Problems with Methods used to Screen Estrogenic Chemicals by Yeast Two-Hybrid Assays

Shigeru Nakano,* Yasuhiro Nagao, Toru Kobayashi, Masaharu Tanaka, Satoshi Hirano, Yoichi Nobuhara, and Toshihiro Yamada

Central Research Institute, Nissin Food Products Co., Ltd., 2247 Noji-cho, Kusatsu, Shiga 525–0055, Japan

(Received July 13, 2001; Accepted October 4, 2001)

We developed a yeast two-hybrid assay using a human-type estrogen receptor (hERα) to screen test chemicals reporting weak estrogenic activity at high concentrations. As a result of exposure to several test chemicals, viable yeast cells showed a decrease in number compared with a control. It was shown that this phenomenon was due to fungicidal effects of the test chemicals within the yeast cell or to inhibition of yeast cell proliferation. Therefore, in performing a yeast two-hybrid assay, it is necessary to monitor yeast cell proliferation by methods such as the measurement of the number of viable cells, and it is necessary to screen test chemicals within a concentration range where these chemicals show neither fungicidal effects towards the yeast cell nor inhibition of yeast cell proliferation.

Key words —— yeast two-hybrid assay, endocrine disruptor, estrogenic chemical

INTRODUCTION

Chemical materials which are suspected to disrupt the human endocrine system are listed in the book entitled "Our Stolen Future" by Theo Colborn *et al*.[1] They describe the effects of these chemicals on our ecosystem. Such chemicals are referred to as endocrine disruptors and constitute a current social problem. However, the endocrine disruptive effects of some of the chemicals they list are not detailed, and we therefore feel it important to scientifically clarify these effects on our ecosystem.

*To whom correspondence should be addressed: Central Research Institute, Nissin Food Products Co., Ltd., 2247 Noji-cho, Kusatsu, Shiga 525–0055, Japan. Tel.: +81-77-561-9114; Fax: +81-77-561-9140; E-mail: s-nakano@mb1.nissinfoods.co.jp

Methods including receptor binding assays,[2–4] a MCF-7 cell proliferation assay (E-screen)[5] and reporter gene assays[6,7] on animal cell lines have recently been developed as *in vitro* screening techniques to detect endocrine disruptors. A yeast two-hybrid assay recently developed by Nishikawa *et al*.[8] has attracted attention because it allows prediction of endocrine disruptors in a relatively short period of time. This screening method is a binding assay between a receptor and a ligand and is based on the ligand-dependent interaction of a nuclear hormone receptor with a co-activator. When a co-activator binds to the ligand-hormone receptor complex, transcription of a reporter gene is induced. The product of the reporter gene should be easily detectable, for example, the gene encoding β-galactosidase was used in this experiment. They tested the estrogenic activity of 517 chemicals and report that 64 of these induced transcription.[9] However, concerns have been raised over the permeability and toxicity of high concentrations of the test chemicals to the yeast cells.[10] The solubility of the test chemicals believed to activate transcription is currently unknown. Therefore, we developed a yeast two-hybrid assay based on the human-type estrogen receptor (hERα) to screen 16 chemicals in which weak estrogenic activity has been reported at high concentrations. We also tested the estrogenic activity and the solubility of these chemicals in our experimental system and found that several of those inhibited yeast cell proliferation and were insoluble at high concentrations that they showed weak estrogenic activity.

MATERIALS AND METHODS

Chemicals —— 17-β-Estradiol (E2), diethylstilbestrol (DES), bisphenol A (BPA), genistein (GS), 4-nonylphenol (NP), Daidzein (DZE), daidzin (DZ), ethyl 4-hydroxybenzoate (EHB), methyl 4-hydroxybenzoate (MHB), 2-hydroxyfluorene (HF), 4,4′-dihydroxybenzophenone (DHBP), 4-chloro-3,5-dimethylphenol (CDMP), 4-chlorophenol (CP), 4-hydroxyacetophenone (HAP), 4-methylphenol (MP), 4-*n*-nonylphenol diethoxylate (NPDE), 4-*tert*-octylphenol diethoxylate (OPDE), *cis*-1,2-diphenylcyclobutane (DPCB), benzyl butyl phthalate (BBP), Di-iso-propyl phthalate (DIPP), Di-*n*-propyl phthalate (DNPP), methylene blue, *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Sigma-Aldrich Japan,

Inc. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan) or Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan). Zymolyase 20T was purchased from Seikagaku Corporation, Ltd. (Tokyo, Japan). Molecular biology reagents were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan), unless otherwise stated. All other chemicals and solvents were of extra pure grade or HPLC grade, obtained from commercial sources and were used without further purification.

Plasmids —— The yeast expression plasmid, pGBT9 was purchased from Clontech Laboratories Japan, Ltd. (Tokyo, Japan). The ligand binding domain (LBD) of the hERα (amino acids 252-600) was amplified by RT-PCR and cloned as described by Nishikawa *et al.*[8] Briefly, total RNA was isolated from MCF-7 cells purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan), and subjected to RT-PCR. The amplified fragments were digested with the restriction enzymes *Eco* RI and *Bam* HI and inserted into the *Eco* RI-*Bam* HI site of pGBT9 so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain (GAL4DBD). The subsequent plasmid was called pGBT9-GAL4DBD-hERLBD. Plasmid, pGAD424-GAL4AD-TIF2RID (generously supplied by Nishikawa *et al.*[8]) was used to express fusion protein with the GAL4 activation domain (GAL4AD) and the receptor interaction domains (RID) of the co-activator, transcriptional intermediary factor 2 (TIF2). Sequences were confirmed by DNA sequencing.

Yeast Strain —— The yeast strain Y190 (*MATa*, *ura3-52*, *his3-D200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4Dgal80D*, *URA3*:::*GAL-lacZ*, *cyhr2*, *LYS2*:::*GAL-HIS3*), obtained from Clontech Laboratories Japan, Ltd., was transformed with pGBT9-GAL4DBD-hERLBD and pGAD424-GAL4AD-TIF2RID using a lithium acetate method.[11,12] Yeast transformants were selected by growth on SD medium, obtained from Clontech Laboratories Japan, Ltd., without tryptophan or leucine, and were confirmed by the detection of plasmids using a colony direct PCR[13] and by a filter-lift assay based on the E2-dependent development of X-Gal.[14]

Yeast Two-Hybrid Assay —— We performed a yeast two-hybrid assay as described by Nishikawa *et al.*[8] with slight modification. Briefly, yeast transformants were grown overnight at 30°C with shaking at 250 rpm in 20 ml of selective SD medium lacking tryptophan and leucine. Fifty μl of overnight culture and 2.5 μl of test chemical in dimethyl sulfoxide (DMSO) or 2.5 μl of DMSO (as a control) were combined in a test tube containing 200 μl of fresh selective medium. After incubation at 30°C for 4 hr, 150 μl of culture was taken for measurement of optical density (OD) at 600 nm using a spectrophotometer, (ImmunoReader NJ-2000, Nippon Intermed, Co., Ltd. (Tokyo, Japan) and for colony formation unit (CFU) analysis. One hundred μl of culture was harvested by centrifugation at 25°C for 5 min at *ca.* 8000 *g*. The cells were resuspended in 200 μl of Z buffer (0.1 M sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 3.5 mM β-mercaptoethanol) containing 1 mg/ml Zymolyase 20 T and incubated at 37°C for 15 min. The reaction was started by the addition of 40 μl of 4 mg/ml ONPG in 0.1 M sodium phosphate (pH 7.0) and incubation followed at 30°C for 30 min. The reaction was stopped by the addition of 100 μl of 1 M Na_2CO_3 and was centrifuged at *ca.* 8000 *g* for 5 min at 25°C. To measure β-galactosidase activity the supernatant was divided into 150 μl aliquots within a 96 well microplate and the OD was measured at 420 and 550 nm using a microplate reader [MTP-100, Corona Electric, Co., Ltd. (Katsuta, Ibaragi, Japan)]. β-galactosidase activity was used to estimate estrogenic activity and was calculated using the following equation:

$$U = 1000 \times ([OD_{420}] - [1.75 \times OD_{550}]) / ([t] \times [v] \times [OD_{600}])$$

where t = time taken for β-galactosidase reaction (min), v = volume of culture used in the assay (ml), OD_{600} = cell density at the start of the assay, OD_{420} = OD by *o*-nitrophenol at the end of the reaction, and OD_{550} = light scattering at the end of the reaction.

CFU Analysis and Methylene Blue Staining —— Serial dilutions of culture were made and 100 μl of each dilution was spread onto 1.5% agar SD plates (lacking tryptophan and leucine). Colonies of yeast were counted after incubation periods of 2 days at 30°C. The number of viable yeast cells in 1 ml of culture was determined from the serial dilution ratio. For methylene blue staining, culture was mixed with equivalent 0.01% methylene blue solution in 0.1 M potassium phosphate (pH 4.6), and the staining state of cells was observed by a phase-contrast microscope [BH-2, Olympus Optical, Co., Ltd. (Tokyo, Japan)].

Solubility of the Test Substance —— One hundred μl of 0.1 M of individual test chemicals was

added to 10 ml of SD medium and shaken at 250 rpm for 30 min at 30°C. Each solution was passed through a filter (Millex-LG, pore size: 0.2 μm, diameter: 4 mm, Nihon Millipore, Ltd., Tokyo, Japan). After the addition of 100 μl of a 10 μg/ml internal standard solution (4-*tert*-Butylphenol or *n*-Propyl 4-hydroxybenzoate) to the constant filtrate, 20 μl of the solution was analyzed by HPLC. The solubility of individual test chemicals in SD medium represents the concentration in their filtrate.

[Column : Inertsil ODS-3 (4.6 mm i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan), mobile phase: 30% to 100% acetonitrile in water at 1.75% / min or 70% to 100% acetonitrile in water at 1.0%/min, flow rate: 1.0 ml/min, column temperature: 50°C, detector: ultraviolet (225, 249 or 265 nm)].

RESULTS AND DISCUSSION

β-Galactosidase activity was only detected in yeast transformants harboring both pGBT9-GAL4DBD-hERLBD and pGAD424-GAL4AD-TIF2RID in the presence of E2 (Table 1). This activity was dependent on the dose of E2. This dose dependency was also observed with the suspected estrogenic chemicals, DES, BPA, GS and NP (Fig. 1). Therefore, we concluded that this yeast two-hybrid assay, based on hERα, was effective in the screening for estrogenic chemicals.

Nishihara *et al.*[9] called the concentration of the test chemical, which showed 10% of the total β-galactosidase activity produced at 10^{-7} M of E2, REC10 (10% relative effective concentration). When the

Table 1. Effect of E2 on β-Galactosidase Activity in Yeast Transformants

Plasmid in yeast (Y190)		β-galactosidase activity [U]	
pGBT9-GAL4DBD-hERLBD	pGAD424-GAL4AD-TIF2RID	E2 (+)	E2 (−)
−	−	0.6± 2.8	2.1±4.2
+	−	0.0± 1.5	2.2±1.7
−	+	19.2± 9.0	15.5±4.8
+	+	500.1±19.4	2.0±1.6

Each value represents the mean ± S.D. of triplicate assays.

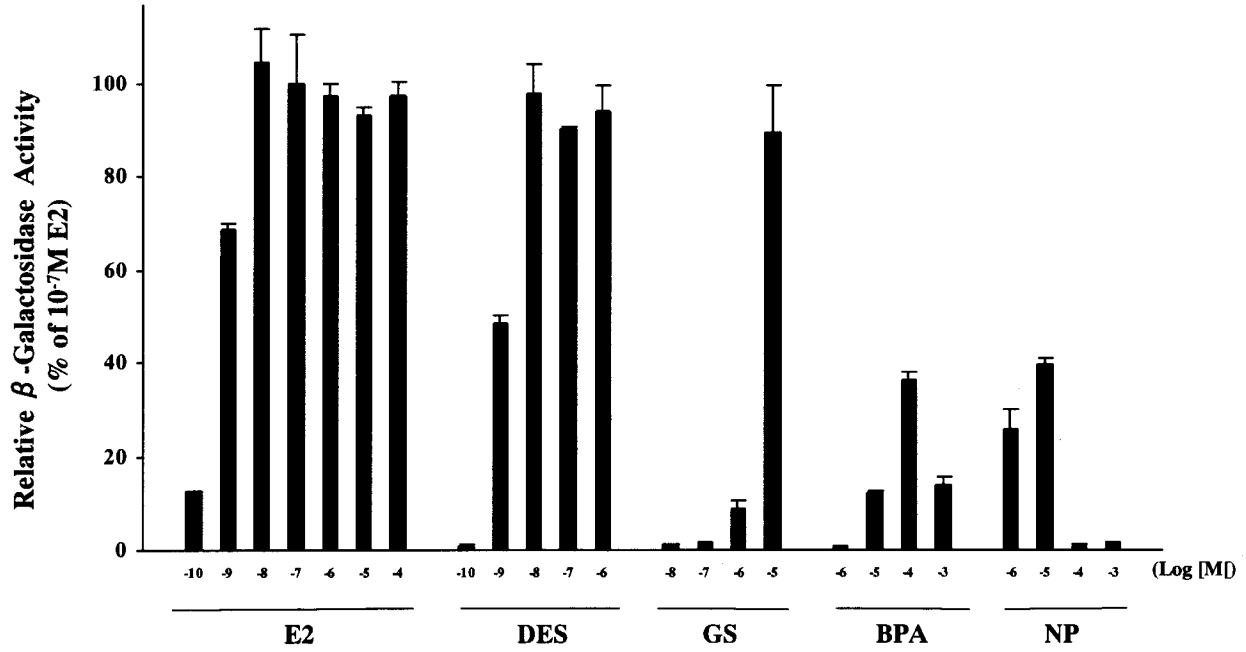

Fig. 1. Yeast Two-Hybrid Assay for Estrogen Receptor Activity

Estrogenic chemicals were added to the yeast cultures in doses ranging at the indicated concentrations. After 4 hr incubation the cultures were assayed for β-galactosidase activity. The values are represented as the relative activity (%) of β-galactosidase activity when E2 is present at a concentration of 10^{-7} M. Each value represents the mean + S.D. of triplicate assays.

activity of the test chemical was higher than 10% activity of 10^{-7} M of E2 within the concentration tested, they judged the chemical to be positive. We consider this criterion to be too severe for conventional primary screening.

Therefore, we performed the screening using a method we developed towards some chemicals with estrogenic activity at a higher concentration. Out of 64 chemicals in which estrogen-like activities were reported, 16 with estrogenic activity at a higher concentration (10^{-4}–10^{-3} M) were identified. REC10 was calculated from the values in Fig. 2(A) as described by Nishihara *et al.*[9] The REC10 of 7 chemicals, EHB, MHB, CDMP, CP, HAP, MP and DNPP, was 4×10^{-5}, 1×10^{-4}, 2×10^{-5}, 7×10^{-5}, 2×10^{-4}, 2×10^{-4} and 5×10^{-4} M, respectively. These values were similar to those reported by Nishihara *et al.*[9] However, the REC10 of 6 chemicals (DZE, HF, DHBP, NPDE, DPCB and BBP) were lower than those they reported, and the REC10 of 3 chemicals (DZ, OPDE and DIPP) could not be calculated (data not shown).

In the yeast two-hybrid assay, yeast cells are grown throughout the screening process. After incubating the cells for 4 hr with shaking, the OD at 600 nm, an index of yeast cell content, was increased on approximately two-fold in the absence of test chemicals (control) and in the presence of 10^{-7} M of E2. However, when exposed to 10^{-3} M of 6 chemicals (HF, CDMP, CP, NPDE, OPDE, and DPCB), the OD was significantly decreased ($p < 0.001$) in comparison with the control [Fig. 2(B)].

In order to determine the number of viable yeast cells, CFU analysis of a yeast suspension was performed after incubating the cells for 4hr with shaking. The results are shown in Fig. 2(C). CFU analysis of the yeast cell suspension treated with each of the 6 chemicals, HF, CDMP, CP, NPDE, OPDE and DPCB showed a dose-dependent decrease in the number of viable yeast cells when the concentrations of the chemicals were 10^{-4}–10^{-3} M ($p < 0.01$

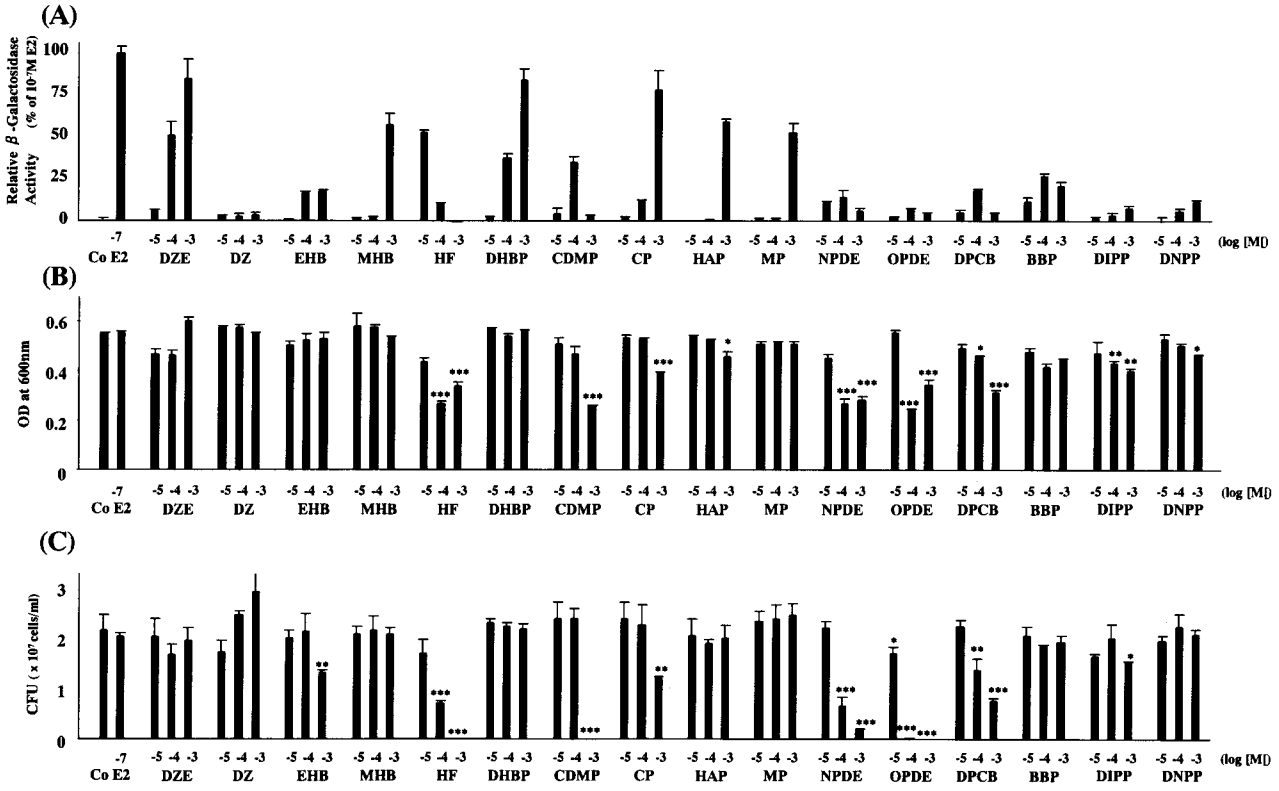

Fig. 2. The Effect of Test Chemicals on β-Galactosidase Activity, OD and CFU in a Yeast Two-Hybrid Assay

Test chemicals in which weak estrogenic activities were reported at high concentration were added to the yeast cultures in doses ranging from 10^{-5}–10^{-3} M. After 4 hr incubation, the cultures were assayed for β-galactosidase activity and measurement of OD and CFU. Each value represents the mean + S.D. of triplicate assays. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (*vs.* control, Dunnet), Co: control.

(A) The effect of test chemicals on β-galactosidase activity: β-galactosidase activity after the addition of 10^{-7} M of E2 was 500-600U. The values are represented as the relative activity (%) of β-galactosidase activity when E2 is present at a concentration of 10^{-7} M.

(B) The effect of test chemicals on OD at 600 nm of the yeast cultures: OD at 600 nm before incubation on control (Co) was 0.25–0.28.

(C) The effect of test chemicals on CFU of the yeast cultures: The values are represented as colony formation units per ml of the yeast cultures.

Table 2. CFU and Solubility of Individual Test Chemicals at REC10

Test chemical	REC10[a)] (M)	CFU at REC10[b)] (%)	Solubility in SD medium (M)	Solubility at REC10[c)]	Ratio of stained cells[d)] (% at 10^{-3} M)
Daidzein (DZE)	1×10^{-3}	96	1.5×10^{-5}	i	< 10
Daidzin (DZ)	3×10^{-3}	100	$> 1.0 \times 10^{-3}$	s	< 10
Ethyl 4-hydroxybenzoate (EHB)	1×10^{-4}	100	$> 1.0 \times 10^{-3}$	s	< 10
Methyl 4-hydroxybenzoate (MHB)	4×10^{-4}	100	$> 1.0 \times 10^{-3}$	s	87
2-Hydroxyfluorene (HF)	1×10^{-4}	41	2.9×10^{-4}	s	< 10
4,4′-Dihydroxybenzophenone (DHBP)	3×10^{-4}	98	$> 1.0 \times 10^{-3}$	s	< 10
4-Chloro-3,5-dimethylphenol (CDMP)	1×10^{-4}	100	$> 1.0 \times 10^{-3}$	s	< 10
4-Chlorophenol (CP)	2×10^{-4}	96	$> 1.0 \times 10^{-3}$	s	< 10
4-Hydroxyacetophenone (HAP)	2×10^{-4}	94	9.5×10^{-4}	s	< 10
4-Methylphenol (MP)	3×10^{-4}	100	$> 1.0 \times 10^{-3}$	s	< 10
4-*n*-Nonylphenol diethoxylate (NPDE)	1×10^{-3}	8	2.3×10^{-6}	i	< 10
4-*tert*-Octylphenol diethoxylate (OPDE)	3×10^{-4}	30	3.8×10^{-5}	i	< 10
cis-1,2-Diphenylcyclobutane (DPCB)	3×10^{-4}	7 5	4.6×10^{-6}	i	< 10
Benzyl butyl phthalate (BBP)	5×10^{-4}	95	3.6×10^{-6}	i	< 10
Di-iso-propyl phthalate (DIPP)	2×10^{-3}	93	4.0×10^{-4}	i	< 10
Di-*n*-propyl phthalate (DNPP)	1×10^{-3}	100	3.0×10^{-4}	i	< 10

a) REC10: The concentration showing 10% activity of 10^{-7} M of E2 (relative activity). The values representing the REC10 were reported by Nishihara *et al*.[9)] *b*) CFU at REC10: The values are represented as the relative CFU (%) at REC10[a)] against CFU when individual test chemicals are present at a concentration of 10^{-5} M. *c*) Solubility at REC10: In individual test chemicals, REC10 was compared with the solubility in the SD medium. i (insoluble), REC10 $>$ solubility ; s (soluble), REC10 $\leq$ solubility. *d*) Ratio of stained cells: The values represent the ratio of cells stained with methylene blue at 10^{-3} M of individual test chemicals.

or $p < 0.001$). Viewing the number of viable yeast cells at a concentration of 10^{-5} M of individual test chemicals as 100%, the 4 chemicals HF, NPDE, OPDE and DPCB in particular decreased the number of cells at a chemical concentration of corresponding to REC10 estimated by Nishihara *et al*.[9)] to 41, 8, 30 and 75%, respectively (Table 2). In addition, when each yeast suspension after incubating for 4 hr was subjected to methylene blue staining and microscopic observation, only those yeast cells treated with HF were markedly stained at 10^{-3} M, and therefore expected to be in the state of cellular death (Table 2). None of the chemicals tested had apparent effects on morphology (sharp and size) of yeast cells (data not shown).

These results indicate that of the chemicals tested, only HF tends to be fungicidal to the yeast cell, while OPDE, NPDE and DPCB tend to inhibit cellular proliferation at high concentrations.

The inhibitory mechanism of OPDE, NPDE and DPCB remains unclear at present. However, analysis of their solubility demonstrated that these three chemicals did not dissolve in the selective growth medium (Table 2). Since the hydrophobicity of these chemicals is very high, they may bind non-specifically to the yeast cell membrane and wall and penetrate into the cells, leading to unexpected inhibition of cellular proliferation.

In the yeast two-hybrid assay, Nishihara *et al*.[9)] gave attention to the toxicity of the test chemicals against the yeast cell by checking the OD of the yeast suspension. We also believe that toxicity against the yeast cell is an essential element in that assay. It is important to perform the screening in the concentration range where toxicity of the test chemicals is not observed. Therefore, it is recommended that the proliferation state of the yeast cells be checked not only by an indirect method such as OD measurement but also by a direct method like measurement of the number of viable cells.

At least, in evaluating the results of a yeast two-hybrid assay under conditions where cell proliferation is inhibited, it is necessary to determine the presence of estrogenic activity of the test chemicals by comparing the results of different analytical methods.

Acknowledgements We are grateful to Dr. T. Nishihara and Dr. J. Nishikawa for providing a plasmid (pGAD424-GAL4AD-TIF2RID).

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