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Note

Yeast Two-Hybrid Detection Systems That Are Highly Sensitive to a Certain Kind of Endocrine Disruptors

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We tested the effects of several combinations of bait and fish components of the yeast two-hybrid detection system for estrogenic activity. A combination of the full-length human estrogen receptor α with the nuclear receptor-binding domain of co-activator steroid receptor co-activator-1 (SRC-1) or transcriptional intermediate factor-2 (TIF-2) was most effective for estrogen-dependent induction of the chromosome-integrated UAS_{GAL}-*CYC1*^{p}-*lacZ* reporter construct among the two-hybrid systems so far tested.

Key words: endocrine disruptor; estrogen; yeast two-hybrid system; human estrogen receptor-α (hERα); steroid receptor co-activator-1 (SRC-1)

Previously we constructed a yeast two-hybrid endocrine disrupter (ED) detection system that employed the human estrogen receptor β (hERβ) ligand binding domain (LBD) as a bait and one of the p160 family co-activators as a fish.[1)] To improve the system, we tested various combinations of human ERα (hERα) derivatives and co-activators for efficient ligand-dependent expression of the reporter gene in *Saccharomyces cerevisiae*.

The fusion gene coding for either the Gal4 DNA-binding domain (Gal4 DBD)-hERα or Gal4 DBD-hERα LBD (carries the residue 311 to 595 of hERα) fusion protein was expressed from the vector plasmid pGAL4DBD-hERα or pGAL4DBD-hERαLBD,[1,2)] respectively, in *S. cerevisiae* strain YRG-2 that had a reporter construct, $UAS_{GAL\,17mers(X3)}$-$TATA_{CYC1}$-*lacZ*, on its chromosome. For construction of pGAL4DBD-hERα, a full-length hERα cDNA was amplified by PCR using a set of primers with either the *Eco*RI or *Sal*I site at the 5′ termini: hERα Forward/*Eco*RI (5′-GAATTCA-TGACCATGACCCTCCACACC-3′); hERα Reverse/*Sal*I (5′-GTCGACGCCAGGGAGCTCTCAGAC-3′). The resulting PCR products were once inserted between the *Eco*RI and *Sal*I sites of pBluescript II KS+ (Stratagene, La Jolla, CA), and their nucleotide sequences were confirmed with DNA sequencing. An hERα cDNA fragment of correct sequence was excised and inserted between the *Eco*RI and *Sal*I sites of pGBT9 (Clontech, Mountain View, CA) to obtain pGAL4DBD-hERα. The YRG-2 strain that expressed Gal4 DBD-hERα showed β-galactosidase reporter activity in response to E2 of over 10^{-10} M, and the highest activity was approximately 14 units/mg protein at 10^{-5} M E2. On the contrary, the same strain that expressed Gal4 DBD-hERα LBD did not show the reporter activity, suggesting that hERα LBD alone is not sufficient for trans-activation of yeast transcription machinery in response to E2, which is consistent with previous reports that the N-terminus AF-1, one of two transcription activation domains in ERα, is essential for transactivation in the yeast one-hybrid assay,[3)] and that AF-1 itself, in conjunction with the hERα DNA binding domain or the Gal4 DNA binding domain, was active in the expression of the reporter gene.[4,5)] REC10 values against various chemicals by this one-hybrid system are listed in Table 1.

Steroid receptor co-activator-1 (SRC-1) is a nuclear receptor (NR) co-activator sharing sequence homology with SRC-2/TIF-2 and SRC-3/p/CIP/AIB-1/TRAM-1/RAC-3/ACTR as a member of the p160 co-activator family. The p160 proteins contain a short conserved NR interaction motif (NR Box) with the core amino acid sequence LXXLL in the NR binding domain (NRBD).[6)] The mammalian steroid hormone receptors interact with NRBD in a steroid-dependent manner in *S. cerevi-*

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Abbreviations: DBD, DNA binding domain; TAD, transcriptional activation domain; hER, human estrogen receptor; LBD, ligand-binding domain; NR, nuclear receptor; ED, endocrine disruptor; AF-1 and AF-2, transcription activating factor-1 and factor-2; SRC-1, steroid receptor co-activator-1; TIF-2, transcriptional intermediate factor-2; NRBD, nuclear receptor binding domain; E2, 17β-estradiol; BPA, bisphenol A; DES, diethylstilbestrol; 4-*tert*-OP, 4-*tert*-octylphenol; 4-NP, 4-nonylphenol; γ-HCH, γ-hexachlorocyclohexane; *p*,*p*′-DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,3,5-trichlorophenoxyacetic acid

Table 1. Evaluation of Estrogenic Activities of the Various Compounds Using Yeast One-Hybrid and Two-Hybrid Systems

Compound	REC10[a]				
	One-hybrid system	Two-hybrid system			
	hERα	hERα LBD + SRC1	hERα LBD + TIF2	hERα + SRC1	hERα + TIF2
17β-Estradiol	4.88×10^{-10}	1.75×10^{-10}	2.13×10^{-10}	2.49×10^{-10}	1.22×10^{-10}
Estrone	2.78×10^{-8}	1.60×10^{-8}	1.58×10^{-8}	1.53×10^{-9}	7.90×10^{-10}
Testosterone	3.15×10^{-3}	N	N	1.54×10^{-5}	1.42×10^{-4}
Coumestrol	7.84×10^{-6}	4.56×10^{-6}	2.54×10^{-6}	5.42×10^{-8}	8.92×10^{-7}
Genistein	1.71×10^{-4}	1.33×10^{-4}	1.81×10^{-4}	2.95×10^{-7}	2.35×10^{-6}
DES	1.52×10^{-9}	3.24×10^{-9}	1.45×10^{-9}	3.59×10^{-12}	1.91×10^{-12}
γ-HCH	N	N	nt	1.72×10^{-5}	1.12×10^{-4}
p,p'-DDT	1.25×10^{-4}	nt	1.03×10^{-4}	2.82×10^{-7}	8.35×10^{-7}
2,4-D	N	2.09×10^{-4}	nt	5.42×10^{-6}	6.33×10^{-5}
2,4,5-T	N	N	nt	1.01×10^{-6}	4.35×10^{-6}
BPA	2.05×10^{-4}	1.47×10^{-4}	2.12×10^{-4}	3.93×10^{-6}	1.42×10^{-5}
4-NP	1.07×10^{-5}	5.60×10^{-6}	4.08×10^{-6}	1.37×10^{-7}	1.46×10^{-6}
4-*tert*-OP	5.18×10^{-6}	1.80×10^{-6}	2.41×10^{-6}	1.82×10^{-7}	1.14×10^{-6}

[a]REC10 (10% relative effective concentration against E2)[16] is the concentration of a tested chemical showing 10% of the highest estrogenic activity of E2 in a given system. N, negative; nt, not tested. β-Galactosidase activities that gave REC10 were 1.4, 2.14, 2.1, 2.8, and 4.1 units/mg protein for hERα one-hybrid system, and for two-hybrid systems of hERα LBD + SRC-1 NRBD, hERα LBD + TIF-2 NRBD, hERα + SRC-1 NRBD, and hERα + TIF-2 NRBD, respectively.

siae[7–9] and yeast two-hybrid systems were constructed to examine the interaction and their responsiveness to estrogenic compounds.[10,11] cDNAs corresponding to SRC-1 NRBD (from residue 231 to 1,094) and TIF-2 NRBD (form residue 670 to 1,750) were almplified and cloned into the *Eco*RI site of pGAD10 (Clontech) to obtain plasmids pGAL4TAD-SRC1NRBD and pGAL4-TAD-TIF2NRBD.[1,12] The two-hybrid system with pGAL4DBD-ERαLBD and either one of these two plasmids in the strain YRG-2 responded to over 10^{-10} M E2 (data not shown). Maximum activities were 22 units/mg protein at 10^{-8} M E2 for SRC-1 NRBD and 30 units/mg protein at 10^{-6} M E2 for TIF-2 NRBD. It should be noted that the SRC-1 and TIF-2 NRBDs employed contained an activation domain, AD1, that contributed much to transcription activation in yeast.[13,14] Nishikawa *et al.* reported similar two-hybrid systems, except for the use of rat ERα LBD as a bait protein.[10] Human and rat ERα LBD sequences are different in 16 amino acid residues in their very C-terminus,[15] and might be different in their interaction with co-activators. REC10 values to various chemicals by our hERα LBD-SRC-1 NRBD and hERα LBD-TIF-2 NRBD two-hybrid systems are listed in Table 1.

When Gal4 DBD with entire hERα was used in combination with either Gal4 TAD-SRC-1 NRBD or Gal4 TAD-TIF-2 NRBD, the sensitivity of the system to E2 improved markedly (Fig. 1, Table 1, and data not shown), indicating that the entire hERα is more effective in E2-dependent transcription activation than hERα LBD. The highest β-galactosidase activity by the systems that employed SRC-1 NRBD and TIF-2 NRBD was 26 units/mg at 10^{-8} M and 41 units/mg protein at 10^{-9} M respectively. These two-hybrid systems were analyzed for their responsiveness to a variety of natural and synthetic steroids and phytoestrogens. We present the net data for the case of SRC-1 NRBD (Fig. 1), because they similarly responded to the chemicals, and the system with SRC-1 NRBD tended to give higher sensitivity to synthetic steroids and xenobiotics (see Table 1). E2 gave β-galactosidase reporter activity at more than 10^{-10} M, whereas DES was more effective, and its least induction concentration was the order of 10^{-12} M. Estrone, coumestrol, and genistein were also effective, and their least induction concentrations reached 10^{-10} M, 10^{-9} M, and 10^{-8} M respectively. Testosterone induced β-galactosidase activity at 10^{-6} M or more. When pesticides, industrial chemicals and alkylphenols were tested (Fig. 1, closed symbols), 4-NP induced reporter activity at more than 10^{-8} M. 4-*tert*-OP, p,p'-DDT, BPA, and 2,4,5-T also induced reporter activity at more than 10^{-7} M. 2,4-D and γ-HCH were less effective and had similar dosage effects similar to testosterone at over 10^{-6} M. REC10 values by this system as well as by the system that employed TIF-2 NRBD are listed in Table 1. These systems showed higher sensitivity to the above synthetic chemicals than the former two-hybrid systems.[1,16]

The use of the entire hERα in combination with the co-activator NRBD resulted in higher response to estrogens than did the use of Gal4 DBD-hERα LBD or Gal4 DBD-hERβ (reference 1 and our unpublished results). This is in part brought about by the synergistic action of AF-1 and AF-2 of hERα, which cooperatively mediates recruitment of co-activator SRC-1.[17] A ligand-dependent direct interaction between the B domain in AF-1 and the C-terminal domains of ERα has also been reported and is supposed to stabilize this cooperative interaction between hERα and SRC-1.[17] E2 is known to be equally effective in binding to the two ER subtypes, hERα and hERβ, but generally induces higher transcriptional activation by hERα than by hERβ in cultured

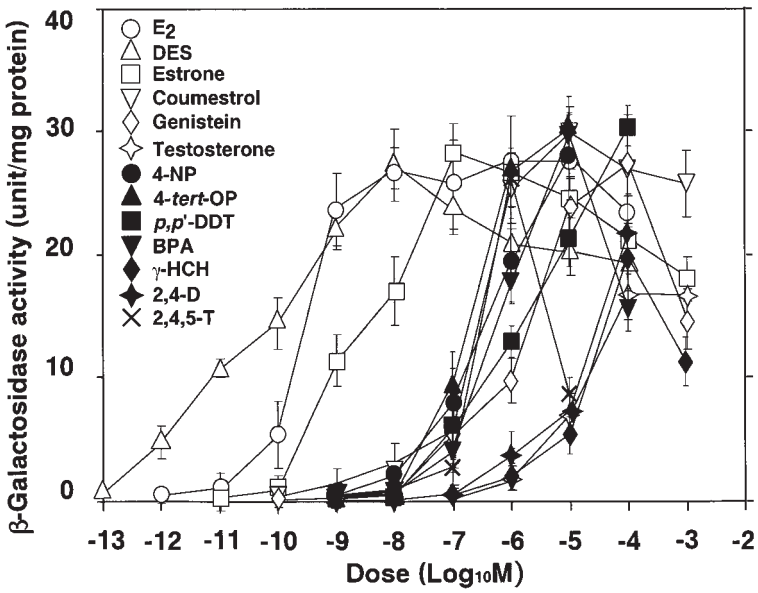

Fig. 1. Dose-Response Curves for Various Chemicals as Determined by the Yeast Two-Hybrid System with pGAL4DBD-hERα and pGAL4TAD-SRC1NRBD.

S. cerevisiae strain YRG-2 with plasmid pGAL4 DBD-hERα was incubated in SD medium supplemented with the indicated concentrations of chemicals.[1] β-Galactosidase activities were measured as described previously.[1] The activity that produced one nmol *ortho*-nitrophenol from *ortho*-nitrophenyl-β-D-galactopyranoside per min was defined as one unit. Symbols are designated in the figure. The standard error of each point is indicated by a vertical bar. Abbreviations of chemicals are indicated in the footnote to the first page.

cells and yeast two-hybrid systems.[1,18] It has also been reported that the property of NR Box in SRC-1 NRBD influences the ligand-dependent binding affinity of NR subtypes to SRC-1.[19]

Recently, Ellison *et al.* reported a yeast detection system for endocrine disruptors, in which hERα and SRC-1a, a subtype of SRC-1, were expressed in yeast that carried a reporter gene *lacZ*, the promoter of which had three estrogen-responsive elements.[20] They found estrogen and SRC-1 dependent reporter gene expression, although the level of the reporter activity was two to three orders lower than those by our system. This is probably because of the inefficiency of trans-activation by SRC-1 in yeast. Our two-hybrid systems that utilize ligand-dependent interaction between the entire hERα and NRBD of SRC-1 or TIF-2 on the basis of Gal4 DBD and TAD functions might be useful for highly sensitive detection of EDs.

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