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Construction of High Sensitive Detection System for Endocrine Disruptors with Yeast *n*-Alkane-assimilating *Yarrowia lipolytica*

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To construct a highly sensitive detection system for endocrine disruptors (EDs), we have compared the activity of promoters with the *n*-alkane-inducible cytochrome P450 gene (*ALK1*), isocitrate lyase gene (*ICL1*), ribosomal protein S7 gene (*RPS7*), and the translation elongation factor-1α gene (*TEF1*) for the heterologous gene in *Yarrowia lipolytica*. The promoters were introduced into the upstream of the *lacZ* or hERa reporter genes, respectively, and the activity was evaluated by β-galactosidase assay for *lacZ* and Western blot analysis for hERα. The expression analysis revealed that the *ALK1* and *ICL1* promoters were induced by *n*-decane and by EtOH, respectively. The constitutive promoter of *RPS7* and *TEF1* showed mostly a high level of expression in the presence of glucose and glycerol, respectively. In particular, the *TEF1* promoter showed the highest β-galactosidase activity and a significant signal by Western blotting with the anti-estrogen receptor, compared with the other promoters. Moreover, the detection system was constructed with promoters linked to the upstream of the expression vector for the hERα gene transformed into the *Y. lipolytica* with a chromosome-integrated *lac*Z reporter gene under the control of estrogen response elements (EREs). It was indicated that a combination of p*TEF1*p-hERα and CXAU1-2XERE was the most effective system for the E_2-dependent induction of the β-galactosidase activity. This system showed the highest β-galactosidase activity at 10^{-6} M E_2, and the activity could be detected at even the concentration of 10^{-10} M E_2. As a result, we have constructed a strongly sensitive detection system with *Y. lipolitica* to evaluate recognized/suspected ED chemicals, such as natural/synthetic hormones, pesticides, and commercial chemicals. The results demonstrate the utility, sensitivity, and reproducibility of the system for identifying and characterizing environmental estrogens.

Keywords: Endocrine disruptors detection system, *Yarrowia lipolytica*, hERα, ERE

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Compounds in food and the environment that bind to the estrogen receptors (ERs) and act as either agonists or antagonists of endogenous steroids have attracted increasing interest. Disturbances of steroid hormone production in the gonads have become a serious problem, since estrogen-like chemicals can cause reproductive dysfunctions in humans and wildlife [26].

The key target of environmental chemicals, so-called endocrine disruptors (EDs), is the nuclear receptors that function as a transcription factor essential for the specific gene expression. The ER is a member of the nuclear receptor superfamily [16, 30], and plays important roles in a variety of biological events during embryonic development and in physiological regulation in adulthood. The ER binds to its ligand, estrogen, and then binds to the estrogen response element (ERE) within the regulatory region of the target gene promoters. Recently, a number of chemicals that bind to the ER have been identified. These chemicals have little structural resemblance to natural estrogens. Therefore, it is important to evaluate the structural essentials for binding to the receptor, particularly for an assessment and prediction of xenoestrogens [4, 32, 33].

One of the urgent works in solving the problem with EDs is to compile a list of suspected substances among the large number of chemicals, using an appropriate screening test method. An *in vitro* screening method, in particular, is a very useful method for the primary selection of suspected EDs. Accordingly, an assay for EDs was developed using

yeast *Saccharomyces cerevisiae* as follows: yeast cells transformed with the plasmids encoding the estrogen (ER), androgen (AR), or progesterone receptor (PR), hormone response element sequence (HREs), and promoters linked to the upstream of reporter genes were evaluated to detect the estrogenic activity of the screening compounds [1, 6, 9]. Furthermore, the yeast two-hybrid system is based on the ligand-dependent interaction between the hormone receptors and various coactivators. The hormonal activity is detected with a chromosome-integrated *lac*Z reporter gene under the control of the Gal4p-binding site [13, 14, 21].

Yarrowia lipolytica is an ascomycetous yeast that can utilize *n*-alkane as the sole carbon source. Because of its stable haploid life cycle, *Y. lipolytica* is advantageous for the molecular genetic studies, and is attracting increasing attention in fundamental research and biotechnology [3, 24]. *Y. lipolytica* is capable of emulsifying hydrocarbons during the degradation process. However, only a few steps in the degradation pathway of its *n*-alkanes and fatty acids have been characterized. Yeast cells grown with *n*-alkanes produce a bioemulsifier known as liposan [5, 34].

In this study, we describe a highly sensitive detection system that works successfully at very low concentration of EDs in *Y. lipolytica*. The system was constructed to detect genes encoding the hERα protein, expressed with the promoters *ALK1*, *ICL1*, *RPS7*, or *TEF1* in the *Y. lipolytica* strains CXAU1-1xERE or CXAU1-2xERE, respectively. The hERα binds to E_2 as the ligand, and then binds to ERE within the regulatory region of the *lac*Z reporter gene promoter. The use of *Y. lipolytica* with a combination of p*TEF1*p-hERα and CXAU1-2xERE could better detect the estrogenic activities of natural hormones and endocrine disruptors at low concentrations. Consequently, the detection system that was constructed with *Y. lipolytica* is a powerful approach to the primary screening of recognized/suspected EDs.

Materials and Methods

Chemicals

17β-Estradiol (E_2), estrone (E_1), and testosterone were purchased from Sigma Chemical Co. Genestein and coumestrol were obtained from Fluka Chemie AG. Diethystilbestrol (DES), bisphenol A (BPA), 4-nonylphenol (4-NP), and 4-*tert*-octylphenol (4-*tert*-OP) were acquired from Tokyo Kasei Kogyo (Tokyo). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,3,5-trichloro-phenoxyacetic acid (2,4,5-T) were supplied by Kanto Chemical Co. (Tokyo). 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (*p,p'*-DDT) and γ-hexachlorocyclohexane (γ-HCH) were purchased from Wako Pure Chemicals (Osaka). All chemicals used were of reagent grade, and used without further purification.

Strains and Media

The reporter gene construct was integrated into the genomic DNA of the *Y. lipolytica* strain CXAU1 (*ade1*, *ura3*) as a wild-type strain [10] to generate CXAU1-1xERE or CXAU1-2xERE, respectively. The estrogen-responsive reporter gene (EREs-$TATA_{ALK1}$-*lacZ*), which contains one or two copies of a consensus EREs oligonucleotide and TATA box in front of the *lac*Z gene, was constructed. The reporter gene was constructed by the following procedure. The gene was amplified by PCR. The primer set for PCR of 1xERE was as follows: forward primer (5'-AGGTCACAGTGACCT-3') and reverse primer (5'-AGGTCACAGTGACCT-3'). The primer set for PCR of 2xERE was as follows: forward primer (5'-AGGTCACAGTGACCTA GGTCACAGTGACCT-3') and reverse primer (5'-AGGTCACAGTG ACCTAGGTCACAGTGACCT-3'). The reporter gene construct, pSUT5-EREs, was inserted into an *E. coli*–*Y. lipolytica* shuttle vector pSUT5 [34] with 1xERE-$TATA_{ALK1}$-*lacZ* or 2xERE-$TATA_{ALK1}$-*lacZ* digested with *Not*I and *Apa*I, respectively (Fig. 1A). The reporter gene construct pSUT5-EREs was then digested with *Nhe*I and *Dra*I to enhance the level of integration at the defective *ura3* locus in the wild-type strain CXAU1 (Fig. 1B). The yeast was transformed by employing an electroporation method using a Gene Pulser (Bio-Rad), and was selected by growing on SD medium lacking adenine. Integration was confirmed by Southern hybridization analysis (data not shown). The YNB medium was used as the minimal medium. An appropriate carbon source was added to YNB as follows: 2% glucose (SD), 2% glycerol (SG), 1% EtOH, or 2% *n*-decane. Adenine or uracil (24 mg/l) was then added if necessary. For the solid medium, 2% agar (WAKO, bacterial grade) was added.

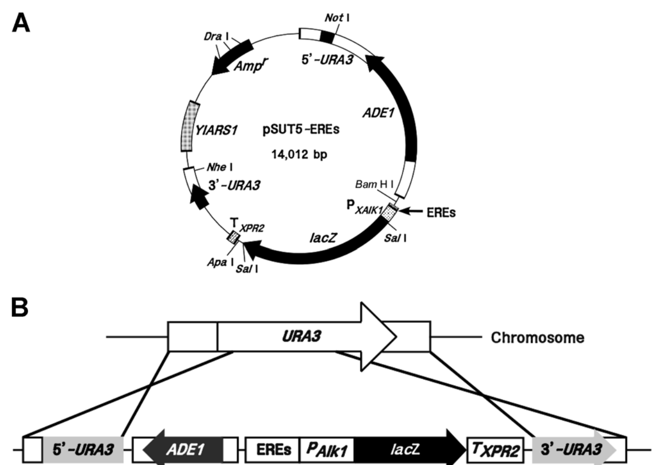

Fig. 1. Construction of the reporter gene pSUT5-EREs.
A. Restriction enzyme site of the reporter gene. **B**. The reporter gene constructs were linearized by digestion with *Nhe*I and *Dra*I to enhance the level of integration at the defective *ura3* locus in *Y. lipolytica* strain CXAU1.

Construction of Vector to Evaluate Promoter Activities Using *lacZ* Gene

The total DNA of the *Y. lipolytica* strain CXAU1 was prepared to use as a template for PCR applications. An *E. coli*–*Y. lipolytica* shuttle vector pSUT5-*lacZ* [29] was used to screen the powerful promoters derived from *Y. lipolytica*. The promoters, *ALK1* [10, 22], *ICL1* [2, 8, 11, 17], *RPS7* [18], and *TEF1* [19, 25], were amplified by PCR using the primer sets listed in Table 1 to create the pSUT5-promoters-*lacZ*. The PCR-amplified fragments were digested with the restriction enzymes listed in Table 1, and inserted into the

Table 1. Synthetic nucleotide oligomers used in this study.

Primer	Sequence
To insert in vector pSUT5-*lac*Z	
ALK1 forward - *Not*I	5'-GCGGCCGCAGATCTGTGCGCCTCT-3'
reverse - *Spe*I	5'-ACTAGTAGTGCAGGAGTATTCTG-3'
ICL1 forward - *Spe*I	5'-ACTAGTCTCGAGATGGACATACTTGTA-3'
reverse - *Stu*I	5'-AGGCCTCACTGGGTTAGTACGGGACAG-3'
RPS7 forward - *Not*I	5'-GCGGCCGCTTACCTGCTACTTGTCTCAACA-3'
reverse - *Spe*I	5'-ACTAGTCATTTTTGTGTTTGTTGAGTGAAGA-3'
TEF1 forward - *Not*I	5'-GCGGCCGCAGAGACCGGGTTGGCGGC-3'
reverse - *Hin*dIII	5'-AAGCTTTTCGGGTGTGAGTTGACAAG-3'
To insert in vector pSUT5-hERα	
ALK1 forward - *Spe*I	5'-ACTAGTAGATCTGTGCGCCTC-3'
reverse - *Spe*I	5'-ACTAGTAGTGCAGGAGTATTCTG-3'
ICL1 forward - *Spe*I	5'-ACTAGTCTCGAGATGGACATACTTGTA-3'
reverse - *Eco*RI	5'-GAATTCCACTGGGTTAGTACGGGACAG-3'
RPS7 forward - *Spe*I	5'-ACTAGTTTACCTGCTACTTGTCTCAACA-3'
reverse - *Bam*HI	5'-GGATCC TTGTGTTTGTTGAGTGAAGA-3'
TEF1 forward - *Bam*HI	5'-GGATCCAGAGACCGGGTTGGCGGC-3'
reverse - *Bam*HI	5'-GGATCCTTCGGGTGTGAGTTGACAAG-3'

multiple cloning site (MCS) of pSUT5-*lac*Z to generate p*ALK1*p-*lac*Z, p*ICL1*p-*lac*Z, p*RTS7*p-*lac*Z, or p*TEF1*p-*lac*Z, respectively (Fig. 2A). All the nucleotide sequences were determined using a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer) and an automated DNA sequencing system (ABI Prism 310).

Construction of Vector to Evaluate Promoter Activities Using hERα Gene

For the construction of pSUT5-hERα, a full-length hERα cDNA was amplified by PCR using a set of primers with the *Sal*I and *Apa*I sites at their forward primer (hERα-*Sal*I) 5'-GTCGACATGACCATGACCCTCCACACC-3' and reverse primer (hERα-*Apa*I) 5'-GGGCCCGCCAGGGAGCTCTCAGAC-3', respectively. The hERα cDNA fragment sequences were confirmed by DNA sequencing analysis. The pSUT5 was created by extracting the *lac*Z fragment by digestion with the *Sal*I and *Apa*I restriction enzymes from the vector pSUT5-*lac*Z, and the hERα cDNA fragment with the correct sequence was inserted between the *Sal*I and *Apa*I sites of pSUT5 to obtain pSUT5-hERα. The promoters, *ALK1*, *ICL1*, *RPS7*, and *TEF1*, were amplified by PCR using the probes listed in Table 1 to insert the promoters into the pSUT5-hERα. These fragments were digested with the restriction enzymes listed in Table 1, and then

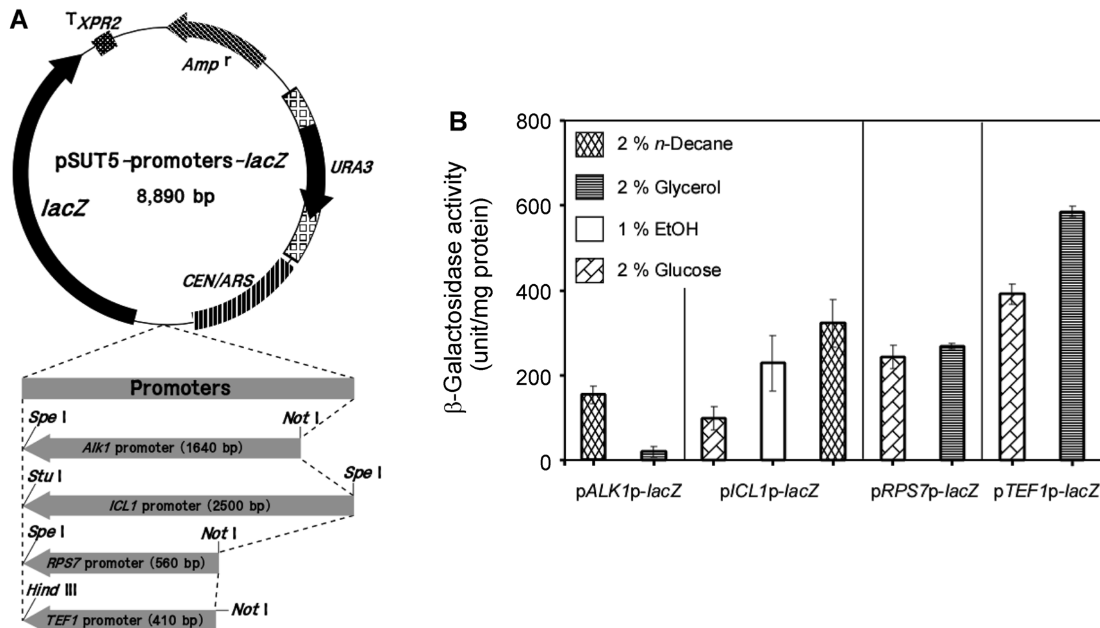

Fig. 2. Construction of the vector to evaluate the promoter activities using the *lac*Z reporter gene.

A. A set of plasmids pSUT5-promoters-*lac*Z were constructed by inserting promoters *ALK1*, *ICL1*, *RPS7*, or *TEF1* into the pSUT5-*lac*Z. **B**. The expression level of *lac*Z reporter genes under the promoters, as measurement by β-galactosidase activity assay. The control was without any carbon sources.

inserted into the corresponding restriction enzyme sites of pSUT5-hERα to construct p*ALK1*p-hERα, p*ICL1*p-hERα, p*RTS7*p-hERα, or p*TEF1*p-hERα, respectively (Fig. 3A). All the nucleotide sequences were determined using a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer) and an automated DNA sequencer (ABI Prism 310).

Western Blot Analysis

Western blot analysis was used to confirm the expression of the hERα gene in yeast. The yeast transformants with p*ALK1*p-hERα or p*ICL1*p-hERα inserted were grown in a SG medium to the logarithmic phase. The cells were then transferred to YNB containing 2% *n*-decane or 1% EtOH as the carbon source to induce culture, and were cultured for 6 h or 12 h, respectively. The yeast transformants with p*RPS7*p-hERα or p*TEF1*p-hERα inserted were grown in SG medium to the logarithmic phase. The expression of hERα in each culture was analyzed by Western blot analysis (Fig. 3B).

Sodium dodecyl sulfate polyacrylamide gel (SDS−PAGE) was performed essentially as described by Sambrook *et al*. [27], where 25-μl aliquots of the supernatant of samples used to measure the enzymatic activity were loaded. The SDS−PAGE gel was blotted onto a Hybond-C (Amersham) nylon membrane according to the supplier's recommendation by semidry electroblotting for 90 min at 180 mA (Sartoblot II-S stabilizer). For blotting, the membranes were incubated for 60 min at RT with an anti-estrogen receptor (synthetic peptides corresponding to amino acids 578−595 of the human estrogen receptor; Sigma) raised against the tested enzymes, diluted 1:5,000 in PBS [phosphate-buffered saline; 0.01 M sodium phosphate (pH 7.5), 0.15 M NaCl, 0.1% Tween 20]. The bound rabbit antibodies were visualized by incubation for 60 min at RT with anti-rabbit Ig [horseradish peroxidase-linked whole antibody (from donkey), Amersham], diluted 1:5,000 in PBS. Western blotting was performed using the ECL detection system (Amersham), according to the manufacturer's protocol.

Growth of Yeast and β-Galactosidase Assay

Yeast culture to screen powerful promoters derived from *Y. lipolytica*. Yeast transformants were precultured in a SD medium for 2 days. The yeast culture was then inoculated into 10 ml of the SD or SG medium at 2% inoculum, and incubated for 24 h. The cells were collected by centrifugation, and washed twice. The yeast transformants, p*ALK1*p-*lac*Z and p*ICL1*p-*lac*Z, were suspended in order to induce culture in 3 ml of YNB containing various carbon sources (2% *n*-decane, 2% glycerol, 1% ethanol, 2% glucose). The control was without any carbon sources. The yeast cells were cultured at 30°C for 6 h (p*ALK1*p-*lacZ*) or 12 h (p*ICL1*p-*lac*Z), respectively. However, *RPS7* and *TEF1*, as the constitutive promoters, did not induce a culture.

Yeast culture to detect the EDs. The β-galactosidase activity to detect the activity of EDs was measured using a method reported elsewhere [13]. The yeast transformants were grown at 30°C for 2 days in 2 ml of the selective SD medium. If necessary, after inducing the culture, a portion (0.2 ml) of the preculture (or induced culture) was diluted in 9.8 ml of fresh SD containing the E_2 or EDs and added to a dimethylsulfoxide (DMSO) solution. The DMSO concentration did not exceed 1% of the culture volume. The yeasts were incubated for 17 h at 30°C with constant vigorous orbital shaking.

β-Galactosidase assay. The cells were collected, washed twice, and suspended in 1 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol). The cells were crushed by vortexing three times for 1 min each time. The crushed cell suspension was removed by centrifugation at 15,000 rpm for 10 min. For the β-galactosidase assays, 0.1 ml of 4 mg/ml ONPG

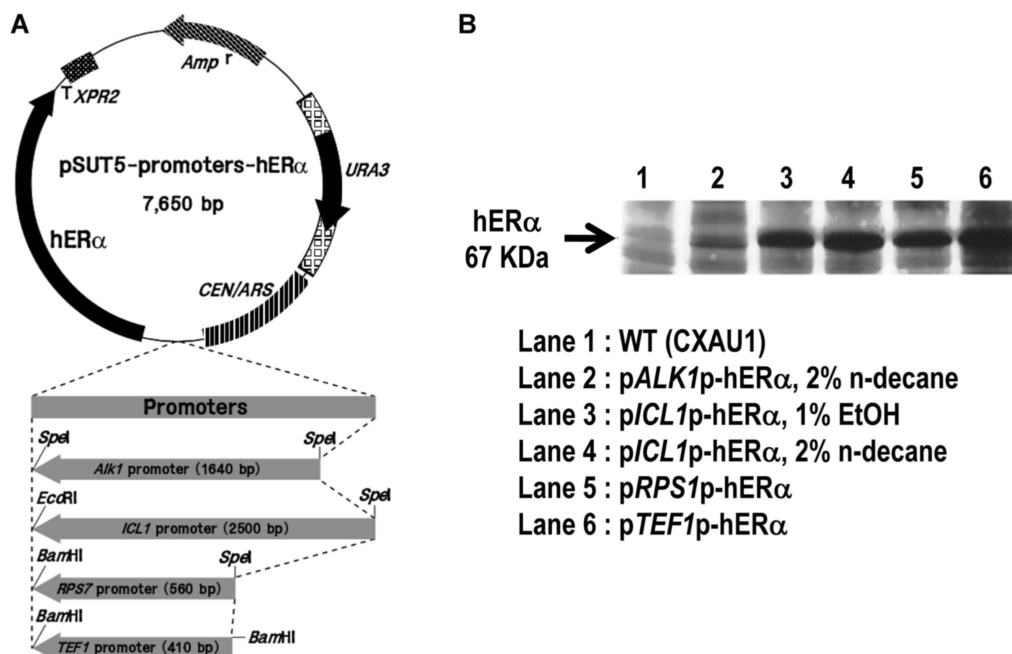

Fig. 3. Construction of the vector to evaluate the promoter activities using the hERa gene.
A. A set of plasmids pSUT5-promoters-hERα were constructed by inserting *ALK1*, *ICL1*, *RPS7*, or *TEF1* into the pSUT5-hERα. **B**. The expression level of the hERα under the promoters, as assessed by Western blot analysis.

(*o*-nitrophenyl-β-D-galactopyranoside dissolved in Z-buffer) was added to 0.2 ml of the cell extract and 0.5 ml of the fresh Z-buffer, and the mixture was incubated at 30°C. When a yellow color had developed, 0.25 ml of a 1 M Na_2CO_3 solution was added to quench the reaction, and the absorbance was measured at 420 nm. One unit of enzyme activity is defined as the activity that produces 1 nmol of *ortho*-nitrophenol per minute. The protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad). The data are representative of three independent experiments, and the experiments were performed in triplicate.

Evaluation of Estrogenic Activities of Various Compounds Using REC10 and RIE

The estrogenic activities of the various compounds were evaluated using *Y. lipolytica* and *S. cerevisiae* detection systems. The results were converted to REC10 (10% relative effective concentration against E_2) [15, 20], which is the concentration of a tested chemical showing 10% of the highest estrogenic activity of E_2 in a given system. When the activity of the test substance was higher than REC10 within the concentration tested, the chemical was judged to be positive. Furthermore, the relative inductive efficiency (RIE) was determined by the REC10 achieved within these yeast detection systems. The RIE is the ratio of the REC10 of E_2 to the REC10 of the tested chemicals, multiplied by 100. By definition, E_2 has an RIE of 100 [6, 7].

RESULTS AND DISCUSSION

Comparison of Promoter Activities with β-Galactosidase Activity

The representative promoter assay of *ALK1*, *ICL1*, *RPS7*, and *TEF1* in *Y. lipolytica* was performed in order to obtain a promoter with high activity to express heterologous genes. As shown in Fig. 2B, the *ALK1* was induced by 2% *n*-decane and repressed by 2% glycerol. The *ICL1* promoter was induced by 1% EtOH and 2% *n*-decane, and repressed by 2% glucose. The *ICL1* promoter gave a higher β-galactosidase activity than did the *ALK1* promoter. The promoters *RPS7* and *TEF1*, as constitutive genes, showed activity only in the presence of 2% glucose and 2% glycerol. In particular, the *TEF1* promoter showed the highest β-galactosidase activity compared with the promoters examined. These results are consistent with what has been reported [18] and showed that the promoters are able to be applied for the induction of target genes in *Y. lipolytica*.

Comparison of Promoter Activities with Expression of hERα

The hERα protein could not be detected by SDS−PAGE followed by CBB staining (data not shown). However, Western blot analysis using the anti-estrogen receptor showed a single polypeptide chain with a M_r of 68 kDa (hERα) (Fig. 3B). The protein was also expressed in the yeast *Y. lipolytica* as well as in *S. cerevisiae*. The results showed that the *ALK1* and the *ICL1* promoters were induced by *n*-decane and by either EtOH or *n*-decane, respectively. In addition, the hERα was expressed significantly by the *RPS7* and *TEF1* promoters, as the constitutive genes, in the yeast cells incubated in the medium containing 2% glycerol. In particular, the *TEF1* promoter resulted in the highest level of expression with the anti-estrogen receptor than with the other promoters examined.

Ligand-Dependent Activation of the Yeast Transformants CXAU1-1xERE and CXAU1-2xERE

The yeast transformants CXAU1-1xERE or CXAU1-2xERE were integrated into the genome of the *Y. lipolytica* strain CXAU1 were retransformed with pSUT5-promoters-hERα construct inserted each of *ALK1*, *ICL1*, *RPS7*, or *TEF1* promoters. Fig. 4 shows the activity by various combinations of pSUT5-promoters-hERα and the reporter gene CXAU1-EREs to E_2 concentrations ranging from 10^{-10} to 10^{-4} M (Fig. 4A and 4B). The hERα reporter gene induced by the

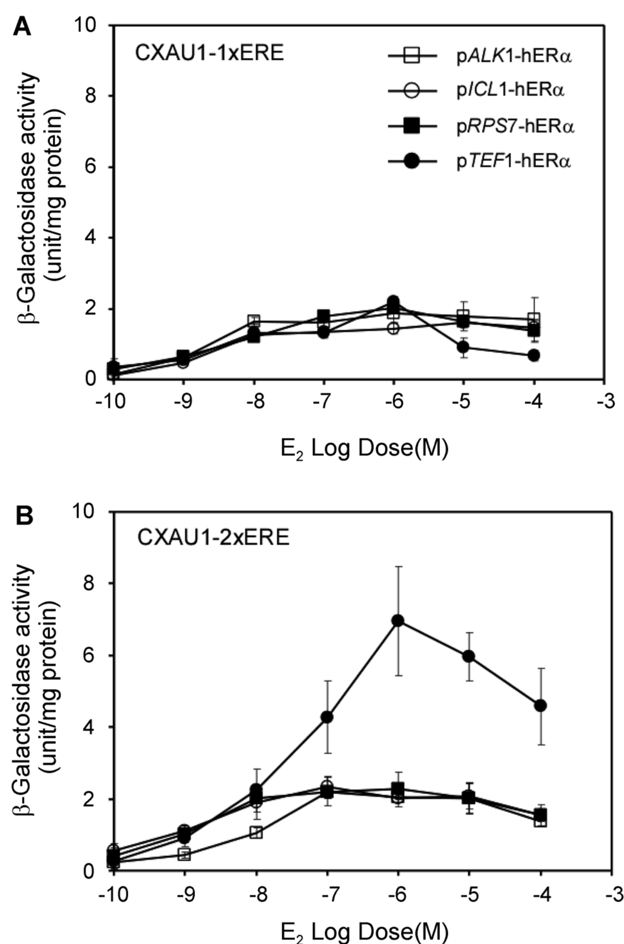

Fig. 4. Comparison of β-galactosidase activities of the yeast transforments CXAU1-1xERE (**A**) and CXAU1-2xERE (**B**) inserted expressing hERa under various promoters in the presence of E_2.

TEF1 promoter showed the highest β-galactosidase activity at 10^{-6} M E_2, and detected the activity at even the concentration of more than 10^{-10} M E_2 in the CXAU1-2xERE strain. In contrast, the CXAU1-1xERE strain that transformed hERα did not show activity in the presence of E_2, suggesting that it carries only 1 copy of the ERE, and so is not able to transact on the yeast transcription machinery in response to E_2. Two copies of the ERE were revealed to be needed on E_2 to transactivate the yeast transcription machinery. Furthermore, the interaction between the EREs and the hERα expressed by the *TEF1* promoter had a greater mediated transcriptional activation in the presence of estrogen.

Applications of the Yeast Detection System to Endocrine Disruptors

The estrogen-like activities of natural and synthetic steroids, pesticides, and industrial chemicals, which are recognized as EDs, were analyzed using the detection system in *Y. lipolytica* with a combination of p*TEF1*p-hERα and CXAU1-2xERE (Fig. 5). The lowest concentrations of chemicals that showed detectable activity of β-galactosidase were 10^{-8} M for DES, 10^{-8} M for estrone, 10^{-7} M for coumestrol, 10^{-8} M for genisteine, and 10^{-7} M for testosterone (Fig. 5A). Of the pesticides tested, *p,p'*-DDT, 2,4-D, 2,4,5-T, and γ-HCH strongly activated the production of β-galactosidase at 10^{-4} M, 10^{-4} M, 10^{-6} M, and 10^{-4} M, respectively (Fig. 5B). Alkylphenols yielded a high β-galactosidase activity at 10^{-7} M or more (Fig. 5B). 4-NP showed a similar activity of β-galactosidase to that yielded at 10^{-6} M. 4-*Tert*-OP showed a high β-galactosidase activity at 10^{-5} M. The estrogenic activities of industrial chemicals such as bisphenol A (BPA) was also analyzed (Fig. 5B). BPA was effective at the concentration of 10^{-4} M. These results suggest that the constructed yeast detection system is applicable to a wide variety of chemicals and is quite useful for the primary screening of potential effectors on the functions of estrogen receptors.

Comparison of Estrogenic Activities with REC10 Values Using This Detection System and Other Detection Systems

The sensitivity of this system was compared with other known systems. Chemicals that gave more than 10% of β-galactosidase activity of that given by some concentration of E_2 (10% relative effective concentration, REC10) were tentatively considered to be positive (Table 2). The results considered to be positive are the following: in the case of *Y. lipolytica*, the combination of p*TEF1*p-hERα and CXAU1-2xERE was 0.7 unit/mg protein; and in the case of *S. cerevisiae*, 1) hERα, 2) hERα LBD+SRC1, and 3) hERβ LBD +SRC1 were 1.4, 2.14, and 7.33 unit/mg protein, respectively (Table 2) [15, 20]. The γ-HCH, 2,4-D, and 2,4,5-T resulted in negative (or were not detected) results when using the

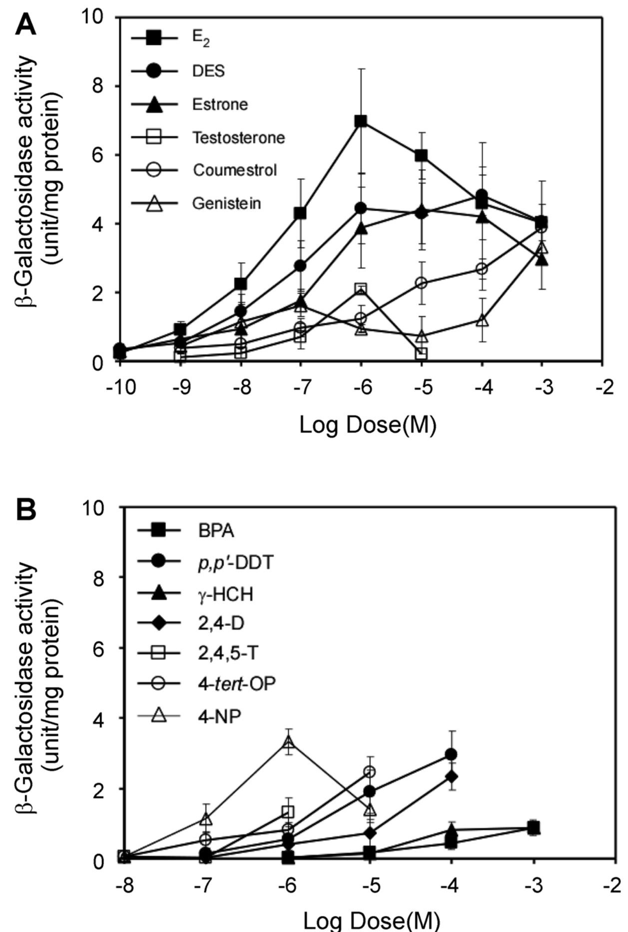

Fig. 5. Dose-response curves for natural and synthetic hormones (**A**), and industrial and pesticide chemicals (**B**) as determined with the combination of p*TEF1*-hERa and CXAU1-2xERE.

hERα assay constructed by the coactivator SRC1 using *S. cerevisiae*. However, all of the chemicals tested were found to be positive by a combination of p*TEF1*p-hERα and CXAU1-2xERE. These findings agree with those in other reports, which demonstrated estrogenic activities using *in vivo* and *in vitro* assays, with the exception of some chemicals that were the first tested [12, 23, 28, 31]. It is clear that the detection system with a combination of p*TEF1*p-hERα and CXAU1-2xERE using *Y. lipolytica* had much more sensitive detection in comparison with the hERα or interaction of hERs LBD and coactivators using *S. cerevisiae* from the estrogenic activities of natural hormones and endocrine disruptors at a lower concentration ranging from 3 orders to 1 order by the REC10 and RIE (Table 2). In conclusion, the combination of p*TEF1*p-hERα and CXAU1-2xERE is the most effective system to detect the xenoestrogen-dependent induction of the reporter gene activity at 2 to 3 orders lower concentration compared with previous yeast reporter systems with *S. cerevisiae*,

Table 2. Evaluation of the estrogenic activities of the various compounds determined using yeast *Y. lipolytica* and *S. cerevisiae* systems.

Compound	*Y. lipolytica* system		*S. cerevisiae* system[a]					
	p*TEF1*p-hERα+ CXAU1-2xERE		hERα		hERα LBD+SRC1		hERβ LBD+SRC	
	REC10[b]	RIE[c]	REC10	RIE	REC10	RIE	REC10	RIE
17β-Estradiol	4.25×10^{-9}	100	4.88×10^{-10}	100	2.49×10^{-10}	100	1.14×10^{-9}	100
Estrone	1.28×10^{-8}	33.2	2.78×10^{-8}	1.76	1.53×10^{-9}	16.3	4.20×10^{-9}	27.1
Testosterone	9.65×10^{-7}	0.44	3.15×10^{-3}	0.0001	1.54×10^{-5}	0.0016	2.12×10^{-5}	0.005
Coumestrol	2.35×10^{-7}	1.81	7.84×10^{-6}	0.0062	5.42×10^{-8}	0.46	3.25×10^{-7}	0.35
Genistein	2.33×10^{-8}	18.2	1.71×10^{-4}	0.0003	2.95×10^{-7}	0.084	1.59×10^{-6}	0.072
DES	2.41×10^{-9}	176.3	1.52×10^{-9}	32.1	3.59×10^{-12}	6,936	3.95×10^{-10}	288.6
γ-HCH	5.84×10^{-4}	0.0007	N	-	1.72×10^{-5}	0.0014	1.03×10^{-4}	0.001
p,p'-DDT	1.29×10^{-5}	0.033	1.25×10^{-4}	0.088	2.82×10^{-7}	0.088	1.95×10^{-5}	0.006
2,4-D	8.45×10^{-5}	0.005	N	-	5.42×10^{-6}	0.0046	1.35×10^{-4}	0.0008
2,4,5-T	3.20×10^{-6}	0.128	N	-	1.01×10^{-6}	0.025	2.92×10^{-6}	0.039
BPA	3.45×10^{-3}	0.0001	2.05×10^{-4}	0.0002	3.93×10^{-6}	0.006	2.75×10^{-5}	0.004
4-NP	3.75×10^{-7}	1.13	1.07×10^{-5}	0.0046	1.37×10^{-7}	0.182	1.86×10^{-6}	0.061
4-*tert*-OP	3.85×10^{-6}	0.11	5.18×10^{-6}	0.01	1.82×10^{-7}	0.14	3.35×10^{-7}	0.34

[a]The *S. cerevisiae* system was constructed to develop a highly sensitive detection system for EDs. We constructed two-hybrid systems with hER LBD+SRC1 that coexpress the Gal4p DNA binding domain/ligand-binding domain of human estrogen receptor (hER) α or β and the Gal4p transactivation domain/ nuclear receptor-binding domain of coactivator SRC1 in *S. cerevisiae* with a chromosome-integrated *lacZ* reporter gene under the control of Gal4p-binding sites. The one-hybrid system hERα was constructed to coexpress the Gal4p DNA-binding domain/full-length hERα in *S. cerevisiae* with a chromosome-integrated *lacZ* reporter gene [14, 15]. [b]REC10 (10% relative effective concentration against E_2) is the concentration of a tested chemical showing 10% of the highest estrogenic activity of E_2 in a given system; [c] RIE (relative inductive efficiency), the reciprocal REC10 ratio, E_2 has an RIE of 100; N, Negative. β-Galactosidase activities that gave REC10 were 0.7, 1.4, 2.14, and 7.3 units/mg protein for p*TEF1*phERα+CXAU1-2xERE, the hERα one-hybrid system, and the two-hybrid systems of hERα LBD+SRC1 and hERβ+SRC1, respectively. Chemicals were recognized/suspected as EDs such as natural/synthetic hormones, pesticides, and industrial chemicals.

because *Y. lipolitica* can emulsify hydrocarbons and most of the EDs have liposolubility. The detection system with *Y. lipolytica* was also identified as more significantly useful for the primary screening than the other *in vitro* screening tests, because it is less time-consuming and more easy to perform than the cell line system with MCF-7 E-screen. Moreover the data demonstrated the utility, sensitivity, and reproducibility of this system for characterizing environmental estrogens. The greatest strength of the system is that it is not necessary to consider endogenous estrogens and receptors, because these are absent from yeast cells. However, it might have false-negative results owing to a difference in the membrane transport of chemicals, because the yeast cell is not an animal cell but a plant cell [22].

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