J. Biochem. **131**, 399–405 (2002)

Employment of the Human Estrogen Receptor β Ligand–Binding Domain and Co-Activator SRC1 Nuclear Receptor–Binding Domain for the Construction of a Yeast Two-Hybrid Detection System for Endocrine Disrupters[1]

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Received October 16, 2001; accepted December 17, 2001

To screen a wide variety of chemicals for endocrine disrupters, and to develop an effective microbial degradation system for them, a good system is needed for the rapid and accurate evaluation of the endocrine-disrupting activities of suspected chemicals and their degradation products. We constructed two-hybrid systems that co-express 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 co-activator SRC1, TIF2, or AIB1 in *Saccharomyces cerevisiae* with a chromosome-integrated *lacZ* reporter gene under the control of Gal4p-binding sites. We found that the combination of the hERβ ligand–binding domain and SRC1 nuclear receptor–binding domain was most effective for the xenoestrogen-dependent induction of reporter activity. The extent of transcriptional activation by known xenoestrogens and phytoestrogens was found to correlate well with their estrogenic activities as measured by the previous system with rat ERα. This system detects estrogenic activity in some chemicals that have not been suspected of being positive. We also applied this assay system to test the microbial degradation products of γ-hexachlorocyclohexane (γ-HCH) by *Sphingomonas paucimobilis*. Among the γ-HCH metabolites, 2,5-dichlorohydroquinone and chlorohydroquinone had estrogenic activities similar to the original chemical, while hydroquinone, a later stage metabolite, showed no activity, suggesting the necessity of evaluating intermediate metabolites in microbial degradation systems.

Key words: co-activator SRC-1, endocrine disrupter, γ-HCH, hERβ, yeast two-hybrid system.

Many industrial chemicals and environmental pollutants affect human health by disrupting normal endocrine function. Such chemicals are called endocrine disrupters (EDs). There is a wide variety of EDs including natural products such as coumestrol and genistein, pesticides such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (*p,p'*-DDT) and γ-hexachlorocyclohexane (γ-HCH), and commercial chemicals such as bisphenol A and some alkyl phenols.

The key targets of EDs are nuclear hormone receptors, which bind to steroid hormones and regulate their target gene transcription. The estrogen receptor (ER) is a member of the nuclear hormone receptor superfamily (*1*), and plays important roles in a variety of biological events during embryonic development and in physiological regulation in adulthood. ER binds to its ligand, estrogen, and then binds to estrogen-response elements within the regulatory region of the target gene promoters. Two ER subtypes, ERα and ERβ, show high degrees of homology in their ligand-binding domains (LBDs) and DNA-binding domains (DBDs), and have a similar range of affinities for 17β-estradiol (E_2) (*2, 3*), although their functional difference is not yet clear. The ER protein is composed of six functionally and physically discriminated regions (A/B, C, D, and E/F). Regions C, D, and E are required for DNA binding, nuclear localization, and estrogen binding, respectively. In addition, there are two domains essential for transcriptional activation, AF-1, which is located in the N-terminal region A/B, and

[1] This work was partially supported by the ECO Molecular Sciences Research Program from RIKEN and involved the use of the facilities of the Biotechnology Center, the University of Tokyo.

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Abbreviations: GAL4 DBD, Gal4p DNA binding domain; GAL4 TAD, Gal4p transactivation domain; hER LBD, human estrogen receptor ligand–binding domain; NR, nuclear receptor; E_2, 17β-estradiol; γ-HCH, γ-hexachlorocyclohexane; ED, endocrine disruptor; AF-1 and AF-2, transcription activating factor–1 and factor–2, respectively; ERE, estrogen response element; AIB1, amplified in breast cancer 1; SRC-1, steroid receptor co-activator 1; TIF2, transcriptional intermediate factor 2; NRBD, nuclear receptor binding domain.

AF-2, which is located in region E and the activity of which is ligand-dependent. Both the DNA-binding and estrogen-binding domains have been reported to contribute to ER dimerization (*1, 4–7*). Upon activation by a ligand, the E domain recruits accessory proteins called co-activators, among which SRC1 (*8*), TIF2 (*9*), and AIB1 (*10*) form the well-defined p160 family. A highly conserved region in the middle of the p160 proteins interacts with the ligand-binding domain of nuclear receptors in a ligand-dependent manner. The three LXXLL motifs in the receptor interaction domain of the co-activators are essential for the interaction with nuclear receptors (*11–13*). Mammalian steroid hormone receptors introduced into the yeast strain *Saccharomyces cerevisiae* can also function as steroid-dependent transcriptional activators (*14–16*). Consequently, two-hybrid systems using the interaction of ERs and co-activators have been developed in yeast (*17–19*).

The ER subtype ERβ is similar to ERα in activity, but differs from it in many aspects, including tissue expression, ligand specificity and affinity, and selectivity in recruiting co-activators (*20*). Although the ligand-dependent activation level of reporter genes with estrogen-response elements on their promoter is generally lower with ERβ than ERα, the increase in their activation by ERβ is larger than by ERα (*21*). The affinity of ERα and ERβ for nuclear receptor boxes of p160 co-activators is greatly influenced by the ligand that is bound to the ERs (*22*). By selectively recruiting co-regulators to ERβ, isoflavone phytoestrogens only activate ERβ–mediated transcription pathways (*23*). Recent progress on the transcriptional regulation by estrogens suggests the necessity of another yeast two-hybrid detection system for endocrine disrupters, in which ERβ and a suitable co-activator are employed. Otherwise, some chemicals that might affect estrogen systems could be missed.

γ-HCH is a halogenated organic insecticide that has been used worldwide. Because of its toxicity and persistence in soil, many countries have prohibited the use of γ-HCH, although, contaminated sites remain throughout the world. Moreover, some countries still use γ-HCH for economic reasons, and thus new sites are continuously being contaminated (*24*). *Sphingomonas paucimobilis* UT26 degrades γ-HCH under aerobic conditions, and a unique degradation pathway of γ-HCH in UT26 has been elucidated (*24*). In this pathway, γ-HCH is transformed to 2,5-dichlorohydroquinone (2,5-DCHQ) by two different constitutively expressed dehalogenases (LinA and LinB) and one dehydrogenase (LinC). In the downstream pathway, 2,5-DCHQ is reductively dehalogenated to produce chlorohydroquinone (CHQ) and hydroquinone (HQ), and then ring-cleaved by enzymes LinD and LinE (*24–26*).

In this study we demonstrate the utility of the interaction between human ERβ and various co-activators in identifying chemicals that interact with the estrogen receptor ligand–binding domain. We used the constructed yeast two-hybrid system to evaluate a number of natural and synthetic steroids, and γ-HCH metabolites by a microbial degradation system. The resulting two-hybrid system is sensitive, specific, and reproducible for detecting estrogenic chemicals.

MATERIALS AND METHODS

Chemicals—17β-Estradiol (E_2), estron, and testosterone were purchased from Sigma Chemical Co. Genestein and coumestrol were purchased from Fluka Chemie AG. Chlorohydroquinone (CHQ), biphenyl (BP), bisphenol A, diethylstilbestrol (DES), 2,2′,3,4′-tetrachlorobiphenyl (TeCB), 4-*tert*-octylphenol (4-*tert*-OP), and 4-nonylphenol (NP) were obtained from Tokyo Kasei Kogyo (Tokyo). 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (*p,p′*-DDT) and γ-hexachlorocyclohexane (γ-HCH) were obtained from Wako Pure Chemicals (Osaka). Hydroquinone (HQ), 2,3-dihydroxybiphenyl (2,3-DHBP), naphthalene, α-naphtol, β-naphtol, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,3,5-trichloro-phenoxyacetic acid (2,4,5-T), 4-heptylphenol (4-HP), and 4-octylphenol (4-OP) were purchased from Kanto Chemical (Tokyo). Phenanthrene, *p*-chlorobiphenyl (*p*-CBP), and indole were purchased from Nacalai Tesque (Kyoto). 2,5-Dichlorohydroquinone (2,5-DCHQ) was obtained from Aldrich Chemicals. 1,1-Bis(4-chlorophenyl)-2,2-dichloroethylene (*p,p′*-DDE), 1,1-dichloro-2,2-bis(*p*-chlirophenyl)ethane (*p,p′*-DDD), and 4-*n*-nonylphenol (4-*n*-NP) were purchased from Dr. Ehrenstorfer GmbH. All chemicals used were of reagent grade, and used without further purification.

Plasmids and Yeast Strains—A set of fusion plasmids pGBT9 (GAL4DBD–hERα LBD) and pGBT9 (GAL4DBD–hERβ LBD) were constructed by inserting hER LBDs (corresponding to amino acid residues 311 to 595 of hERα or residues 213 to 477 of hERβ) (*27*) into pGBT9 (Clontech) carrying a DNA region encoding the Gal4p DNA-binding domain (GAL4DBD). Another set of fusion plasmids, pGAD-SRC1, pGAD-TIF2, and pGAD-AIB1, were constructed by inserting the cDNAs of the nuclear receptor (NR)–binding domains (NRBD) of co-activators (corresponding to amino acid residues 231 to 1094 of SRC1, 670 to 1750 of TIF2, and 180 to 819 of AIB1) into pGAD10 (Clontech) carrying a DNA region encoding the Gal4p transactivation domain (GAL4TAD) (*28*). *Saccharomyces cerevisiae* YRG-2 (*MATa ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, GAL80-538, LYS::UAS$_{GAL1}$-TATA$_{GAL1}$-HIS3, URA3::UAS$_{GAL4\ 17mers(X3)}$-TATA$_{CYC1}$-lacZ*) (Stratagene) was transformed with two plasmids (one from each of the above sets) by electroporation (*29*), and transformants were selected on SD medium lacking leucine and tryptophan (*30*). DNA enzymes were used according to the manufacturers' protocols and DNA manipulation was done according to Sambrook *et al.* (*31*).

Growth of Yeast and β-Galactosidase Assay—β-Galactosidase activities were measured essentially by following the method used previously (*29*). Yeast transformants were grown overnight at 30°C with vigorous orbital shaking in 2 ml of selective SD medium. A portion (0.2 ml) of the overnight culture was diluted into 9.8 ml of fresh SD containing E_2 or EDs added in dimethylsulfoxide (DMSO) solution. The concentration of DMSO did not exceed 1% of the culture volume. Yeast cells were cultured for 11 h at 30°C. After incubation, the cells were collected by centrifugation at 3,500 rpm for 5 min. To prepare crude extracts, 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) and glass beads were added, and the cells were disrupted by vortexing three times for 1 min each time. Cell debris was removed

by centrifugation at 15,000 rpm for 10 min. For β-galactosidase activity assays, 0.1 ml of 4 mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside dissolved in Z-buffer) was added to 0.2 ml of cell extract and 0.3 ml of fresh Z-buffer, and the mixture was incubated at 30°C. When a yellow color developed, 0.25 ml of 1 M Na_2CO_3 was added to terminate the reaction, and the absorbance at 420 nm was measured. One unit of enzyme activity is defined as the activity that produces 1 nmol of *ortho*-nitrophenol per min. The protein concentration was measured with a Bio-Rad Protein Assay (Bio-Rad). Experiments in Figs. 3 to 5 included measurements of E_2 as a positive control, although the results are omitted from the figures. The data are representative of three independent experiments, and the respective experiments were performed in triplicate.

Cellular Distribution of γ-HCH and Its Metabolites—Yeast transformants were grown overnight at 30°C with vigorous shaking in 20 ml of SD medium. A portion of the overnight culture (4 ml) was diluted into 196 ml of fresh SD medium containing 10^{-4} M 2,5-DCHQ, CHQ, or HQ. Yeast cells were grown for 11 h at 30°C with vigorous shaking at 120 rpm, and collected by centrifugation (5,000 rpm, 10 min) at 4°C. Ten milliliters of Z buffer and glass beads were added to the cell pellet, and the cells were broken by vortexing five times for 1 min each time. After the removal of cell debris by centrifugation at 5,000 ×*g*, the resultant supernatant was centrifuged at 100,000 ×*g* for 1 h at 4°C. The supernatant was saved as the 'cytosol' fraction. The amounts of γ-HCH metabolites in the culture medium and cytosol were measured after extraction with the same volume of ethylacetate using a gas chromatograph–mass spectrometer (Shimadzu, model QP5000) as previously described (*25*).

RESULTS

Ligand-Dependent Interaction between hER LBD and Co-Activators in Yeast—The genes coding for GAL4DBD-hERLBD and GAL4TAD-NRBD fusion proteins were expressed from vector plasmids in *S. cerevisiae* strain YRG-2, which has a reporter construct, $UAS_{GAL4\ 17mers(X3)}$-$TATA_{CYC1}$-*lacZ*, on its chromosome. When the above two fusion proteins associate with each other through the ligand-dependent interaction between hER LBD and NRBD, the GAL4TAD recruits the basal transcriptional machinery to the *CYC1* promoter, resulting in the production of β-galactosidase. The β-galactosidase activity, therefore, reflects the strength of the interaction between hER LBD and NRBD, or the ability of estrogen-like chemicals that make hER LBD to associate with NRBDs of the co-activators, because the protein–protein interaction between hER and the co-activator is strictly dependent on the presence of estrogens (*17–19*).

Figure 1 illustrates the response of various combinations of hER LBDs and NRBDs of co-activators TIF2, SRC1, and

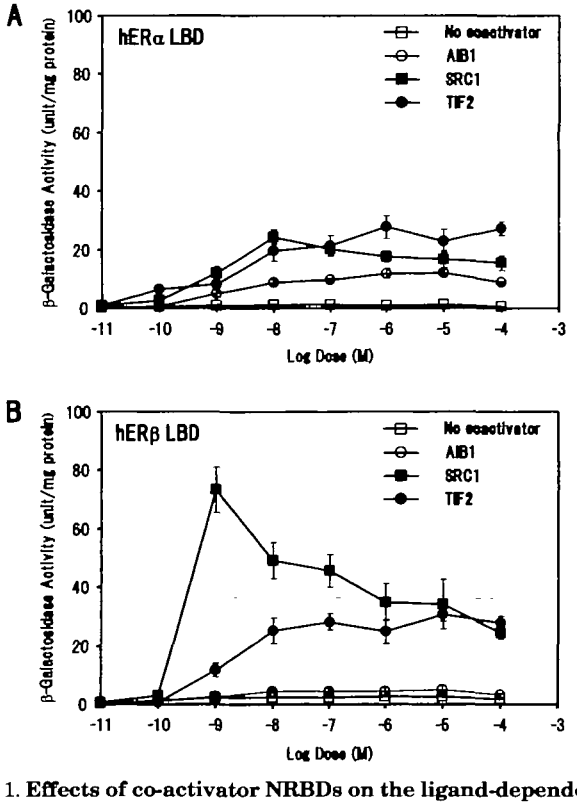

Fig. 1. **Effects of co-activator NRBDs on the ligand-dependent activation of hER LBDs in yeast.** *S. cerevisiae* strain YRG-2 was transformed with pGBT9 containing inserts of ER LBDs (A, hERα LBD; B, hERβ LBD) and with pGAD10 containing inserts of co-activator NRBDs. Transformants were incubated in the presence of the indicated concentrations of E_2, and β-galactosidase activities were measured as described in "MATERIALS AND METHODS."

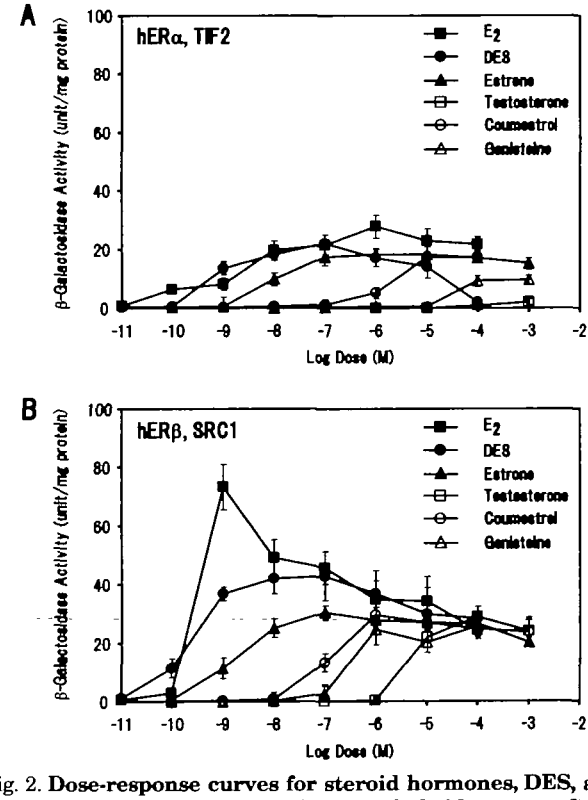

Fig. 2. **Dose-response curves for steroid hormones, DES, and phytoestrogens as determined by two-hybrid systems.** *S. cerevisiae* strain YRG-2 with plasmids pGBT9 (GAL4 DBD–hERα LBD) and pGAD-TIF2 (panel A), and with pGBT9 (GAL4 DBD–hERβ LBD) and pGAD-SRC1 (panel B) were incubated in the presence of the indicated concentrations of estrogenic compounds. β-Galactosidase activities were measured as described in "MATERIALS AND METHODS."

AIB1 to 10^{-11} to 10^{-4} M E_2 (Fig. 1, A and B). When hERα LBD was the ligand-binding and activating component, the use of TIF NRBD showed the most sensitive response with some β-galactosidase activity detected at 10^{-10} M E_2 (Fig. 1A). At the same E_2 concentration, SRC1 showed only very weak activity and AIB1 showed none. At higher E_2 concentrations, both TIF2 and SRC1 were similarly effective and gave higher reporter activities. These two co-activator NRBDs were also effective in the system in which rat ERα LBD was employed (*17*). When hERβ LBD was used, the combination with SRC1 NRBD resulted in low β-galactosidase activity at 10^{-10} M E_2, but strikingly high activity at 10^{-9} M E_2 (Fig. 1B). The decrease in the reporter activity at higher E_2 concentration was not due to growth inhibition by the added ligand (data not shown). TIF2 NRBD was less effective and AIB1 NRBD did not function at all under the conditions tested. Without the expression of GAL4TAD-NRBD, the expression of GAL4DBD-hERβLBD or GAL4 DBD-hERαLBD alone did not produce β-galactosidase activity at a detectable level.

The ligand specificity of the two effective combinations of hER LBD and co-activator NRBD were analyzed by examining the effect of a variety of natural and synthetic steroids and phytoestrogens (Fig. 2, A and B). For the combination of hERα LBD and TIF2 NRBD, E_2 is most effective among the chemicals tested at 10^{-10} M (Fig. 2A). The order of effectiveness was E_2>DES>estrone>coumestrol>genisteine>testosterone. In the case of hERβ LBD and SRC1 NRBD, the order of effectiveness of the above chemicals was similar, although DES produced higher β-galactosidase activity than E_2 at 10^{-10} M (Fig. 2B). This latter combination gave higher β-galactosidase activity for the chemicals tested and seemed more sensitive than the former combination. The lowest concentration that gave detectable β-galactosidase activity was 10^{-10} M for DES, 10^{-9} M for estrone, 10^{-7} M for coumestrol, 10^{-6} M for genisteine, and 10^{-5} M for testosterone. These concentration levels are one to two orders lower than those for the combination of hERα LBD and TIF2. The latter two-hybrid system that employs hERβ LBD and SRC1 NRBD is more sensitive to estrogenic chemicals than the former system and seems to be more suitable for screening chemicals for endocrine-disrupting activity, although it might screen with less specificity because of its relatively high sensitivity to testosterone. We further analyzed how this system responds to many other chemicals.

Applications of the Yeast Detection System to Endocrine Disrupters—We analyzed the estrogen-like activity of pesticides and industrial chemicals using the above system with hERβ LBD and SRC1 NRBD (Figs. 3 and 4). Of the pesticides tested, *p,p'*-DDT, 2,4-D, and 2,4,5-T activated the production of β-galactosidase at concentrations as low as 10^{-6}, 10^{-5}, and 10^{-7} M, respectively (Fig. 3A). *p,p'*-DDD and *p,p'*-DDE, which are *p,p'*-DDT metabolites, had little detectable effect on this system. Alkylphenols yielded high β-galactosidase activities at 10^{-6} M or more (Fig. 3B). The 4-NP used consisted of *p*-NP (75%) and *m*- and *o*-NP (25%), and gave β-galactosidase activity similar to that yielded by 4-*n*-NP at

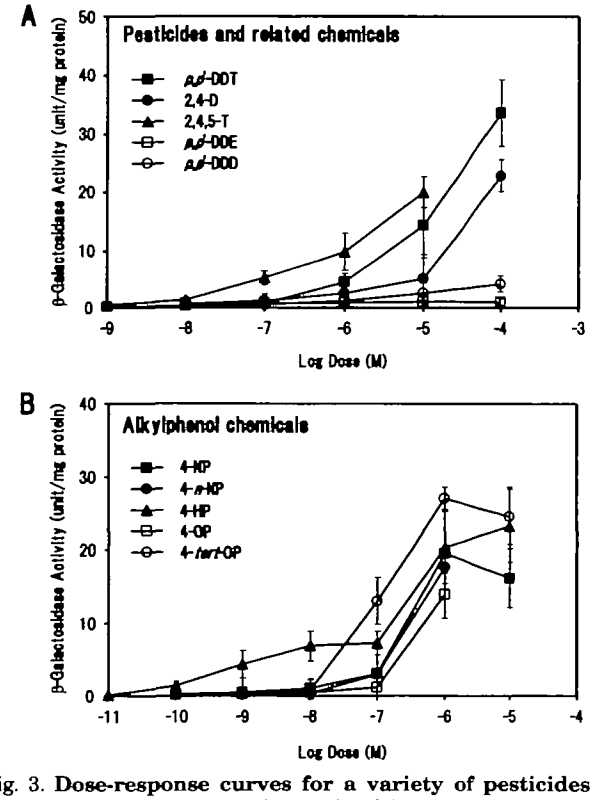

Fig. 3. **Dose-response curves for a variety of pesticides (A) and alkylphenols (B) as determined by a yeast two-hybrid system with hERβ LBD and SRC1 NRBD.** The experimental procedures were the same as in Fig. 2. Chemicals were added to the yeast culture as indicated.

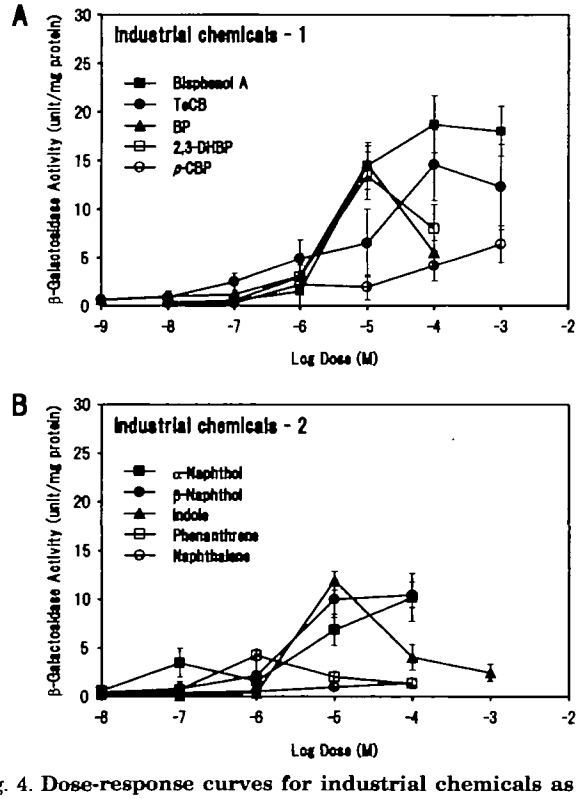

Fig. 4. **Dose-response curves for industrial chemicals as determined by a yeast two-hybrid system with hERβ LBD and SRC1 NRBD.** The experimental procedures were the same as in Fig. 2. Estrogenic chemicals were added to the yeast culture as indicated (panels A and B).

more than 10^{-7} M. 4-*tert*-OP showed high β-galactosidase activity at concentrations above 10^{-7} M, whereas 4-OP was only effective at concentrations greater than 10^{-6} M. 4-HP activated β-galactosidase production at the very low concentration of 10^{-10} M. The estrogenic activities of industrial chemicals were also analyzed (Fig. 4, A and B). Bisphenol A, BP, 2,3-DHBP, TeCB, α-naphtol, β-naphtol, and indole were effective at concentrations above 10^{-6} M. Among these chemicals, bisphenol A and TeCB showed maximum β-galactosidase activity at 10^{-4} M, and BP, 2,3-DHBP and indole showed maximum induction at 10^{-5} M. The latter three chemicals showed much reduced activity at higher concentrations, presumably because of their inhibitory effect on yeast growth. α-Naphtol and β-naphtol showed their highest induction at 10^{-4} M. *p*-CBP gave some β-galactosidase activity at 10^{-3} M, but was judged negative in this system.

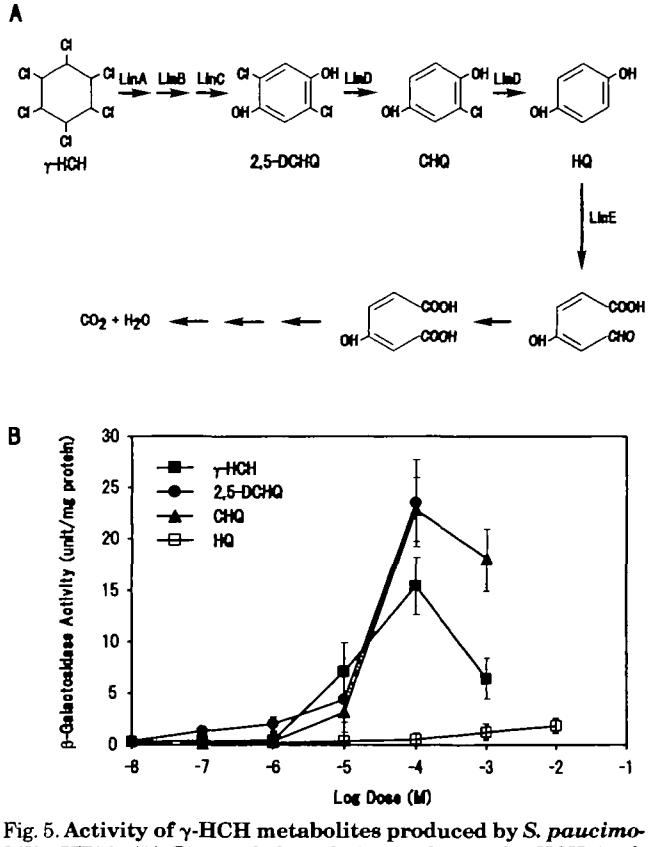

Fig. 5. **Activity of γ-HCH metabolites produced by *S. paucimobilis* UT26.** (A) Proposed degradation pathway of γ-HCH in *S. paucimobilis* UT26 (*20–22*). (B) Dose-response curves for γ-HCH metabolites determined by the two-hybrid system with hERβ LBD and SRC1 NRBD. The experimental procedures were the same as in Fig. 2. γ-HCH and its metabolites were added to the yeast culture as indicated.

Phenanthrene gave some activation at 10^{-6} M, but less at concentrations over 10^{-6} M. Naphthalene induced little β-galactosidase activity in this system. These results indicate that the constructed yeast detection system is applicable to a wide variety of chemicals and is very useful for the primary screening of potential effectors of estrogen receptor functions.

Application of the Yeast Detection System to Microbial Metabolites of γ-HCH—The microbial degradation of EDs is a potentially useful method for eliminating undesirable chemicals from the environment. *S. paucimobilis* UT26 degrades γ-HCH (γ-BHC), a highly chlorinated pesticides that causes serious environmental problems under aerobic conditions (*24*). γ-HCH is converted to hydroquinone (HQ) *via* 2,5-dichlorohydroquinone (2,5-DCHQ) and chlorohydroquinone (CHQ) by the successive action of the *linA*, *linB*, *linC*, and *linD* gene products (*24–26*) (Fig. 5A). To determine how far the degradation must proceed in order to inactivate the estrogenic activity of γ-HCH, we measured the estrogenic activities of its metabolites (Fig. 5B). 2,5-DCHQ induced little β-galactosidase activity even at 10^{-7} M. Both 2,5-DCHQ and CHQ were more effective at 10^{-4} M than the original chemical, but HQ, a dechlorinated metabolite, showed little induction activity even at higher concentrations. Since it is possible that the observed difference might be due to differences in their permeability across the plasma membrane, we examined their cytosolic concentrations as described in "MATERIALS AND METHODS." The amount of HQ in the cytosolic fraction was less the amounts of 2,5-DCHQ and CHQ, but still reached half their values (Table I). On the contrary, the β-galactosidase activity induced by HQ was only about 1/40 of that induced by 2,5-DCHQ and CHQ, suggesting that the low β-galactosidase activity induced by HQ is not likely to be due to its low membrane permeability.

DISCUSSION

In this paper, we present a useful yeast two-hybrid assay system for the detection of potentially estrogenic chemicals. The system is based on the estrogen-dependent interaction between the hER ligand–binding domain and the co-activator nuclear receptor–binding domains. We tested six combinations of ligand-binding domains of two hER species and three co-activators for their response to E_2 and found that the combination of hERβ LBD and the co-activator SRC1 NRBD was most effective for inducing reporter enzyme activity. This combination was also effective in detecting several representative estrogenic chemicals. We decided to examine this ERβ LBD–co-activator NRBD combination further for its capability to screen xenoestrogens. The reason that hERβ LBD gave a higher reporter activity than hERα LBD is not clear. This was observed even in the presence of

TABLE I. **Intracellular distribution of γ-HCH metabolites.**

Samples	*A* (μmol)	Cytosol (μmol)	Total protein in cell extract mg	β-Gal. activity (unit/mg protein (relative))
2,5-DCHQ	16.7 ± 4.10	0.41 ± 0.13	2.7 ± 0.3	21.70 ± 5.86 (1)
CHQ	19.6 ± 2.38	0.53 ± 0.09	2.5 ± 0.1	20.23 ± 6.35 (0.93)
HQ	18.4 ± 3.61	0.28 ± 0.04	2.8 ± 0.3	0.53 ± 0.17 (0.024)

A: amount of given metabolites in the supernatant after cultivation. Cytosol: metabolites in the 100,000 ×*g* supernatant. Total protein in cell extract: amount of protein in the cell extract from 10 ml of culture. β-Galactosidase activity was measured in cell extracts after 11 h incubation. Means ± SD of 3 independent experiments are shown.

E_2, which is equally effective in binding to the two ER subtypes and generally gives higher transcriptional activation to hERα than to hERβ in cultured cell systems (*20*). It is probably that the employment of only LBDs from ERs induced them to get rid of accessory domains that suppress or contribute to their interaction with the nuclear receptor boxes of co-activator NRBDs (*32*).

To compare the sensitivity of our system with others that have been reported, chemicals that gave more than 10% (7.33 units/mg protein) of the β-galactosidase activity produced by 10^{-9} M E_2 (10% relative effective concentration, REC10) were judged tentatively to be positive (*33*). Estrone, testosterone, coumestrol, genistein, DES (Fig. 2), *p,p'*-DDT, 2,4-D, 2,4,5-T (Fig. 3A), 4-NP, 4-*n*-NP, 4-HP, 4-OP, 4-*tert*-OP (Fig. 3B), bisphenol A, TeCB, BP, 2,3-DHBP (Fig. 4A), α-naphthol, β-naphthol, indole (Fig. 4B), γ-HCH, 2,5-DCHQ, and CHQ (Fig. 5B) were found to test positive in our detection system. These findings are in good agreement with those in previous reports, which demonstrated their estrogenic activity by *in vivo* and *in vitro* assays, with the exception of some chemicals tested first here (*34–37*). Among the chemicals evaluated as negative in our system, DDT metabolites *p,p'*-DDE and *p,p'*-DDD induced a very small amount of β-galactosidase activity, and these chemicals reportedly antagonized the binding of androgen to the androgen receptor (*38*). Testosterone, 2,4-D, 2,4,5-T, *p,p'*-DDT, and BP were not classified as positive by Nishihara *et al.* (*33*), who used a yeast two-hybrid system similar to ours. This discrepancy might be due to the difference in the incubation periods of the recombinant yeast cells in the presence of estrogenic chemicals, or due to the difference in the two-hybrid constructs. Although the source animals from which the ER cDNAs are derived differ [we used human ERs instead of rat ERs (*33*)], the difference in ER subtypes seems to be more influential, "because the selectivity and affinity of their ligand-dependent association with co-activators are different, in spite of their sequence similarity (*2, 3*)." In the presence of xenoestrogens, ERβ tends to bind to co-activators SRC-1a and TIF2 at their much lower concentrations and has a greater ability to potentiate reporter gene activity than ERα in transiently transfected HeLa cells expressing SRC-1e and TIF2 (*21*). ERβ showed a 30-fold greater binding affinity to genistein compared with ERα (*23*). Isoflavone phytoestrogens repress the expression of a TNF-α promoter region through the action of ERβ but not ERα, although E_2 represses this promoter more potently by binding to ERα (*23*), ERβ has different binding affinities than ERα for the nuclear receptor (NR) boxes of co-activators, which varied depending on the estrogens employed (*22*). Ligand-bound ERβ but not ERα showed an especially high affinity for NR box IX of SRC-1a, a longer splice variant of SRC-1. Thus the data suggest that the interactions of ERβ with estrogenic chemicals are not the same as those of ERα. ERβ tends to bind to co-activators SRC-1a and TIF2 at much lower xenoestrogen concentrations (*21*).

The estrogenic activities of alkylphenols reportedly depend on their structural features in both the position (from high to low, *para>meta>ortho*) and branching (*tertiary>secondary = normal*) of the alkyl groups joined to a phenyl ring (*39*). In agreement with the results reported by Edwin and John (*39*), Fig. 3B also shows that 4-*tert*-OP has higher estrogenic activity than 4-*n*-OP, but that a number of 4-NP hydrocarbon isomers do not give higher activity than 4-NP, probably because insolubility. In our system, α-naphthol and β-naphthol gave detectable activities. Indole, a natural metabolite of tryptophan that has not been classified as positive, was positive in our system. Polycyclic aromatic hydrocarbons such as phenanthrene and naphthalene were judged to be negative in this system, and showed only weak induction of β-galactosidase. The observed differences in the response to these aromatic chemicals probably reflect the structural differences between the two types of hER LBD (*20*), which await more detailed analyses.

We also evaluated the estrogenic activities of microbial degradation products of γ-HCH, which itself has estrogenic activity. 2,5-DCHQ and CHQ had the same or higher estrogenic activities than the original chemical, but HQ, a later stage metabolite, had no activity (Fig. 5B). These results imply that bacterial degradation systems for toxic chemicals must be carefully assessed to ensure that they do not produce secondary toxic chemicals.

In conclusion, our two-hybrid detection system for potentially estrogenic chemicals, which employs the ligand-binding domain of hERβ and the NR-binding domain of co-activator SRC1, is efficient and sensitive enough to evaluate a broad range of chemicals and has different binding specificities for xenobiotics than the previous yeast system with rat ERα. We expect that our system will provide a useful tool that complements the current yeast detection systems employing ERα.

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