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Note

A Genetic Test for Yeast Two-Hybrid Bait Competency Using RanBPM

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ABSTRACT

We describe a simple genetic test for assessing the competency of Gal4-based baits prior to a yeast two-hybrid screen, which allows determination of whether a bait protein is expressed appropriately for an interaction to be detected. The novel test, based on interaction with the protein RanBPM, is easier and more predictive than other methods such as Western blotting, allowing identification of ~80% of incompetent baits prior to screening.

SINCE the development of the yeast two-hybrid assay (Fields and Song 1989), tens of thousands of two-hybrid screens have been carried out. While such screens have been widely successful, revealing new pathways, proteins, and functions for known proteins, individual screens often fail due to lack of interactions, which can be caused by bait-specific or library-specific factors. Bait-specific factors include poor expression, incorrect localization, or degradation of the bait fusion protein (a protein of interest fused to a transcription factor binding domain). Often Western blotting is used to test the competency of a bait fusion before screening, allowing verification that the protein is correctly expressed in the yeast cells. However, this approach is not ideal. First, the Western-blotting procedure is time-consuming and requires antibodies to either the transcription factor binding domain or the bait protein of interest. Second, this method is unable to reveal information about localization within the cell—a protein may be highly expressed but not localized to the nucleus where it is required for the assay. Finally, the bait protein-binding domain fusion may be expressed at a level that is high enough for a successful screen, but too low to detect by Western blotting.

For LexA-based two-hybrid screening, also called the interaction trap method, a bait competency test is available. This test relies on the ability of transcriptionally inactive LexA fusions to repress transcription when bound to specifically positioned LexA operators (Brent and Ptashne 1984). The reporter plasmid uses a galactose-inducible *GAL1-LacZ* reporter with LexA operators inserted into the *GAL1* UAS. Cells carrying the reporter plasmid and bait plasmid are tested for reduced LacZ activity after the addition of galactose. While this method can be useful in bait characterization, it has fallen out of use because the failure of a bait in the repression assay does not necessarily correlate with a poor screen outcome (Golemis *et al.* 2008). For Gal4-based two-hybrid screens, no genetic test has been developed.

In the interest of improving the efficiency of Gal4-based two-hybrid screening, we reasoned that a protein that interacts with the Gal4-binding domain (Gal4BD) could be used to test the competency of a Gal4BD–bait fusion protein prior to a screen, which would allow the determination of whether the bait is expressed appropriately for an interaction to occur. Such a competency test could be carried out easily with a bait of interest using a mating-based two-hybrid assay. We report here the design and testing of such a bait competency test using a novel Gal4-interacting protein, RanBPM. Comparing the results of the bait competency test with those of Western blotting, we show that the RanBPM competency test is superior in both ease of testing and predictive ability.

RanBPM and CSN5 interact with the Gal4-binding domain: We identified several Gal4 activation domain (Gal4AD) fusion proteins that gave positive two-hybrid interaction results with vectors expressing Gal4BD alone, making them good candidates for competency test proteins. These proteins included CSN5 and an N-terminal truncated version of RanBPM (ΔN-RanBPM, containing amino acids 51–654 of mouse RanBPM). (Figure 1A). CSN5, a part of the COP9 signalosome, was previously found to interact with Gal4BD (Nordgard *et al.* 2001), but this interaction has not been demonstrated for RanBPM. To assess their interaction capabilities, we tested both proteins for interaction with 34

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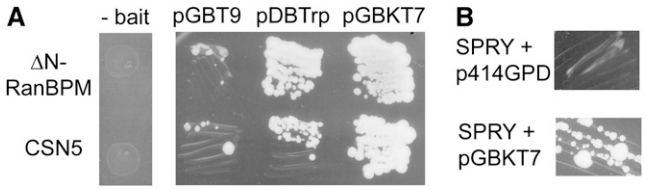

Figure 1.—Yeast two-hybrid interactions of Gal4BD-interacting proteins. (A) Interaction of CSN5 or ΔN-RanBPM with Gal4BD expressed from vectors pGBT9 (Clontech), pDBTrp [a version of pDBLeu (Invitrogen) with a Trp1 selective marker replacing Leu2)], and pGBKT7 (Clontech). Strains AH109 and Y187 (Clontech) were used, and CSN5 and ΔN-RanBPM Gal4AD fusion proteins were in pGADT7rec (Clontech). Growth shown is on SD medium −Trp/−Leu/−His + 3 mm 3-AT. Also shown ("- bait", left) are control self-activation tests of yeast expressing Gal4AD-CSN5 or ΔN-RanBPM alone (SD medium −Leu/−His + 3 mm 3-AT). (B) Interaction of the SPRY domain of RanBPM with Gal4BD from vector pGBKT7 or control vector p414GPD (SD medium −Trp/−Leu/−His + 3 mm 3-AT).

Gal4-bait fusion proteins that we knew were competently expressed because they had yielded hits in previous two-hybrid screens. CSN5 interacted with 44% of the baits, and ΔN-RanBPM interacted with 91% of the baits tested.

RanBPM is a 90-kDa ubiquitously expressed protein of unknown function. It was originally identified in a two-hybrid screen using Ran (Nakamura *et al.* 1998), where the interacting protein was found to be a truncated 55-kDa version of RanBPM (Nishitani *et al.* 2001). Full-length RanBPM contains a proline-rich N-terminal region (which is removed in ΔN-RanBPM), a consensus SPRY domain, and LiSH/CTLH motifs (Murrin and Talbot 2007). The SPRY domain has been implicated in mediating protein–protein interactions (Hilton *et al.* 1998), and previous studies indicated the SPRY domain alone could mediate interactions between RanBPM and some proteins (Rao *et al.* 2002; Wang *et al.* 2002; Cheng *et al.* 2005; Yuan *et al.* 2006). We found that the SPRY domain alone (amino acids 51–289 of mouse RanBPM) was sufficient for interaction with Gal4BD (Figure 1B), but did not use this construct further since the interaction was not as robust as with ΔN-RanBPM.

TABLE 1

RanBPM interaction correlated with two-hybrid success

	No hits	Hits	Total
Negative	21	4	25[a]
Weak positive	3	6	9[b]
Strong positive	1	40	41[c]

Screens were scored as having hits if at least one reproducible interacting protein (as confirmed by individual two-hybrid retests) was identified.

[a] Sixteen baits were in pGBT9, 4 in pDBTrp, and 5 in pGBKT7.

[b] Three baits were in pGBT9 and 6 in pGBKT7.

[c] Fourteen baits were in pGBT9, 6 in pDBTrp, and 21 in pGBKT7.

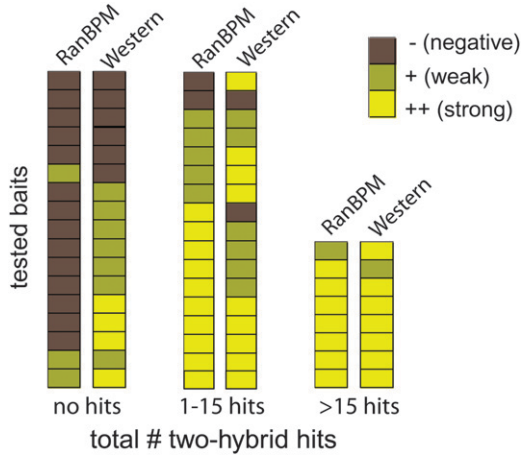

Figure 2.—Correlation of two-hybrid outcome with bait competency tests. Paired RanBPM and Western blot results are shown for each of the 42 baits tested. Tested baits are grouped into three categories, depending on two-hybrid screen outcome: no hits, 1–15 hits, or >15 hits. Competency test outcome is indicated by a shaded box, with gray indicating no RanBPM interaction or bait protein expression, yellow-gray indicating poor interaction or expression, and yellow indicating strong interaction or expression.

ΔN-RanBPM interaction as a predictor of yeast two-hybrid screen outcome: To examine the ability of ΔN-RanBPM interaction to predict yeast two-hybrid screen outcome, we compared the RanBPM interaction results with results from two-hybrid screens for 75 bait proteins (Table 1). Each protein was expressed in a Gal4-binding domain fusion vector—pGBT9 (low expression), pDBTrp (medium expression), or pGBKT7 (high expression)—and tested for interaction with RanBPM using a mating strategy (see supporting information, File S1). Interactions were scored in three categories: negative (no colonies apparent), weak positive (colonies appearing after 5 days), and strong positive (colonies appearing at or before 5 days). Of the 75 tested baits, 25 failed to pass competency testing, and 46 of the remaining 50 baits (92%) gave hits. When we tested the 25 eliminated baits for interaction in two-hybrid screens, we found that a significant number (21 of the 25, or 84%) failed to give hits. The total number of bait proteins that failed competency testing but gave hits during screening was 4, which gives a false negative rate of 8% (*i.e.*, four "negatives" of 50 screens that gave hits). The false positive rate was also 8% (*i.e.*, four baits did not yield hits, of 50 baits that scored as "competent").

Comparison of the RanBPM interaction test and Western blotting: Western blotting, which tests the expression level of a bait fusion, is often used as a test of two-hybrid bait competency. We chose a subset of the proteins examined above, 42 baits in total, to analyze by Western blotting so that we could compare the methods (Figure 2 and Table 2). Because one of our aims was to determine the abilities of the two methods in predicting incompetent baits, about half of the baits, a total of 17,

TABLE 2

Comparison of RanBPM interaction and Western blotting as predictors of two-hybrid success

	No hits	Hits	Total
	RanBPM interaction		
Negative	14	2	16
Positive	3	23	26
	Western blotting		
Negative	6	2	8
Positive	11	23	34

were proteins that had failed to yield hits in two-hybrid screening. Western blot results were scored in three categories: negative (no expression), weak (low-level expression), and strong (high-level expression).

Western blotting and RanBPM interaction gave similar rates of false negatives (8% in both cases): of the 25 baits that yielded hits during screening, only 2 baits failed to interact or failed to yield a positive immunoblot. However, Western blotting had a much higher false positive rate than the RanBPM interaction test: 32% of proteins that showed expression by Western blot failed to give hits, compared with 12% for the RanBPM test with this group of baits. With Western blotting, with the 17 baits that failed to give hits, only 6 scored as "incompetent" (*i.e.*, no expression), while 11 showed some degree of expression. In comparison, with the RanBPM test for the same 17 baits, 14 scored as "incompetent," with the remaining 3 showing a weak interaction.

Conclusions: Over 30 different proteins have now been reported to interact with RanBPM. All of these interactions were initially identified by two-hybrid screens, but most were further confirmed by co-immunoprecipitation or pull-down approaches in heterologous systems, suggesting that RanBPM may be an inherently promiscuous or "sticky" protein. As the function of RanBPM remains unknown, it is unclear whether this promiscuity is biologically relevant or rather represents an artifact of assays that use highly overexpressed proteins. In HeLa cells, RanBPM was found in a large protein complex of >670 kDa (Nishitani *et al.* 2001), which would support the idea that the protein is involved in multiple interactions, protein aggregation, or scaffolding functions.

Independent of its biological role, our results with 75 baits showing that ~90% of productive Gal4BD-fused baits interact with ΔN-RanBPM and that ~80% of nonproductive baits fail to interact with ΔN-RanBPM clearly indicate the utility of this truncated protein in assessing bait competency. It should be noted that these data were generated using a specific combination of bait and prey vectors (pGBT9, pDBTrp, pGBKT7, and pGBKT7rec) and strains (AH109 or mated AH109/Y187 diploids) and that the percentages are specific to these vectors and strains. However, preliminary results using other vectors and strains (data not shown) suggest that the method is transferable.

Our studies indicate that the RanBPM interaction test not only is easier than Western blotting, requiring merely a series of streaks on plates, but also is a better predictor of success in two-hybrid screening. Using the RanBPM interaction test, our screen success rate (the percentage of screens yielding hits) was ~90%, while this rate was only 66% when we used Western blotting as an indicator of bait competency. In particular, we found that a significant number of baits showed abundant expression by Western blotting, but failed to produce hits in two-hybrid screens.

We expect that the RanBPM interaction test will be useful for researchers carrying out high-throughput two-hybrid screens with multiple baits, as well as for investigators testing individual bait proteins (for example, to identify the site of interaction with a known binding partner). Identification of residues and domains important for an interaction often involves mutation or truncation of a bait protein, where loss of interaction due to protein instability or mislocalization can occur. Testing baits for interaction with ΔN-RanBPM, a relatively simple procedure, can provide a valuable control for such interaction studies.

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FILE S1

METHODS

Strains and vectors: For two-hybrid studies, strains AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ, MEL1*) and Y187 (*MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ, *gal80*Δ, *met-, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ, MEL1*) were used (Clontech). All libraries were from the Matchmaker two-hybrid system (Clontech) and were either purchased or made from cDNA using the Matchmaker Library Construction and Screening Kit (Clontech). Gal4AD fusion proteins were in pGADT7rec (Clontech). The Gal4AD-ΔN-RanBPM fusion protein contained amino acids 51-654 of mouse RanBPM fused to a N-terminal Gal4AD, while the Gal4AD-RanBPM SPRY domain construct contained amino acids 51-289 of mouse RanBPM fused to Gal4AD. Bait proteins were cloned in frame with Gal4BD in pGBT9 (Clontech), pGBKT7 (Clontech), or pDBTrp (a version of pDBLeu (Invitrogen) with a Trp+ selection marker) using homologous recombination in yeast.

Interaction testing via the yeast two-hybrid assay: Gal4AD plasmids containing ΔN-RanBPM, CSN5, or RanBPM-SPRY in vector pGADT7rec were expressed in strain Y187 and patched on YPD plates. On top of patches, Gal4BD empty vectors or Gal4BD bait fusion proteins expressed in strain AH109 were patched. Yeast were mated overnight at 30˚, then patched onto SD -Trp/ -Leu plates to select for diploid cells that contained both Gal4AD and Gal4BD plasmids. Colonies that grew on SD -Trp / -Leu were then patched on SD -Trp/-Leu/-His/ + 3 mM 3-aminotriazole (3-AT). RanBPM interaction was scored for no interaction (no growth on reporter plates), poor interaction (slight growth after five days incubation), or strong interaction (growth within five days).

Two-hybrid screening: Two-hybrid screens were carried out by mating or by library transformation with AH109 cells expressing the bait plasmid. For mating screens, AH109 yeast expressing the bait plasmid were grown overnight in 70 ml SD -Trp media. Cells were centrifuged, resuspended in 5 ml SD -Trp, and mixed with 1 ml of a thawed library aliquot containing Y187 yeast expressing the Gal4AD-fusion plasmid, and 45 ml 2 X YPD media (2% yeast extract, 4% peptone, 4% dextrose). Cells were incubated at 30˚ for 20-24 hours with gentle agitation to mate. After mating, the mixture was centrifuged and rinsed two times in 0.5 X YPD, and the cell pellet was resuspended in 5 ml 0.5 X YPD. The library was plated on 35 150 mm plates (SD -Trp/-Leu/-His/ + 3 mM 3-AT) and incubated at 30˚ for approximately 7-10 days. For transformation screens, AH109 yeast expressing the bait plasmid were grown overnight in 60 ml SD -Trp media. Cells were diluted to an OD_{600} of 0.22 in 150 ml media and grown for several hours to reach log phase (OD_{600} ~ 0.75). Cells were pelleted, washed in distilled water, and resuspended in 2.5 ml TE/LiAc (550 µl 10xTE; 550 µl 1 M LiAc; 3.9 ml dH_2O). Cells were pelleted at 10,000 rpm for 1 minute, then resuspended in 2 ml TE/LiAc. For transformation of library DNA, yeast cells (prepared above) were mixed with PEG,

1xTE, LiAc, salmon sperm DNA, and ~80 μg library DNA using a standard high-efficiency transformation procedure. Transformants were plated and incubated as described above with the mating screen.

Western blot analysis: Baits expressed in strain AH109 were grown to log phase (OD_{600} of ~.8) in SD –Trp media. Yeast (~ 18 OD units) were lysed in 200 μl 2x Laemmli Sample Buffer by glass bead disruption (425-600 μm beads, Sigma). For lysis, samples were vortexed 1 minute, boiled 3 minutes, vortexed 7 minutes, boiled 2 minutes, then centrifuged at 14,000 rpm for 5 minutes. Equal amounts of total protein were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane using standard procedures. Western blotting was performed using an anti-Gal4BD monoclonal antibody (sc-577, Santa Cruz Biotechnology). The secondary antibody used was an IRDye 800CW goat anti-rabbit IgG (Li-COR), and proteins were visualized using an Odyssey infrared imaging system (Li-COR).