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A new version of the two-hybrid assay for detection of protein–protein interactions

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The yeast two-hybrid system originally developed by Fields and Song is a powerful *in vivo* assay to detect protein–protein interactions (1). It can be used to test interactions between known proteins, or to identify proteins that interact with a protein of interest (2). In this system, one protein is fused to a DNA binding domain (DBD), while the other is fused to a transcriptional activating domain (AD). If the two proteins interact in a yeast cell, a functional transcriptional activator is reconstituted, the activity of which is monitored by the expression of a reporter gene containing cognate sites for the DBD. A number of different DBDs and ADs have been successfully used in this system, including the DBDs of GAL4 and LexA, and the acidic ADs (AADs) of GAL4 and VP16 (1–6). The reporter gene is commonly *Escherichia coli lacZ*, whose expression is detected by a color assay (1–6), or a yeast selectable gene, such as *HIS3* (3–5) or *LEU2* (6).

Here, we describe a new version of the two-hybrid system in which the target protein is expressed as a fusion with the DBD of the human oestrogen receptor (ER) in a yeast strain containing an integrated *URA3* reporter gene driven by one or three ER response elements (*EREs*). This sytem utilizes an uracil growth selection, and is particularly useful for large screening of AAD-tagged cDNA libraries. In contrast to the existing *HIS3*- and *LEU2*-based systems, it can be assayed quantitatively and makes possible a negative selection scheme by using the 5-FOA selection procedure (7). Two DBD and AAD fusion vectors (pBL1 and pASV3, respectively) have been constructed (Figs 1 and 2). Both plasmids are derivatives of YEp90 (8) and contain the promoter region and the transcription terminator of the yeast *PGK* gene. pBL1 carries the *HIS3* marker, and directs the synthesis of DBD fusion proteins that contain, at their amino termini, an epitope tag corresponding to the F region of ER [ER(F); aa 553–595; (9)] and the DBD of ER [ER(C); aa 176–282; (10)] which also includes a dimerization function and a nuclear localization signal (NLS; 11). A derivative without a NLS, pBL2, which is identical to pBL1 except that it contains a shorter ER DBD (aa 176–250) has also been constructed. pASV3 carries the *LEU2* marker and sequences encoding an initiation codon, the NLS of the yeast ribosomal protein L29 [aa 22–32; (12)], and the VP16 AAD (aa 411–490). AAD fusion vectors, pASV1 and pASV2, which differ from pASV3 only in the reading frame, are also available (Fig. 2). These pASV plasmids contain a direct repetition of two *lox* sites, and the corresponding λ phage vectors, λASV1-3, have been created to facilitate construction of large VP16-tagged cDNA libraries that can then be converted to a plasmid library by using the *cre-lox* site-specific recombination system (13). The two previously described reporter strains PL1(α) and PL3(α), which contain an integrated *URA3* reporter gene controlled by one or three EREs (8), respectively, are used as host strains for the two-hybrid assay. These strains are auxotrophic for histidine and leucine, allowing selection for pBL1 and pASV3 plasmids, and also auxotrophic for uracil and tryptophan. An interaction between two fusion proteins is detected by plating cotransformants on synthetic medium lacking uracil. In many cases, however, a residual *URA3* expression due to the ability of the DBD fusion protein to transactivate on its own is sufficient to allow growth without uracil. This can be overcome by incorporating into the plates 6-azauracil (6-AU), an inhibitor of the *URA3* gene product, which restores uracil auxotrophy when added to the medium at concentrations ranging from 3 to 30 μg/ml. A quantitative measurement of the *URA3* gene activation can be obtained by determining the activity of the reporter gene product, orotidine-5′-monophosphate decarboxylase (OMPdecase). The activity of this enzyme can be easily measured in cell-free extracts and gives a sensitive and reliable indication of the level of *URA3* expression (8,14). Results of two-hybrid assays performed with a pair of proteins previously shown to interact are presented in Table 1: the cDNA encoding the DEF region of the human RARα receptor (aa 154–462) was fused to the ER DBD in pBL1 [resulting in DBD-RARα(DEF)]. The entire coding sequence of the mouse RXRα receptor (aa 1–467), and the C-terminal E region of RXRα (aa 263–467) were fused to the VP16 AAD in pASV3 [resulting in AAD-RXRα and AAD-RXRα(E), respectively]. The DBD and AAD fusion vectors were introduced either alone or in combination into PL3(α), and the transformants were assayed for growth on uracil-deficient medium and for OMPdecase activity (Table 1). An interaction between DBD-RARα(DEF) and either AAD-RXRα or AAD-RXRα(E) was detected, in agreement with the previous finding that RARα and RXRα can form heterodimers *in vitro* and in animal cells (15,16). The previously identified dimerization interfaces (15) which are present in the E region of RARα and RXRα, are most probably responsible for these heterodimeric interactions.

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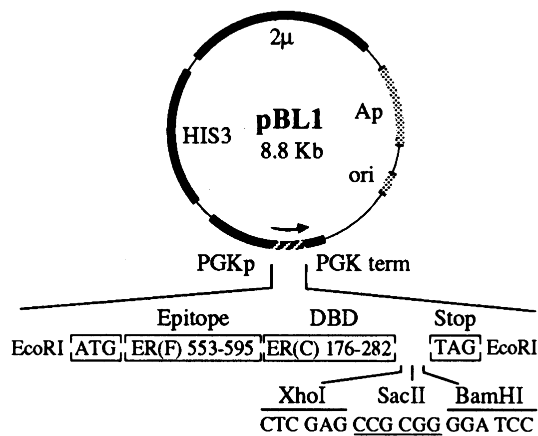

Figure 1. The DNA binding domain fusion vector pBL1. Expression of the DBD fusions in yeast are driven from the *PGK* promoter. Sequences for replication and selection in yeast and in *E.coli* are indicated. The sequence of the polylinker region is shown as in frame triplets.

Table 1. RARα and RXRα interact in yeast

DBD hybrid[a]	AAD hybrid[a]	Ura[b]	OMPdecase activity[c]
DBD	AAD	–	0.5
DBD-RARα(DEF)	AAD	–	0.3
DBD	AAD-RXRα	–	0.5
DBD-RARα(DEF)	AAD-RXRα	+	25.6
DBD	AAD-RXRα(E)	–	0.5
DBD-RARα(DEF)	AAD-RXRα(E)	+	17.9

[a]DNA-binding and activation domain hybrids were co-expressed in the PL3 reporter strain [*MATα ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::(ERE)$_3$-URA3*].
[b]Ability of the transformants to grow on medium lacking uracil plus 10 μg/ml 6-AU.
[c]Transformants were grown exponentially on selective medium containing uracil. Cell-free extracts were prepared and assayed for OMPdecase activity. Enzyme activities are expressed in nmol of substrate/min/mg protein. The reported values are means of duplicates made on three independent transformants. The standard errors are typically 10% of the mean.

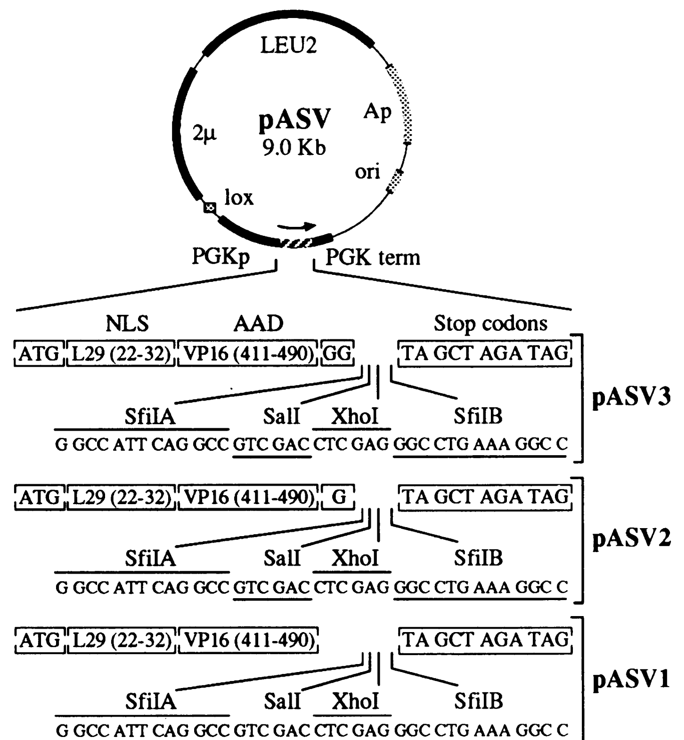

Figure 2. The acidic activating domain fusion vectors pASV1-3. The pASV1-3 vectors direct the synthesis of AAD fusion proteins under the control of the *PGK* promoter. The initiation codon, the L29 NLS, the VP16 AAD, the two extra nucleotides (GG; in pASV3), the sequence of the polylinker region (shown as in frame triplets), and the stop codons (in the three reading frames) are depicted. pASV1 and pASV2 are identical to pASV3 except that they contain no extra nucleotide (pASV1) or only one extra G (pASV2) between the last nucleotide of the VP16 AAD and the first G of the SfiIA site. In addition to the sequences for replication and selection in yeast and in *E.coli*, these pASV vectors contain a direct repetition of two *lox* sites flanking a *Not*I restriction site (see text).

Using this new version of the two-hybrid system, we have recently isolated mouse cDNAs encoding proteins that interact with a number of nuclear receptors (E.vom B., R.L., and P.C., manuscript in preparation). Since interacting clones are identified by a growth selection, this system is highly sensitive. Both weak and strong interactions can be detected by modulating the stringency of the *URA3* selection with different concentrations of 6-AU. Moreover, the OMPdecase assay gives a sensitive measurement of these interactions. The *URA3*-based system has the further advantage to use a target promoter driving the *URA3* reporter which is different from those commonly used in the other two-hybrid systems, such as the *GAL1* promoter. Therefore, combining the *URA3*/ER-based system with another system, such as the *HIS3*/LexA-based system (3), via a mating assay (4), provides a means to improve the elimination of false positives (17). The general strategy is the following. Once positives have been identified in the *URA3*/ER-based system ($His^+Leu^+Ura^+Trp^-$), an extended growth of each clone in the absence of *HIS3* selection is performed to allow segregation of the *HIS3* DBD fusion vector. $His^-Leu^+Ura^-Trp^-$ isolates are then mated to yeast strain L40 [*MATa trp1-901 leu2-3,112 his3-Δ200 ade2 LYS2::(LexAop)$_4$-HIS3 URA3::(LexAop)$_8$-lacZ*; 3] containing *TRP1* BTM116 derivative plasmids that express the target sequence or other (un)related proteins as LexA fusions ($Trp^+Leu^-His^-Ade^-Ura^+$). Diploids from the mating ($Trp^+Leu^+Ade^+Ura^+$) are selected on minimal medium containing histidine and screened for the ability to grow without histidine and to produce β-galactosidase. Library plasmids from parental strains that failed to activate transcription with unrelated DBD fusions in the mating assay are chosen for further analysis. Finally, the *URA3*/ER-based two hybrid system described here has also the advantage to use a reporter gene that allows selection against transcriptional activation (7). Therefore, it constitutes an appropriate system for the isolation of compounds or genes for proteins that inhibit protein–protein interactions.

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REFERENCES

1 Fields,S. and Song,O. (1989) *Nature* **340**, 245–246.
2 Fields,S. and Sternglanz,R. (1994) *Trends Genet.* **10**, 286-292.
3 Votjek,A.B.,Hollenberg,S.M. and Cooper,J.A. (1993) *Cell* **74**, 205-214.
4 Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) *Cell* **75**, 805-816.
5 Hannon,G.J., Demetrick,D., and Beach,d. (1993) *Genes Dev.* **7**, 2378-2391.
6 Zervos,A.S., Gyuris,J., and Brent,R. (1993) *Cell* **72**, 223-232.
7 Boecke,J.D., Lacroute,F., and Fink,G.R. (1984) *Mol. Gen.Genet.* **197**, 345-346.
8 Pierrat,B., Heery,D., Lemoine,Y., and Losson,R. (1992) *Gene* **119**, 237-245.
9 Ali,S., Lutz,Y., Bellocq,J.-P., Chenard-Neu,M.-P., Rouyer,N., and Metzger,D. (1993) *Hybridoma* **12**, 391-405.
10 Kumar,V., and Chambon,P. (1988) *Cell* **55**, 145–156.
11 Ylikomi,T., Bocquel,M.-T., Berry,M., Gronemeyer,H., and Chambon,P. (1992) *EMBO J.* **11**, 3681–3694.
12 Underwood,M.R., and Fried,H.M. (1990) *EMBO J.* **9**, 91–99.
13 Elledge,S.J., Mulligan,J.T., Ramer,S.W., Spottswood,M., and Davis,R.W. (1991) *Proc. Natl. Acad. Sci.* **88**, 1731–1735.
14 Wolcott,J.H., and Ross,C. (1966) *Biochem. Biophys. Acta* **122**, 532–534.
15 Leid,M., Kastner,P., Lyons,R., Nakshatri,H., Saunders,M., Zacharewski,T., Chen,J. Y., Staub,A., Garnier,J-M., Mader,S., and Chambon,P. (1992) *Cell* **68**, 377–395.
16 Nagpal,S., Friant,S., Nakshatri,H., and Chambon,P. (1993) *EMBO J.* **12**, 2349–2360.
17 Bartel,P., Chien,C.T., Sternglanz,R., and Fields,S. (1993) *Biofeedback* **172**, 920–923.