is usually part of a process of deactivation and elimination, but proestrogens (e.g., certain phytoestrogens and organochlorines) may undergo metabolic activation to potent estrogens in various body compartments. Although the range of compounds tested for biotransformation in the RCBA was limited, these studies provide valuable information on the fate of test compounds in this type of system. The corollary to the absence of significant steroid and xenobiotic biotransformation by the recombinant yeast cells is that, in their present form, they could not be utilized for the detection of proestrogens. Proestrogen detection in vitro has been accomplished by co-incubation of metabolizing and estrogen detecting systems (28,47). In this regard, transformed yeast cells would be suitable for combination with an appropriate metabolizing system for the detection of species-specific proestrogens since there would be little interference from yeast cell xenobioc metabolism. By contrast, other mammalian cell assay systems such as MCF-7 breast cancer cells will also have the potential for significant steroid and xenobiotic biotransformation; in isolation, such intrinsic metabolism may confound interpretation of results from a simple screen for estrogenic activity. Although cytochrome P450 activities are poorly maintained in vitro by isolated cells, steroid metabolizing enzymes, including aromatase, hydroxysteroid oxidoreductases, and estrogen sulfa-
tase, have been described in human breast cancer cells. Thus, a comprehensive knowledge of intrinsic metabolic activity is required before a test system can be fully validated and accepted as a good standard method.

Conclusion

The RCBA is a highly sensitive human estrogen receptor-based screening assay for the rapid detection of estrogens. The estrogen potency and relative inductive efficiency of 53 chemicals has been evaluated with this system. Potency values produced with the RCBA were similar to literature values, but were generally higher than those produced with a murine uterotrophic assay. The mechanism through which TCDD stimulates β-galactosidase activity in the RCBA warrants further investigation. Because the RCBA cannot detect proestrogens and has limited sensitivity to antiestrogens, further development is required to broaden the application to the detection of such compounds. The RCBA may be suitable for use by regulatory bodies, such as the FDA in the United States and the Environment Agency in the United Kingdom, as a simple, rapid, and inexpensive method of evaluating intrinsic estrogen activity of wide-ranging chemical classes and may provide a preliminary means of evaluating the risk presented by such chemicals to the public, wildlife, and the environment.

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