Vectors for N- or C-terminal positioning of the yeast Gal4p DNA binding or activator domains

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The yeast two-hybrid system detects in vivo protein-protein interactions leading to the reconstitution of transcription factor activity. The DNA binding domain (DBD) and the activation domain (AD) of this transcription factor are both expressed within the same cell, but on different fusion proteins (the "bait" and "prey" fusions, respectively). Active transcription factor is then only reconstituted when these DBD- and AD-bearing fusions associate noncovalently in the yeast nucleus. The transcription factor activity is monitored through the reporter genes integrated in the genomes of the yeasts used for two-hybrid screening (1).

The strength of the two-hybrid approach is that it reveals protein-protein interactions in the context of the living cell. More confidence can be placed on such interaction data if they are known to involve protein fusions that are still functional—in other words, that neither the DBD or AD domain addition nor the targeting of the protein fusion to the nucleus has caused loss of functionality. Existing two-hybrid vectors generally position the DBD or AD domain at the N-terminus of the protein of interest (1,2). However, there are occasions when it is preferable to fuse this domain to the protein's C terminus (e.g., when this allows preservation of the biological function or provides less self-activation in the two-hybrid system than the corresponding N-terminal fusion). To this end, vectors have been described for placing the LexA DBD at the C-terminus (3). Here we describe plasmids that allow both N-terminal and C-terminal fusions to be made with the same vector. These, a slight modification of the pOBD2 and pOAD vectors from the latest development of the Fields two-

hybrid system (2), facilitate fusion of the Gal4p DBD or Gal4p AD to either the N or C terminus of any protein of interest. This in turn allows rapid identification of which fusion construct is most suitable for two-hybrid screening. We show an example where positioning the Gal4p DBD at a protein C terminus generates a functional protein fusion, suitable for use in two-hybrid screening, but where the corresponding N-terminal DBD addition causes both loss of functionality and self-activation in the two-hybrid system.

Vector pBDC (Figure 1) was constructed by introducing a unique *Nru*I restriction endonuclease site upstream of the Gal4p DBD gene in the *TRP1* vector pOBD2 (2). pBDC is slightly more versatile than the parent pOBD2 in that it can be used to position this DBD at either the N or C terminus of any open reading frame (ORF). Generation of N-terminal DBD fusions is through in vivo recombination in yeast

between *Nco*I plus *Pvu*II-cut pBDC (or pOBD2) and the ORF of interest as a linear fragment [the latter generated by two sequential PCR amplifications, using primers with the sequence extensions described in an earlier report (2)]. Generation of C-terminal DBD fusions is also through a recombination in yeast between linearized pBDC and the ORF as a PCR product, but in this case the pBDC is linearized with *Nru*I (Figure 1) and the ORF-encoding DNA is created using PCR primers with different sequence extensions. The first PCR used to generate the latter DNA uses primers that possess 3′ sequence homologies to the ORF of interest, but 5′ homologies to the vector [forward primer gcttgaagcaagcctcg ATG *** *** *** *** *** *** ***; reverse primer: cagtagcttcatctttcg*** *** *** *** *** *** *** (ATG *** ***, etc., corresponding to the codons of the ORF and lowercase letters the sequences of the pBDC vector)]. The product of this first PCR is then used as the template in a second round of PCR that uses primers completely homologous to regions flanking the *Nru*I site of pBDC (forward primer: caactccaagc-ttgaagcaagcctcgatg; reverse primer: cgatagaagacagtagcttcatctttcg).

In a similar way, vector pADC (Figure 1) can be used to position the Gal4 AD at either the N or C terminus of an ORF. N-terminal AD fusions are generated by an in vivo recombination between *Nco*I plus *Pvu*II-cut pADC [or pOAD (2)] and the ORF as a linear
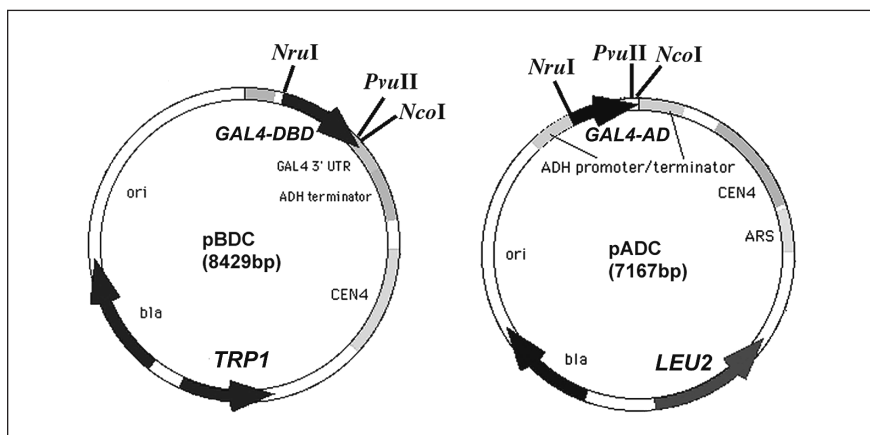

Figure 1. Vectors pBDC and pADC. These plasmids were derived from pOBD2 and pOAD, respectively (2), by changing the pOBD2 sequence at -9 to -4 with respect to the Gal4-DBD initiator codon to TCGCGA and the pOAD sequence at -13 to -8 relative to the Gal4p AD to AAGCTT. Each of these sequence changes introduced a unique *Nru*I restriction site.

PCR product [generated in two sequential amplifications, using primers with sequence extensions described in an earlier report (2)]. Generation of fusions where the AD is positioned at the ORF C terminus is through in vivo recombination between *Nru*I-cut pADC (Figure 1) and an ORF-encoding fragment. The latter is again generated by two sequential PCR amplifications, the first using primers with 3′ sequence homologies to the ORF, but 5′ sequence homologies to the vector (forward primer: agcatacaatccaag ATG *** *** *** *** *** *** *** *; reverse primer: cgctttatccatctttgcaaaggc *** *** *** *** *** *** *** *). The product of this first reaction is then used as the template in a second round of PCR, using primers homologous to the regions flanking the pADC *Nru*I site (forward primer: caagctataccaagcatacaatccaag-atg; reverse primer: ggaattaattccgctt-tatccatctttgcaaaggc).

Figure 2 shows a situation where positioning the Gal4p DBD at a protein N terminus generates a fusion that self-activates in the two-hybrid system and that has totally lost functionality, but where the corresponding C-terminal fusion does not self-activate and retains biological activity. Heat shock protein 90 (Hsp90) is a chaperone essential for the activity of several key signaling and regulatory proteins in eukaryotic cells. It binds these proteins when they are already substantially folded. Then, in response to the appropriate activatory signals, it induces the protein associations that are needed for these "client" proteins to attain full activity (4,5). Placing the Gal4p DBD at the Hsp90 N terminus produces a fusion that is incapable of providing Hsp90 function in yeast (6), probably because this domain addition interferes with intra- and inter-molecular interactions at the Hsp90 N terminus that are critical in the Hsp90

chaperone cycle (7). On the premise that a protein fusion that is also a functional chaperone would be more likely to display the true interactions of Hsp90 in the two-hybrid system, we investigated if it might not be preferable to express Hsp90 with a C-terminal DBD extension. The PJ694α *Saccharomyces cerevisiae* strain used for two-hybrid screening (2) was transformed, as described above, so that it expressed either N- or C-terminal Gal4p DBD fusions to the major isoform of yeast Hsp90 (Hsc82). To determine if these fusions can confer Hsp90 function, we recovered the *TRP1* plasmids that carried these *DBD-HSC82* and *HSC82-DBD* fusion genes from the original PJ694α transformants. We then retransformed them into another yeast, a strain that can be used to test for Hsp90 functionality (PP30[pHSC82] (MAT**a** *trp1-289, leu2-3,112, his3-200, ura3-52, ade2-101oc, lys2-801am, hsc82::KAN-*

MX4, hsp82::KANMX4 [pHSC82]) (8). Yeast deleted for both Hsp90 genes is normally inviable, but strain PP30 [pHSC82] is viable because it carries the *HSC82* gene for Hsc82 on a *URA3* episomal plasmid (pHSC82). By introducing Hsp90 genes into PP30 [pHSC82] on *LEU2*, *TRP1*, or *HIS3* vectors and then selecting for the loss of the original pHSC82 *URA3* vector [using medium containing 5-fluoroorotic acid (FOA), a compound toxic to *URA3* gene-bearing cells], it is possible to determine if these introduced genes can confer the essential Hsp90 function. Growth on FOA reveals the introduced Hsp90 gene is functional, whereas the absence of FOA growth shows it is nonfunctional (8,9). Our pBDC-derived plasmid encoding Hsc82 with a C-terminal DBD extension (Hsc82-DBD) could confer such Hsp90 function, whereas the plasmid coding for Hsc82 with an N-terminal DBD extension (DBD-Hsc82) could not (Figure 2a).

Expression of the nonfunctional DBD-Hsc82 fusion also resulted in appreciable self-activation in the two-hybrid system. This was apparent from the capacity of this fusion to confer high expression of the Gal1p-regulated *HIS3* and *LacZ* genes in the PJ694α two-hybrid strain (2) [evident from the strong growth in the presence of high levels of 3-aminotriazole (3-AT; an inhibitor of the His3p histidine biosynthetic pathway enzyme); Figure 2, b and c; and high *LacZ* expression (Figure 2d)]. This high level of self-activation precludes the use of this nonfunctional DBD-Hsp82 fusion as "bait" in two-hybrid screening. In contrast, the functional Hsc82-DBD fusion (Figure 2a) did not self-activate (growth of PJ694α containing this construct being inhibited at 0.5 mM 3-AT; Figure 2, b and c). However, it was expressed at similar levels to the DBD-Hsc82 fusion (Figure 2f). The latter Hsc82-DBD fusion was recently used in a study of Hsp90's interactions with a newly discovered Hsp90 system co-chaperone, *Aha*I (10). PJ694α cells containing this fusion have also been mated to the array of AD-protein fusions corresponding to at least 80%–90% of the proteins of yeast [expressed in a strain of the opposite mating type, PJ469a (2)]. In the latter screen, no strong interactors with Hsc82-DBD were detected, which is consistent with the probable transience of most chaperone interactions. There were, however, a number of relatively weak Hsp90-protein interactions. Many of the latter were to known accessory proteins (co-chaperones) of the Hsp90 chaperone system, consistent with detection of biologically meaningful protein-protein interactions (manuscript in preparation).
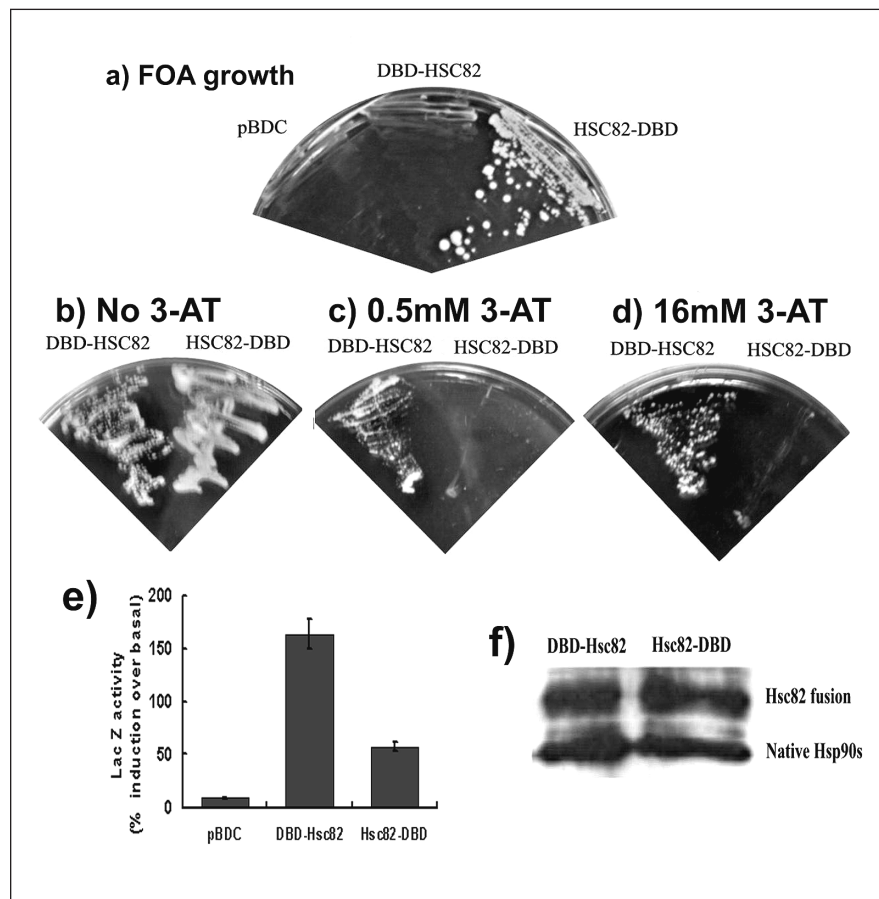
Figure 2. Characterization of the consequences of *DBD-HSC82* and *HSC82-DBD* fusion gene expression in yeast. (a) FOA growth of yeast strain PP30[pHSC82] transformed with empty pBDC and pBDC-derived plasmids bearing *DBD-HSC82* and *HSC82-DBD* fusion genes. (b–d) Growth of PJ694α cells bearing the same *TRP1* vectors in the presence of the indicated levels of 3-AT. (e) *LacZ* expression levels in the same PJ694α cells. (f) A Western blot probed with an anti-yeast Hsp90 rabbit polyclonal antiserum (9) showing the similar levels of *DBD-Hsp82* and *Hsp82-DBD* expression in these cells (the signal from the native Hsp90s of these yeasts provides an internal control for equal gel loading).

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There are probably other instances where, when an N-terminal domain fusion is found to self-activate in the two-hybrid system, the use of the corresponding C-terminal fusion will overcome this problem and permit a two-hybrid screen.

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