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Getting more from the two-hybrid system: N-terminal fusions to LexA are efficient and sensitive baits for two-hybrid studies

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ABSTRACT

Two-hybrid methods detect interactions between two proteins fused at the C-termini of, respectively, a DNA-binding domain and the activation domain of a transcriptional activator. Thus the N-terminus of none of these proteins is available for interaction. We have tested whether a bait protein with a reverted polarity (i.e. N-bait-LexA-C) is suitable for two-hybrid interaction. We show that such constructs give a specific interaction signal, and document two cases where the sensitivity is dramatically increased. Such constructs might lead to the identification of partners missed during classical two-hybrid screens.

In deciphering the functions of a protein, screening libraries with the two-hybrid method has become current practice (1,2). In all cases, a plasmid is used that expresses the protein of interest (the 'bait') fused at the C-terminus of a DNA-binding domain protein (DBD). This type of construction potentially jeopardizes interactions that require a 'free' N-terminus for a protein to interact with its molecular partners. We have addressed this problem by inverting the polarity of the DBD–bait fusion protein within a LexA-based two-hybrid system that is exquisitely sensitive (3). We show here that such bait–LexA fusions are suitable for two-hybrid studies and display positive and specific responses. Even more, a known interaction that is not detected with Lex–bait fusion is detected with a bait–Lex fusion, suggesting that such N-terminal fusions might be more sensitive.

A 1274 bp *Sph*I–*Sph*I fragment of plasmid pVJL10, a derivative of pBTM116, contains a truncated ADH promoter, the LexA coding region, a polylinker and an ADH terminator. *Sph*I sites were blunt-ended and the cassette was inserted between the *Pvu*II sites of plasmid YEplac112 (4). The *Hin*dIII–*Pst*I fragment containing the LexA ORF and the multicloning site was replaced by a fragment generated by PCR and encoding a multicloning site followed by the LexA ORF and a stop codon. The multicloning site allows the generation of fusions at the N-terminus of LexA according to the sequence: GAA TTC GGA TCC GTC GAC GGT GGT ATG, where ATG is the original LexA initiation codon. Notice that (i) *Eco*RI, *Bam*HI and *Sal*I are available as cloning sites and (ii) two glycines are used as spacers between the fused proteins. The resulting plasmid was named pFBL23.

We tested the suitability of this plasmid for two-hybrid interactions. Ras and Ral are GTPases of the Ras family that interact with different sets of proteins. Ras (wild type or as its activated V12 allele that exhibits a reduced GTPase activity) interacts with its effectors cRaf1 and RalGDS, whereas the N17 dominant negative mutant that has lost its ability to bind GTP cannot (3,5,6). Conversely, this mutant has increased affinity for the GEF (guanine nucleotide exchange factor) CDC25 as compared to wild type and V12 proteins. Similarly, the Ral GTPase (wild type or the activated V23 allele) interacts with its effector RLIP76, whereas the dominant negative Ral (N28) mutant cannot (7,8). This allele should interact with RalGDS since it acts as a Ral GEF. No allele of Ral interacts with the Ras effector cRaf1 nor the Ras-GEF CDC25; similarly no Ras allele interacts with RLIP76, the effector of Ral. The activated and dominant negative alleles of H-Ras and RalB proteins were expressed as C-terminal (Fig. 1A) or N-terminal (Fig. 1B) fusions with LexA, and their ability to interact with cRaf-1, RalGDS and RLIP76 were assessed. In the following text, plasmids pBTM116 and pVJL10 used to express C-terminal fusion proteins will be referred to generically as pLex plasmids.

Figure 1 shows that (i) pFBL23 gives no background and (ii) activated alleles of Ras and Ral interact with their respective effectors, independently of the polarity of the fusion protein. These interactions are specific: Ras(V12) does not bind to RLIP76, neither does Ral(V23) to Ras effectors. In both polarities, Ras wt and Ral wt behave as activated alleles, i.e. they bind to their respective effectors (data not shown). (iii) Ral(N28) does not interact with RLIP76, neither does Ras(N17) with cRaf-1 nor RalGDS. These results are independent of the polarity of the constructs, and show that genetics can be used with pFBL23 as well as with classical two-hybrid plasmids (3,7). (iv) Finally, we tested the ability of the dominant negative alleles Ras(N17) and Ral(N28) to interact with their respective GEFs CDC25 and RalGDS. Ras(N17) expressed from pLex as well as from pFBL23 gives a positive specific signal with CDC25. Importantly, whereas the expected interaction of Ras(V12) with CDC25 (9) is barely detectable when Ras is expressed from pLex (Fig. 1A), a strong signal is obtained when it is expressed from pFBL23. Similarly, the interaction between Ral(N28) and RalGDS is undetectable when Ral(N28) is expressed from pLex (Fig. 1A), whereas a strong and specific response is obtained with pFBL23 (Fig. 1B).

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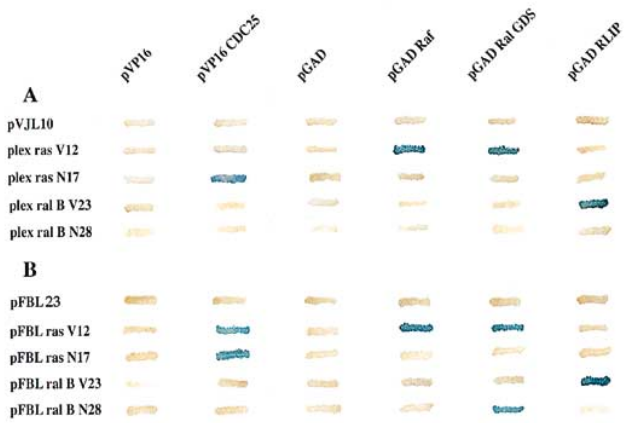

Figure 1. X-Gal assay with yeast diploids harboring various plasmids. L40 and AMR70 cells were transformed with LexA expressing plasmids (pBTM116- and pFBL23-based plasmids) and with activation domain expressing plasmids (pVP16- and pGAD-based plasmids), and transformed cells were selected for trp1 and leu2 complementation, respectively. pVJL10 and pFBL23 plasmids express LexA with no fused protein. pVP16 and pGAD express VP16 activation domain and GAL4 activation domain with no fused protein, respectively. Mating was performed on complete medium, and diploids were selected on medium lacking tryptophan and leucine (10). LacZ expression tests and histidine prototrophy tests were performed according to standard procedures (3). β-Galactosidase tests were stopped after 4–6 h, histidine prototrophy tests gave results consistent with LacZ expression tests (data not shown).

Table 1. β-Galactosidase activity measured in permeabilized cells

Protein expressed as fusion with		β-galactosidase activity ± S.D.	
DNA-binding domain	Activation domain	C-terminal	N-terminal
none	none	0.044 ± 0.016	0.161 ± 0.130
Ras V12	none	0.127 ± 0.008	0.062 ± 0.049
Ras V12	Raf	130.354 ± 23.073	112.700 ± 5.647
Ras V12	CDC25	2.295 ± 0.037*	15.405 ± 1.086*
RalB N28	none	0.131 ± 0.162	0.136 ± 0.013
RalB N28	Ral GDS	0.092 ± 0.012**	0.316 ± 0.000**

β-Galactosidase was measured in permeabilized cells (10) and 1 ml/sample was used per assay, with O.N.P.G. as substrate. Units are calculated as $OD_{420} \times 1000/time \times OD_{600}$. For each couple of plasmids, experiments were performed in triplicate. Statistical differences between activity values obtained with C- and N-terminal fusions were assessed using a paired Student t-test. Only those values identified by * or by ** were found to be significantly different (P <0.001). 'C-terminal' refers to a fusion protein where the bait is C-terminal to LexA. 'N-terminal' refers to fusion proteins where the bait is N-terminal to LexA, i.e. the protein is expressed from pFBL23.

β-Galactosidase activities arising from the interaction of some couples of proteins were quantified (10). Results are shown in Table 1 and can be summarized as follows. First, whether the DBD-fusion protein is expressed from pLex or pFBL, most β-galactosidase activities are not significantly different (as assessed by a paired Student t-test). This is true for all of the controls, where activity is below the threshold of detection in the X-Gal paper test (Fig. 1). This is also the case when strong interactions are involved, such as the Ras(V12)::Raf interaction which displays 130 and 112 U β-galactosidase whether Ras is expressed from pLex or from pFBL23, respectively. Secondly, in between these two extremes (no interaction, very strong interaction), the paper test seems to reflect roughly β-galactosidase activity: Ras(V12)::CDC25 interaction gives a light blue color (Fig. 1) and 2.3 U β-galactosidase when Ras is expressed from pLex, as opposed to an intense blue color and 15.4 U when Ras(V12) is expressed from pFBL23. Finally, when weaker interactions are at stake, pFBL23 provides, for the two examples that we document, an increment in sensitivity. Ral(N28)::RalGDS interaction gives 3.4 times more β-galactosidase activity when Ral(N28) is expressed from pFBL23 compared to Ral(N28) expressed from pLex (Table 1). In this case, expression from pFBL23 allows detection in a paper test, as opposed to expression from pLex (Fig. 1). Ras(V12)::CDC25 interaction gives 6.7 times more β-galactosidase when Ras(V12) is expressed from pFBL23 compared to Ras(V12) expressed from pLex. The differences between these sets of data obtained with the pLex and pFBL plamsids were found to be statistically significant using a paired Student t test (P <0.001).

In conclusion, a fusion protein composed of a bait at the N-terminus and LexA at the C-terminus elicits specific answers in two-hybrid studies. It offers the possibility to detect interactions requiring a 'free' N-terminus of one of the partners. We document two cases where it even exhibits an enhanced sensitivity, although this conclusion should not be generalized without further examples. The plasmid pFBL23, that allows expression of such fusion proteins, should constitute a useful tool and contribute to extend applications of two-hybrid approaches.

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