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Journal of Cellular Biochemistry 105:1399–1409 (2008)

Journal of Cellular Biochemistry

Establishment of a Transgenic Yeast Screening System for Estrogenicity and Identification of the Anti-Estrogenic Activity of Malachite Green

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ABSTRACT

Endocrine disruptors refer to chemical compounds in the environment which interfere with the endocrine systems of organisms. Among them, environmental estrogens pose serious problems to aquatic organisms, in particular fish. It is therefore important and necessary to have a fast and low-cost system to screen the large number of different chemical compounds in the aquatic environment for their potential endocrine disrupting actions. In this study, a screening platform was developed to detect xenoestrogens in the aquatic environment using the fission yeast *Schizosaccharomyces pombe*, and applied for compound screening. The aim was to demonstrate any significant potential differences between the fish screening system and the human screening system. To this end, a yeast expression vector harboring a fish estrogen receptor α and a reporter vector containing the estrogen responsive element fused with the *Escherichia coli LacZ* gene were constructed. After transformation with these two vectors, the transformed yeast clones were confirmed by Western blotting and selected on the basis of the β-galactosidase activity. In this transgenic yeast system, the natural estrogen (estradiol) and other known xenoestrogens such as diethylstilbestrol, bisphenol A, genistein and dichloro-diphenyl-trichloroethane exhibited dose-dependent activities. Using this system, more than 40 putative endocrine disruptors including phytoestrogens, pesticides, herbicides, industrial dyes and other industrial chemicals were screened. Ten of them were demonstrated to exhibit estrogenic actions. Industrial dyes such as malachite green (MG) that disrupt thyroid hormone synthesis are extensively used and are widely distributed in the aquatic environment. Using this system, MG did not show any estrogenic action, but was demonstrated to exhibit anti-estrogenic activity. J Cell. Biochem. 105: 1399–1409, 2008.

KEY WORDS: ENDOCRINE DISRUPTION; TRANSGENIC YEAST; ESTROGENICITY SCREENING; MALACHITE GREEN; ANTI-ESTROGEN

A number of hormonally active agents such as the synthetic estrogen diethylstilbestrol (DES), the pesticide dichloro-diphenyl-trichloroethane (DDT), the dioxins, and the plant secondary metabolites phytoestrogens have been demonstrated to exert adverse actions on living organisms. Among these adverse effects the estrogenic activity of these compounds is an important indicator of their endocrine disrupting actions. Due to the discharge of effluents into aquatic surface waters, fish and aquatic animals are considered the prime risk organisms of endocrine disruption by environmental chemicals. There are numerous reports indicating populations of fish being affected by the adverse actions of environmental estrogens [Gross-Sorokin et al., 2006; Porte et al., 2006].

Hormonal regulation of gene transcription is controlled at various levels including receptor, receptor-related proteins and transcription factors, DNA methylation, state of histone acetylation, etc. Estrogenic compounds might impact any of these proteins/processes to modulate gene expression and tissue response. Most of the chemicals known to disrupt estrogen function found to date are able to bind to estrogen receptors (ER) [Korach et al., 1978; Nelson et al.,

Abbreviations used: BPA, bisphenol A; DBP, dibutyl phthalate; DES, diethylstilbestrol; E_2, estradiol; EDCs, endocrine disrupting chemicals; EMM, Edinburgh minimal medium; ER, estrogen receptor; ERE, estrogen response element; gfERα, goldfish ERα; MG, malachite green; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OP, 4-*tert*-octylphenol.

Components of this transgenic yeast system are available upon request free of charge to individuals from non-profit organizations for non-commercial research activities.

Grant sponsor: The Chinese University of Hong Kong.

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Received 26 July 2007; Accepted 4 September 2008 • DOI 10.1002/jcb.21960 • 2008 Wiley-Liss, Inc.

Published online 3 November 2008 in Wiley InterScience (www.interscience.wiley.com).

1984; Connor et al., 1997], and the complex of estrogen and its receptor will eventually find its way into the nucleus to bind to DNA sequences known as estrogen response elements (EREs) located in the promoter region of the estrogen-responsive genes. Based on this principle, a screening system for human application had been developed using the human ER [Arnold et al., 1996; Yoo et al., 2002]. Comparison between human and fish ER screening system has also been reported [Petit et al., 1995, 2000] indicating that estrogenic compounds might have different binding profiles between fish ER and human ER. For the purpose of identifying compounds with estrogenic activities relevant to aquatic organisms, a plasmid containing the goldfish ERα (gfERα) was constructed in a yeast expression vector in the present study. Together with another construct containing an ERE cloned upstream of a reporter gene, a transgenic yeast system was established in fission yeast. The genetically engineered yeast cells were demonstrated to provide a fast, convenient, inexpensive and high throughput system for screening compounds exhibiting estrogen-like or anti-estrogen activities. The aim of this study is to demonstrate any significant differences between the human and fish screening systems.

Malachite green (MG), a triarylmethane dye used traditionally in aquaculture to treat fish eggs and adult fish for fungal and ectoparasite problems, is used extensively worldwide because of its low cost, easy accessibility and high efficacy. Most toxicity studies on this compound have been conducted in fish. Its biological effects such as the pathological changes in the liver and physiological changes in blood have also been reported [Srivastava et al., 2004]. Besides these actions, MG and its metabolites may affect thyroid hormone function by inhibition of thyroid peroxidase [Doerge et al., 1998], indicating that MG has the potential to disrupt the endocrine system. In the present study, its estrogenicity was investigated using the established transgenic yeast system. This is the first report demonstrating that this compound possesses anti-estrogen activities.

MATERIALS AND METHODS

YEAST STRAIN AND YEAST HANDLING

The fission yeast *Schizosaccharomyces pombe* ED665 (h-ade-M201 leu1-32 ura4-D18) was kindly provided by Dr. Hee-Moon Park (Department of Microbiology at Chungnam National University of Korea, Daejeon, Korea). Edinburgh minimal medium (EMM) (US Biological, Swampscott, MA) without the minimal essential components (leucine and uracil) was used to culture the yeast for selection purpose. Only yeast cells containing both the ER expression vector (with production of uracil) and the interaction vector (with production of leucine) can grow up in this medium. According to the manufacturer's protocol, 32.3 g of EMM powder was dissolved in 1 L of distilled water and sterilized by filtration. For solid culture, an additional 2% agar was added to the medium. Single colony of ED665 was cultured in EMM at 300 rpm in a 30°C shaker. Solid culture was performed on an agar plate in a 30°C incubator. Transformation was performed using the small-scale LiAc yeast transformation method (Yeast Protocols Handbook, Clontech, Mountain View, CA).

PLASMID CONSTRUCTS

To construct the vector for yeast expression, a pair of primers (gfERα-F: TAT AGT CGA CTA TGT ACC CTA AGG AGG AG; gfERα-R: TAT ACC CGG GTC AGG GGT CTG GAC TCT G) was used to amplify the entire gfERα open reading frame according to a published sequence [Choi and Habibi, 2003]. The PCR was carried out in a volume of 50 μl containing 200 μM dNTP, 0.2 pmol primers, and 2.5 units of high fidelity polymerase (Roche, Bale, Switzerland). After an initial denaturation at 94°C for 30 s, the reaction was annealed at 55°C for 30 s and extended at 72°C for 2 min. After 25 cycles of amplification, a further 7 min extension was performed. The PCR products were analyzed on 1% agarose gel. After staining with ethidium bromide, the target band was purified by the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The amplicon was then digested with the restriction enzymes *Sma* I and *Sal* I (Promega, Madison, WI) separately. The digested product was ligated into the *Sal* I/*Sma* I sites of the yeast expression vector pREP42-MH N (kindly provided by Dr. Iain M. Hagan in the Department of Pathology, Stanford University Medical Center, Palo Alto, CA) to generate the recombinant pREP42-MH N-gfERα plasmid containing the gfERα insert (Fig. 1, panel A). The presence of a c-myc tag in front of the gfERα insert provided a means of detecting the expression of gfERα in the transformed yeast cells by Western analysis using an antibody against the c-myc tag. DNA sequencing (Tech Dragon, Hong Kong) was performed to confirm that the sequence was free from any mutation.

In the preparation of the reporter plasmid construct, three copies of a consensus ERE sequence AGGTCACAGTGACCT (Fig. 1, panel B) [Murdoch et al., 1990] were cloned upstream of the *LacZ* gene in the *Sal* I/*Xho* I sites of pRW95-3 (kindly provided by Dr. Michael Schweizer in the Department of Biological Sciences at Heriot-Watt University, Edinburgh, United Kingdom) [Wolf et al., 1996]. Then the *Sal* I/*Sca* I fragment of ERE-LacZ was inserted into the *Sal* I/*Sma* I sites of the pJK148 interaction vector (kindly provided by Dr. Jef D. Boeke in the Department of Molecular Biology and Genetics at Johns Hopkins University, Baltimore, MD) to generate the recombinant pJK148-ERE-LacZ plasmid (Fig. 1, panel B). This recombinant reporter plasmid contains the ERE necessary for interaction with the expressed gfERα to drive the transcription of the *LacZ* gene. The amount of the *LacZ* gene product could be ascertained in a subsequent β-galactosidase enzyme activity assay.

MTT ASSAY

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) assay was performed as described with minor modifications [Smail et al., 1992]. Briefly, 100 μl of MTT (5 mg/ml in phosphate buffered saline) were added to the yeast cells after treatment with the test compounds for 6 h. After incubation at 30°C for 30 min, the supernatant was removed and the colored MTT-formazan product extracted by DMSO was read at 570 nm.

WESTERN BLOTTING

Yeast (*S. pombe*) protein was prepared using protocols of the Sanger lab's small-scale extraction method (http://www.sanger.ac.uk).

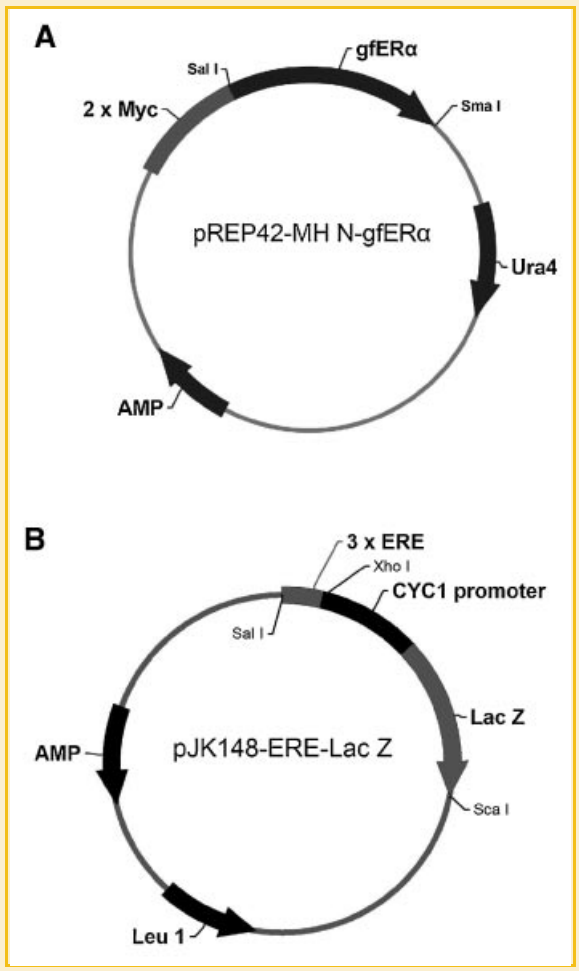

Fig. 1. Plasmid constructs of the transgenic yeast system. Panel A: The expression plasmid. Panel B: The reporter plasmid. Detailed methods of constructing the plasmids are described in the Materials and Methods Section.

After growing up a 50 ml culture to an OD_{595} of 0.25 (5×10^6 cells/ml) in EMM with adenine, the cells were harvested by centrifugation at 3,000 rpm for 5 min, and resuspended in 20 μl of buffer (10 mM sodium phosphate pH 7, 0.1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium metavanadate, 4 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). After homogenization by 1.0 ml of acid washed glass beads (0.5 mm diameter) (Sigma) with vortexing for 1 min, the protein concentration in the samples was then determined using the bicinchoninic acid method (Pierce, Rockford, IL). Fifteen micrograms of protein was loaded onto a 10% polyacrylamide gel. After SDS–PAGE the proteins were transferred onto PVDF membrane using a semi-dry method. In the Western blotting, the first antibody used was mouse anti-myc (Promega), and the second antibody was rat anti-mouse IgG conjugated with alkaline phosphatase (Promega). After incubation and blotting, the PVDF membrane was incubated in a color development buffer (1% v/v 5-bromo-4-chloro-3-indolyl phosphate dipotassium salt and 1% v/v nitroblue tetrazolium) until a satisfactory signal was achieved. The reaction was terminated by rinsing the blot in large quantities of distilled water. The Western bands were then scanned for analysis.

LIQUID ASSAYS FOR β-GALACTOSIDASE

The reporter assay of β-galactosidase was modified from a method of the Sanger Institute (Fission Yeast Handbook). Recipes of the reagents and the procedures are described as follows. All the phytoestrogens (Table I) and other test compounds (Table II) were purchased from Sigma.

The yeast cells were grown up in the selective EMM with OD_{595} in the region of the mid log (0.1–0.9). After the cells were distributed into 48-well plates, the test compounds at various concentrations were added to the yeast cells and incubated for 6 h. Then the cells were spun down and 0.8 ml cold Z buffer (0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.01M KCl, 0.001M $MgSO_4$, 0.03M β-mercaptoethanol added freshly just before use) was added for resuspension. After adding 6 μl of 0.1% SDS from a Pasteur pipette and 6 μl of chloroform, the contents were vortexed vigorously to lyse the cells. After equilibration for 5 min at 30°C, 200 μl of an *o*-nitrophenyl-β-D-galactoside (Sigma) solution (4 mg/ml in water) was added to each tube. The reaction was allowed to proceed until the solution turned yellow. Then the reaction was stopped with 0.5 ml of 1M Na_2CO_3. The lapsed time in min was noted, the cell debris was spun down and OD_{420} of the supernatant was read for calculating the enzyme activity in "Miller units" defined according to the following formula [Kaiser et al., 1994]:

$$\beta\text{-galactosidase unit} = OD_{420}/(OD_{595}\text{ of assayed culture} \times \text{volume assayed} \times \text{time})$$

DATA ANALYSIS

For the liquid assays of β-galactosidase, every dose was performed in quadruplicates in several independent experiments, and the standard error was calculated. Data were analyzed statistically using unpaired *t*-test or one-way ANOVA.

TABLE I. Evaluation of the Estrogenicity of Some Common Phytoestrogens by the Transgenic Yeast System

Phytoestrogen	EC_{50} (M)	Fold induction
Biochanin A	4.49×10^{-7}	1.85 ± 0.03
Chalcone	No activity	—
Fisetin	2.02×10^{-5}	3.20 ± 0.18
Flavone	No activity	—
Formononetin	4.16×10^{-7}	2.56 ± 0.26
Genistein	2.0×10^{-6}	4.17 ± 0.17
Kaempferol	1.19×10^{-5}	4.12 ± 0.21
Luteolin	1.87×10^{-5}	4.63 ± 0.40
Naringenin	4.92×10^{-6}	2.11 ± 0.04
Naringin	No activity	—
Quercetin	No activity	—
Resveratrol	No activity	—
Estradiol	4.28×10^{-9}	4.62 ± 0.21

Quadruplicate assays for each data point in four independent experiments were performed and the results are expressed as mean values ± SEM. Estradiol was used as positive control.

TABLE II. Assessment of the Estrogenicity of Some Commonly Encountered Environmental Pollutants

Name of compound	Estrogenicity testing using fish ER	Estrogenicity testing using human ER
Estradiol	4.28×10^{-9} M	
Pesticides		
Alachlor	−	++(1)[a]
	−(2)[a]	−(3)[c]
Aldrin	−	++(4)[b]
	++(5)[b,d]	−(6)[c]
		−(3)[c]
Asana	−	ND
Aldicarb	−	−(3)[c]
Benomyl	−	−(1)[a]
		−(7)[c]
		++(3)[c]
Dieldrin	−	−(6)[c]
	−(8)[b]	+(3)[c]
	−(9)[d]	−(11)[b]
	−(10)[b]	−(12)[b]
	−(14)[d]	+(13)[a]
	+(2)[a]	+(15)[c]
	++(5)[b,d]	+(16)[c]
		+(17)[a]
		−(18)[c]
Equity Mirex Mix	−	ND
Endosulfan	7.74×10^{-6} M	
Endrin	−	−(6)[c]
		+(3)[c]
Heptachlor	7.34×10^{-6} M	−(3)[c]
Kepone	−	++(19)[c]
	+(14)[d]	−(20)[c]
	+(21)[d]	−(22)[c]
	+(23)[a]	−(24)[b,c]
	+(2)[a]	++(4)[b]
		++(25)[b]
Malathion	−	+(26)[b]
Methyl parathion	−	−(3)[c]
	−(2)[a]	
Methomyl	−	
cis-Nonachlor	−	+(1)[a]
trans-Nonachlor	−	+(1)[a]
		++(3)[c]
Dyes		
Basic fuchsin	−	ND
Brilliant blue G	−	ND
Brilliant blue R	−	ND
Brilliant green	−	ND
Ethyl violet	−	ND
Gentian violet	−	ND
Leucocrystal violet	−	ND
Leuco-malachite green	−	ND
Malachite green	−	ND
Malachite green oxalate salt	−	ND
Metanil yellow	−	ND
Methyl green	−	ND
Sudan I	−	ND
Victoria blue B	−	ND
Victoria blue R	−	ND
Victoria pure blue	−	ND
Industrial chemicals		
Trifluralin	−	ND
4-*tert*-Octylphenol	+	
Dibutyl phthalate	8.95×10^{-6} M	
Di(2-ethylhexyl)phthalate	−	ND
Herbicides		
Atrazine	−	−(3)[c]
	−(2)[a]	−(27)[b]
	−(8)[b]	−(28)[c]
	−(29)[d]	−(30)[c]
		−(17)[a]
Metribuzin	−	ND
Diclofop-methyl	−	
	+(2)[a]	
Cyanazin	−	ND

Using our transgenic yeast system, compounds at 10^{-9}, 10^{-7}, and 10^{-5} M were screened. The EC_{50} values of the more potent ones (i.e., endosulfan, heptachlor and dibutyl phthalate) are shown according to the results from Figure 5. Estradiol was used as positive control. The other compounds were either inactive or of very low activity. Our experimental results were compared with those in the literature using fish ER or human ER. The numbers in the brackets show the reference sources. The minus (−), plus (+) and double plus (++) signs stand for negative, positive and potent effects of the compounds, respectively.
ND, not determined.
Sources of cited information in this table: (1) Klotz et al. [1996]; (2) Petit et al. [1997]; (3) Lemaire et al. [2006]; (4) Scippo et al. [2004]; (5) Okoumassoun et al. [2002]; (6) Tully et al. [2000]; (7) Yamada et al. [2005]; (8) Gale et al. [2004]; (9) Tollefsen et al. [2002]; (10) Tollefsen et al. [2003]; (11) Arcaro et al. [1998]; (12) Arnold et al. [1996]; (13) Arnold et al. [1997]; (14) Smeets et al. [1999]; (15) Andersen et al. [2002]; (16) Legler et al. [1999]; (17) Graumann et al. [1999]; (18) Ramamoorthy et al. [1997]; (19) Wu and Safe [2007]; (20) Safe et al. [2002]; (21) Loomis and Thomas [1999]; (22) Yoon et al. [2000]; (23) Nimrod and Benson [1997]; (24) Shelby et al. [1996]; (25) Soto et al. [1994]; (26) Chen et al. [2002]; (27) Roberge et al. [2004]; (28) Legler et al. [2002]; (29) Sanderson et al. [2001]; (30) Balaguer et al. [1999].
[a]Yeast estrogen screen assay.
[b]Receptor binding assay.
[c]Reporter gene assay.
[d]Vitellogenin assay.

RESULTS

CLONE SELECTION AND WESTERN BLOTTING

After transformation of the yeast cells, more than 10 colonies were randomly chosen for evaluation through measurement of β-galactosidase activity to select the higher expressors for compound screening. For every colony, the control group (without estradiol (E_2) treatment) and the treatment group (incubation with 10^{-7} M E_2) were conducted in parallel and the β-galactosidase activities measured are shown in Figure 2 panel A. Western blotting against c-myc was performed to check the expression level of the gfERα. From Figure 2 panel A, three yeast clones (viz. colony numbers 201, 207, and 208) were selected for further analysis because of their relative high signal to noise ratio. After Western blotting, all the three clones showed clear bands of the expected size (63 kDa) corresponding to the myc-tagged gfERα, while the negative control (ED665, non-transformed yeast cells) did not show any band (Fig. 2, panel B). Finally, clone 201 was chosen to conduct the subsequent screening assays because of its higher potency and stable expression.

VALIDATION OF THE TRANSGENIC YEAST SYSTEM

The transgenic yeast system was validated using a natural estrogen (E_2), a synthetic estrogen (DES), a phytoestrogen (genistein) and an industrial estrogenic compound bisphenol A (BPA) (Fig. 3). All these known estrogenic compounds exhibited a dose-dependent response in the present system. E_2, DES and genistein gave EC_{50} values of 4.28×10^{-9} M, 8.4×10^{-10} M and 2.0×10^{-6} M, respectively. For E_2, the EC_{50} value obtained in the present system is similar to that obtained from a fish cell reporter gene assay using cultured rainbow trout gonad cells [Ackermann et al., 2002]. For BPA, although the potency is relatively weak as indicated by its large EC_{50} value, its efficacy is the highest among the four compounds as indicated by the large amplitude of the dose–response curve.

ASSESSMENT OF THE ESTROGENICITY OF PHYTOESTROGENS USING THE TRANSGENIC YEAST SYSTEM

Using the present system, the estrogenic activities of 11 common phytoestrogens were evaluated in the concentration range of

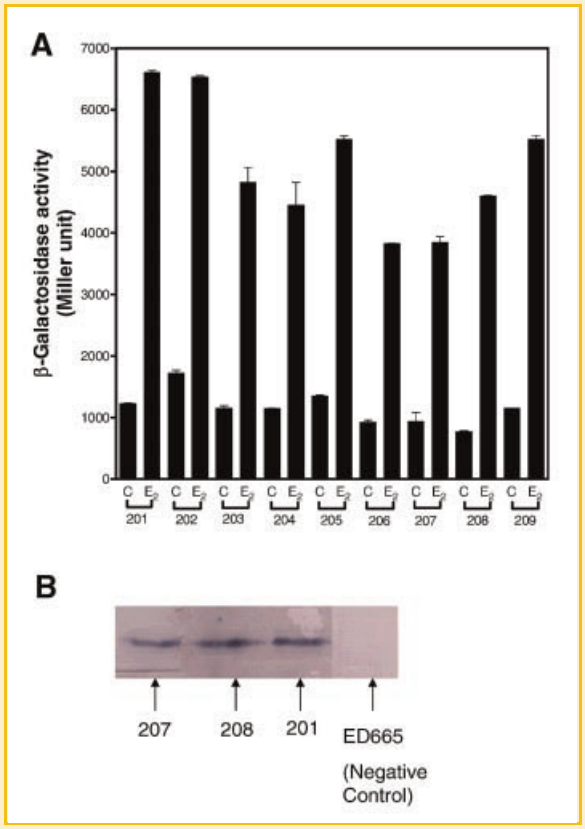

Fig. 2. Yeast clone selection. Various yeast clones (from colony 201–209) were evaluated by E_2 stimulation using the β-galactosidase liquid assay. Quadruplicate assays for each data point in three independent experiments were performed and the results are expressed as mean values ± SEM. C stands for the control (no E_2 added), and E_2 stands for the estradiol treatment at 10^{-7} M (panel A). Panel B shows the Western blotting of the expressed gfERα in the yeast cells by the anti-myc antibody. The numbers under the arrows represent the different yeast clones. ED665, which is the yeast cell without transformation, was used as the negative control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

10^{-10} to 10^{-4} M. Among the phytoestrogens tested, 7 of them showed different degrees of estrogenicity while the rest had no activity. The results are summarized in Table I showing the EC_{50} values and the fold induction.

ASSESSMENT OF THE ESTROGENICITY OF PUTATIVE ENVIRONMENTAL ENDOCRINE DISRUPTORS USING THE TRANSGENIC YEAST SYSTEM

Putative environmental endocrine disruptors including pesticides, herbicides, dyes and other industrial chemicals were evaluated using this yeast system. The estrogenic activities of a multitude of such compounds commonly encountered were tested at three dosages (10^{-9}, 10^{-7}, and 10^{-5} M) and the results are summarized in Table II and in Figure 4. The negative signs indicate that at all the three concentrations tested, no estrogenic activity was detected. The positive signs indicate that some estrogenic activities were detected in the initial screening to be followed by more detailed analysis in Figure 5. Among the 16 pesticides tested, only endosulfan and

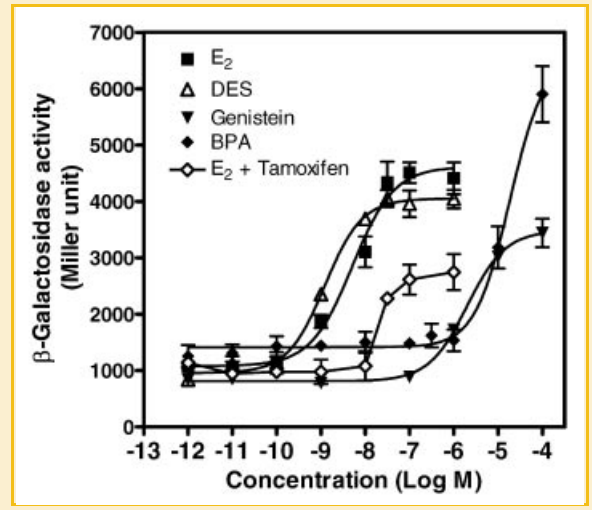

Fig. 3. Validation of the transgenic yeast system using E_2, DES, genistein, BPA and a combination of E_2 and tamoxifen (10^{-5} M). Quadruplicate assays for each data point in three independent experiments were performed and the results are expressed as mean values ± SEM.

heptachlor gave positive results. All the 16 extensively used dyes were found to possess no estrogenic activities. The four common herbicides also gave negative results. Among the four common industrial chemicals, 4-*tert*-octylphenol (OP) and dibutyl phthalate (DBP) gave positive results.

Subsequent detailed studies of endosulfan and heptachlor revealed an obvious dose-dependent relationship (Fig. 5, panels A,B). In the detailed studies, OP gave an obvious increase in β-galactosidase activity at 10^{-6} M. However higher concentrations (10^{-5} and 10^{-4} M) of this compound exhibited much smaller or no activity (Fig. 5, panel C), which was probably due to its toxic action at the higher concentrations (Fig. 5, panel E). DBP did not induce any increase in β-galactosidase activity at concentrations less than 3×10^{-6} M. However definitive estrogenic activities were demonstrated at higher concentrations (10^{-5} M and 3×10^{-5} M) (Fig. 5, panel D).

DEMONSTRATION OF THE ANTI-ESTROGENIC ACTIVITY OF MALACHITE GREEN USING THE TRANSGENIC YEAST SYSTEM

Besides evaluating the estrogenic activities of various compounds, the present transgenic yeast system could also be used to assess the anti-estrogenic activities of the test compounds by the concomitant inclusion of a standard estrogen in the assay. Using E_2 as the standard estrogen, among the compounds shown in Table II, only MG was demonstrated to exhibit anti-estrogen activity. Various concentrations of MG were initially used to test for its potential estrogenicity. The results showed that MG could not induce any increase in β-galactosidase activity up to a concentration of 10^{-6} M tested (Fig. 6, panel A). At 10^{-5} M, there appeared to be a small reduction in β-galactosidase activity but this was probably due to the toxic action of MG on the yeast cells at the elevated concentration (Fig. 6, panel B). MG did not possess any toxic action on the yeast cells up to 10^{-6} M indicating that the lack of estrogenicity of this compound was not caused by the inhibiting

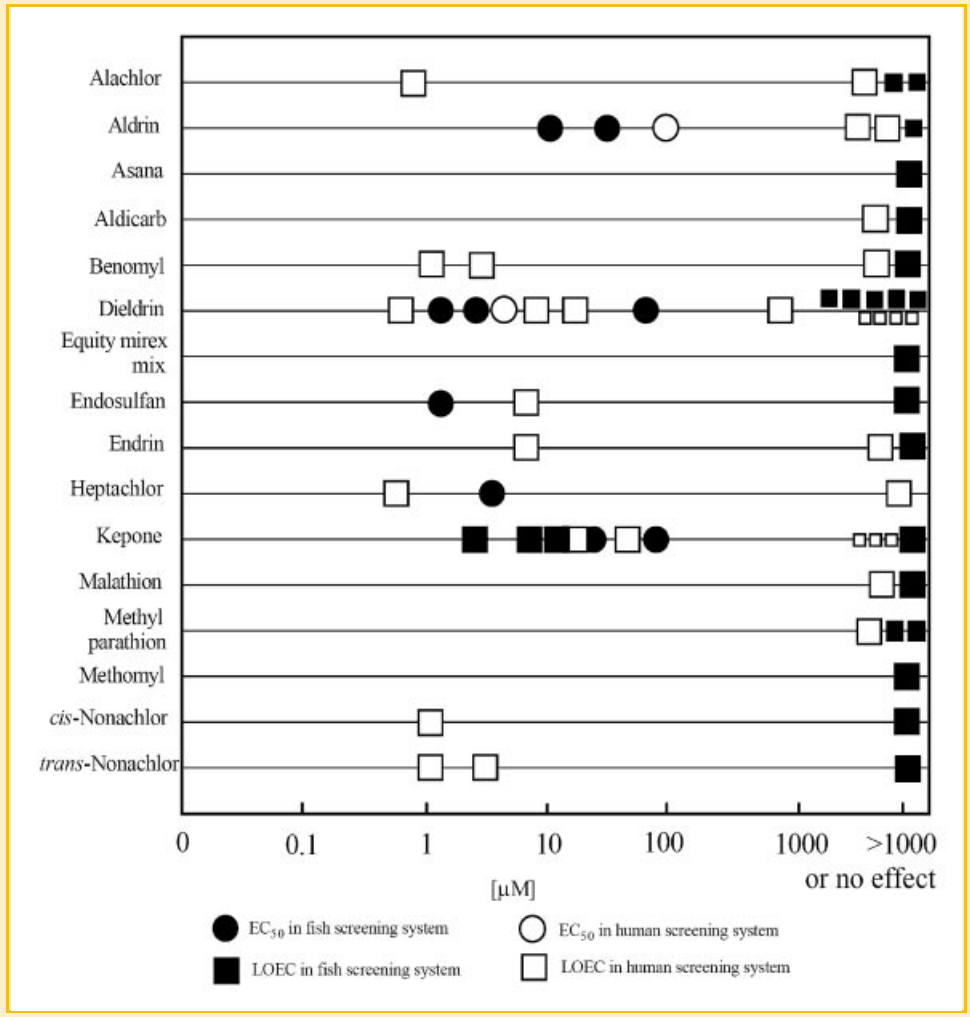

Fig. 4. Assessment of the estrogenicity of some commonly encountered environmental pollutants. Our experimental results were compared with those in the literature using fish ER or human ER. The methods and references can be found in Table II. LOEC: The lowest observed effect concentration; EC_{50}: half maximal effective concentration.

toxic effects. Interestingly, when the system was tested with a combination of E_2 and MG, the estrogenic action of E_2 was partially antagonized by MG (Fig. 6, panels C,D). At the EC_{50} value of E_2 in which there is a definitive increase in β-galactosidase activity induced by E_2, MG could antagonize the estrogenic action of E_2 in a dose-dependent manner (Fig. 6, panel C). The dose-dependent estrogenic action of E_2 is shown in Figure 6 panel D. In the presence of a fixed concentration of MG at 10^{-7} M, there was a reduction in the efficacy of the estrogenic action of E_2 (as indicated by a smaller amplitude of the dose–response curve) without affecting the potency of E_2 (as indicated by no change in the EC_{50} value of the dose–response curve).

DISCUSSION

Endocrine disruptors, or endocrine disrupting chemicals (EDCs), pose a threat to both human beings and aquatic organisms. However, the actions of EDCs are different between human and aquatic organisms. Fish can be regarded as primary risk organism to endocrine disruption by xenoestrogens. Firstly, their sex differentiation is very labile and exposure to external hormones—if applied during an appropriate life span—can completely reverse the functional sex [Zhong et al., 2005]. Secondly, they are directly exposed to effluents discharged into the environment. Thus, the identification of environmental contamination with binding activity to the fish ER is of great importance and some existing data indicated differences to the binding of the human ER. In order to develop a screening system for binding to fish ER, a fast (the whole operation taking less than 2 days) and low-cost (each sample costing approximately 60 U.S. cents) yeast screening system was developed using a fish ER. The present system is amenable to high-throughput operation which is a great advantage in its ability to handle a large number of compounds or samples at the same time. This rapid screening system could therefore be applied to initial studies for the effective and rapid identification from a huge repertoire of putative estrogenic compounds for further detailed studies such as by the in vivo induction of vitellogenin biosynthesis in fish [Li et al., 2005].

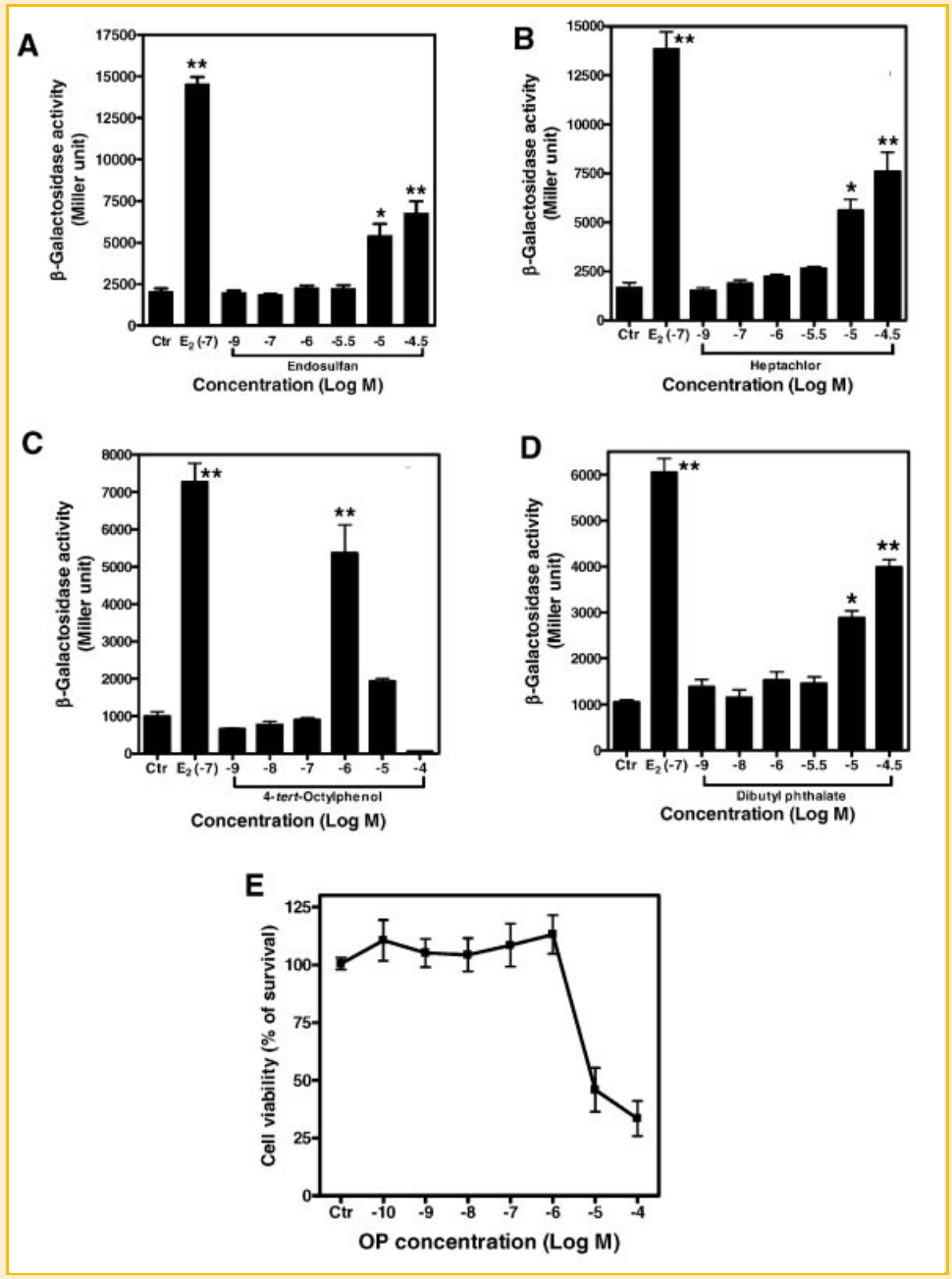

Fig. 5. Estrogenicity of endosulfan (panel A), heptachlor (panel B), 4-*tert*-octylphenol (panel C) and dibutyl phthalate (panel D) demonstrated by the transgenic yeast system. Panel E shows that 4-*tert*-octylphenol (OP) is not toxic to the yeast cells at concentrations up to 10^{-6} M. Quadruplicate assays for each data point in four independent experiments (for panels A–D) or six independent experiments (for panel E) were performed and the results are expressed as mean values ± SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$ compared with the respective control group by one way ANOVA.)

ESTROGENICITY OF PESTICIDES

Pesticides are comprised of a group of very different chemical substances. Endosulfan is a contact and stomach poison that has been used to control insects. It is used in countries throughout the world to control pests on fruits, vegetables, tea, and non-food crops. Endosulfan has a high acute toxicity in many species of animals [Kuvarega and Taru, 2007]. The estrogenic action of endosulfan is controversial. On one hand, endosulfan was reported to act as an ER agonist in cell proliferation assay using MCF-7 cells [Andersen et al., 2002], although the receptor binding affinity on the human ER is rather low ($IC_{50} > 2 \times 10^{-4}$ M) [Andersen et al., 1999]. On the other hand, endosulfan did not induce any vitellogenin production in carp in vivo [Smeets et al., 1999]. Both the β-galactosidase activity in the recombinant yeast assay and luciferase activity in MCF-7 cells using human ER also gave no obvious positive results. In the present study where a fish ER was adopted, the results showed that endosulfan

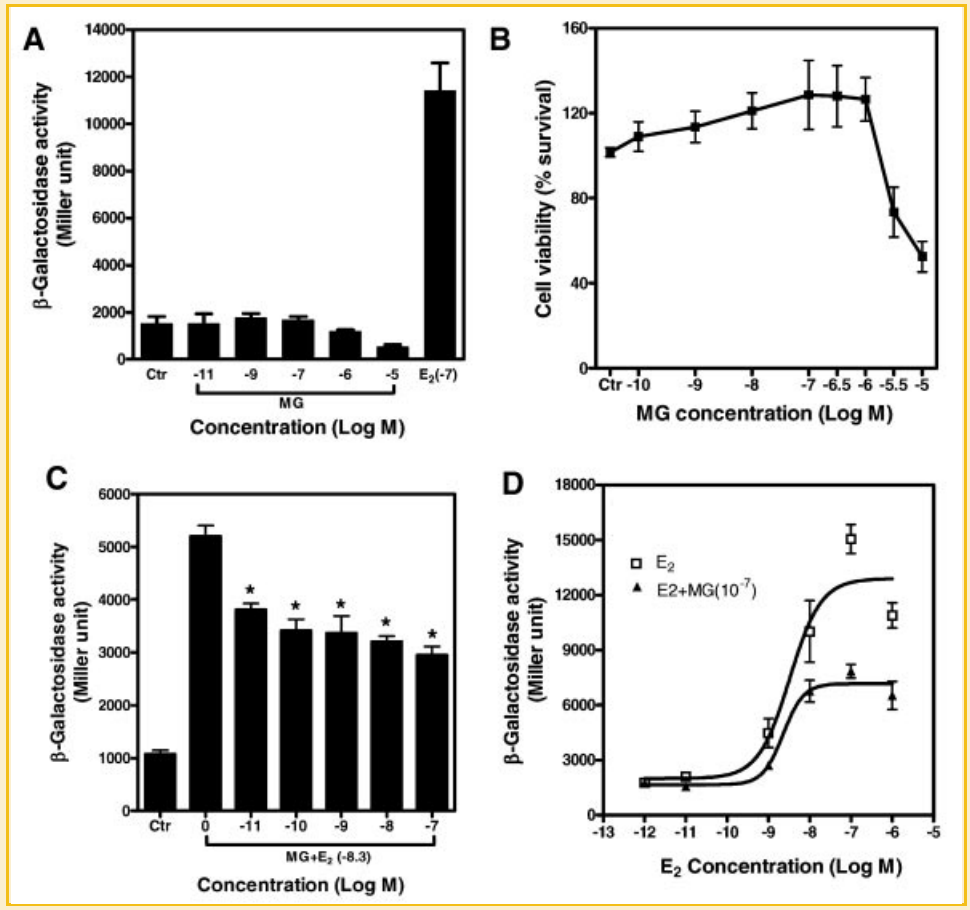

Fig. 6. Anti-estrogenic activities of malachite green (MG) using the transgenic yeast system. Treatment with MG alone (panel A), with a combination of a fixed E_2 concentration and various MG concentrations (panel C), and with a combination of a fixed MG concentration and various E_2 concentrations (panel D) are shown. Panel B shows that MG is not toxic to the yeast cells at concentrations up to 10^{-6} M. Quadruplicate assays for each data point in four independent experiments (for panels A,C,D) or six independent experiments (for panel B) were performed and results are expressed as mean values ± SEM. ($^*P < 0.05$ compared with the respective control group by one way ANOVA.)

exhibits significant estrogenic activity. These conflicting results of endosulfan argue for a need to re-visit the estrogenic actions of putative endocrine disruptors, particularly in the extrapolation of experimental results across different species.

Heptachlor is used as a pest-controlling agent in agriculture, or as a destroyer of household pests and disease-carrying insects. Due to its stability and persistence, high concentrations of its residues have been found in soil, food, human tissues, and wildlife. Also because of its possible carcinogenicity in animal studies, heptachlor is considered to pose a threat to human health [Okoumassoun et al., 2003]. Previous studies on heptachlor have demonstrated its weak estrogenic actions in sensitive assays such as receptor binding [Guillette et al., 2002] and cell proliferation [Wong and Matsumura, 2006]. Heptachlor also showed up as an estrogenic compound in the present transgenic yeast assay, indicating the effectiveness of this system in picking up weak estrogenic activities despite its simple procedure and high throughput nature.

ESTROGENICITY OF DBP AND OP

Although the estrogenicity of DBP has been extensively studied, there is no consistent conclusion. Most reports using in vitro systems derived from mammalian ER showed that DBP had no estrogenic activity [Milligan et al., 1998; Mylchreest et al., 1998; Hashimoto et al., 2000; Yamasaki et al., 2002; Seidlova-Wuttke et al., 2004]. Although some other reports showed conflicting results [Harris et al., 1997; Hashimoto et al., 2003], the general consensus is that in mammals DBP has no estrogenic activity. In other vertebrates, whether DBP is an estrogenic compound or not is uncertain. For example, using the vitellogenin assay, no significant induction of vitellogenesis was observed in male and female medaka exposed to DBP [Nozaka et al., 2004]. However in salmon, DBP showed some weak estrogenic activity by monitoring a more sensitive endpoint (steroid-binding proteins) [Tollefsen et al., 2002]. The present result using a fish ER showed that DBP does possess obvious estrogenic actions. This result demonstrated the potential estrogenic action of DBP on fish. The different behavior between mammalian ER and fish ER clearly demonstrates the need for a specific system for screening estrogenic compounds applicable to aquatic organisms.

OP is a non-ionic surfactant used as a detergent, emulsifier and wetting agent. It is generally believed to be an estrogenic compound, as indicated from results of the recombinant yeast assay using human ER at very low concentrations [Isidori et al., 2006], in

reporter gene assays using cell lines [Paris et al., 2002], and in vitellogenin assays in zebrafish and rainbow trout [Van den Belt et al., 2003]. Results from the present study confirmed these positive results, indicating the reliability of the present assay system. At higher concentrations of OP (10^{-5} and 10^{-4} M in Fig. 5, panel C), the apparent estrogenic activity decreased with the dose, but this is due to the toxicity of OP on the yeast cells (Fig. 5, panel E) [Isidori et al., 2006].

ESTROGENICITY OF TRIARYLMETHANE DYES

MG is a triarylmethane dye widely used in health, textile and other industries. In aquaculture, MG has been used extensively to treat fish eggs and adult fish for fungal and ectoparasite problems. Because MG is reduced into leuco-malachite green and deposited in the fatty tissues of fish at 2.5–2,000 μg/kg level [Bergwerff and Scherpenisse, 2003], human might be exposed to MG through fish consumption. Due to its possible toxic effects on human and extensive use in aquaculture, the toxicity of MG is widely studied. The results show that the LC_{50} values in fish ranged from 30.5 to 383 μg/L [Bills et al., 1977]. Biological effects such as pathological changes in liver and physiological changes in blood have been the focus of most studies. Apart from these effects, MG is also suspected to be a possible carcinogen by the U.S. Food and Drug Administration [Srivastava et al., 2004]. It has also been reported to be a disruptor of thyroid hormone function by inhibition of thyroid peroxidase [Doerge et al., 1998]. Besides the disrupting effects on the thyroid, there is no report on the estrogenicity of MG and its metabolites. The present study shows that MG has no intrinsic estrogenic activity but could act as an anti-estrogen. This novel finding provides a new avenue for further studies on the biological actions of MG. It also indicates that endocrine disruptors could conceivably interfere with the endocrine system through their anti-estrogenic actions.

In conclusion, we have developed a transgenic yeast screening system harboring a fish ER which was applied to screen a variety of different compounds for their estrogenicity, and the results were compared with those from a human screening system reported in the literature. The finding indicates the necessity for using a fish ER system for aquatic toxicological studies in estrogenicity screening. The novel action of malachite green as an anti-estrogen was also demonstrated.

ACKNOWLEDGMENTS

This work was supported by Direct Grants and a Strategic Investments Scheme from The Chinese University of Hong Kong. Acknowledgments are also given to Dr. Hee-Moon Park in the Department of Microbiology at Chungnam National University of Korea, Dr. Iain M. Hagan in the Department of Pathology at Stanford University Medical Center of the United States, Dr. Michael Schweizer in the Department of Biological Sciences at Heriot-Watt University of the United Kingdom, and Dr. Jef D. Boeke in the Department of Molecular Biology and Genetics at Johns Hopkins University of the United States for the provision of yeast strain and the parent plasmid vectors as described in the Materials and Methods Section.

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