

# Identification of proteins that interact with a protein of interest: Applications of the yeast two-hybrid system

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## Abstract

The yeast two-hybrid system is a molecular genetic test for protein interaction. Here we describe a step by step procedure to screen for proteins that interact with a protein of interest using the two-hybrid system. This process includes, construction and testing of the bait plasmid, screening a plasmid library for interacting fusion proteins, elimination of false positives and deletion analysis of true positives. This procedure is designed to allow investigators to identify proteins and their encoding cDNAs that have a biologically significant interaction with your protein of interest. (Mol Cell Biochem 172: 67–79, 1997)

*Key words:* protein interaction, two-hybrid system, yeast, screening, false positive elimination

## Introduction

Interactions between proteins are central to the function of many, if not all, cellular mechanisms. Processes such as, DNA synthesis, transcriptional activation, protein translation, protein localization, and signal transduction all involve protein complexes. Fields and Song [1] were the first to develop a molecular genetic screen that detects protein:protein interactions. This screen, performed in the yeast *Saccharomyces cerevisiae*, is commonly referred to as the two-hybrid system, and is now a standard procedure for molecular biologists. The system utilizes the product of the yeast gene *GAL4*; a protein with two functional domains that activates transcription of genes involved in galactose metabolism. The DNA binding domain (BD) of the *GAL4* protein interacts with DNA sequences within the promoter region of *GAL1* and the transcriptional activating domain (AD) of the *GAL4* protein stimulates transcription. Fields and Song constructed separate plasmids carrying the sequences coding for (A) the DNA binding domain of *GAL4* (*GAL4*<sub>BD</sub>) and (B) the transcriptional activating domain of *GAL4* (*GAL4*<sub>AD</sub>), fused in frame to DNA sequences coding for the protein products of the yeast genes

*SNF1* and *SNF4*, respectively. They introduced these two plasmids into a yeast strain containing a 'reporter gene'; the *lacZ* gene from *E. coli* fused to the *GAL1* promoter. Interaction between *SNF1p* and *SNF4p* allowed the *GAL4* activating domain to activate the transcription of the *lacZ* gene. The resulting  $\beta$ -galactosidase activity was detected by the formation of blue yeast colonies on medium supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal), and also by assay of cell free extracts.

The two-hybrid system has the potential to identify any two proteins that interact. Further development of the system allowed investigators to use a protein of interest as 'bait' to identify cellular proteins that interact [2]. The DNA sequence coding for the product of your favourite gene (*YFG*) is fused to *GAL4*<sub>BD</sub> and introduced on a selectable plasmid into an appropriate yeast strain. There it will bind to the *GAL1* promoter and function as the bait. The yeast cells are then transformed with a cDNA library constructed in a *GAL4*<sub>AD</sub> vector. The transformed cells are plated onto medium that selects for the presence of both plasmids and are then screened for  $\beta$ -galactosidase activity. Interaction between the *GAL4*<sub>AD</sub> fusion protein and the bait provided by the *GAL4*<sub>BD</sub>:*YFG* fusion protein

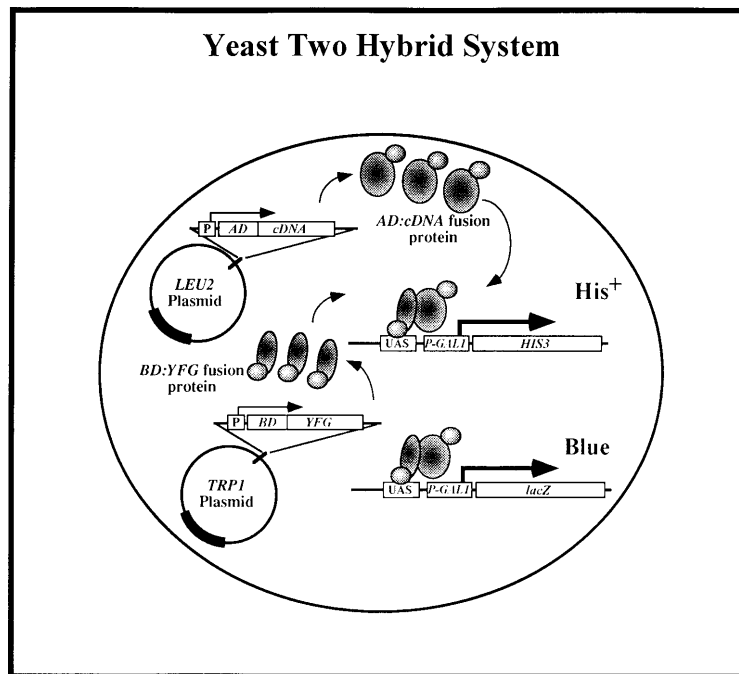


Fig. 1. The Yeast Two-Hybrid System. The yeast cell contains two plasmids. One plasmid encodes the *GAL4* DNA binding domain (*BD*) fused to your favourite gene (*YFG*), and expressed from promoter *P*. This plasmid carries the *TRP1* gene for selection in yeast and produces the *BD:YFG* fusion protein. The other plasmid encodes the *GAL4* transcription activation domain (*AD*) fused to a cDNA sequence expressed from promoter *P*. This plasmid carries the *LEU2* gene for selection in yeast and produces the *AD:cDNA* fusion protein. The yeast strain contains two integrated reporter gene constructs. These reporter genes consist of the *GAL1* promoter (*PGAL1*) fused to either a selectable yeast gene (*HIS3*) or the *E. coli lacZ* gene. The DNA binding domain of the *BD:YFG* fusion protein binds to the upstream activating sequence (UAS) of *P-GAL1*. Interaction between the *BD:YFG* and *AD:cDNA* fusion proteins stimulates transcription of the reporter genes, indicated by the arrows. Transcription and translation of *HIS3* allows selection on synthetic complete medium lacking histidine. These  $\text{His}^+$  yeast colonies will also have  $\beta$ -galactosidase activity and turn blue in the presence of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (*X-Gal*).

will activate transcription of *GAL1-lacZ* and result in a blue yeast colony. The gene coding for the interacting protein can be recovered from the blue yeast colony. It is this ability, to identify both an interacting protein and the gene which codes for it, that makes the two-hybrid system so powerful. This approach has been used by researchers, too numerous to list here, to identify genes coding for proteins that interact with transcription factors, protein kinases, phosphatases, receptors, cytoskeletal proteins as well as proteins involved in cell cycle regulation, apoptosis and a number of human genetic diseases.

Several versions of the basic two-hybrid system have been described in the literature [2–4]. We use a version which employs the selectable reporter gene *GAL1-HIS3*. In this system, the binding domain plasmid carries the yeast *TRP1* gene and a sequence derived from *YFG* fused to *GAL4<sub>BD</sub>* (see Fig. 1). The activating domain plasmid carries the yeast *LEU2* gene and a sequence from a cDNA library fused to *GAL4<sub>AD</sub>*. These plasmids are transformed into a yeast strain which requires tryptophan, leucine, and histidine (*trp1*, *leu2*, *his3*) and contains a functional copy of *HIS3* controlled by the *GAL1* promoter (*GAL1-HIS3*). If the binding domain and activating domain fusion proteins interact, the *GAL1-HIS3*

reporter gene will be activated and the yeast cell will grow on Synthetic Complete (SC) medium lacking the amino acids Trp, Leu, and His. Thus there is positive selection for the presence of activating domain plasmids encoding proteins that interact with the binding domain *YFG* fusion protein.

The genotypes and specific attributes of the yeast strains and plasmids used in our laboratory are listed in Tables 1 and 2. With the exception of DGY63 these yeast strains carry at least one additional reporter gene. Protein:protein interactions which result in a  $\text{His}^+$  phenotype should also activate these other reporter genes.

#### Requirements of the two-hybrid system

The steps involved in accomplishing a two-hybrid screen with your favourite protein gene (*YFG*) are as follows: (1) Construction of the *BD:YFG* fusion plasmid. (2) Transformation of the *BD:YFG* fusion plasmid into yeast. (3) Testing the *BD:YFG* fusion plasmid in yeast for auto-activation of reporter genes. (4) Testing the *BD:YFG* fusion plasmid for expression of *YFGp*. (5) Construction of the *AD:cDNA* fusion

Table 1. Characteristics of yeast strains

| Yeast strain       | Genotype   | Reporter genes   | 3-AT conc. | Plasmid selection          | Reference  |
|--------------------|--|--|------------|----------------------------|------------|
| Y190               | <i>MATa, ade2-101, gal4Δ, gal80Δ, his3, leu-3, 112, trp1-Δ901, ura3-52, URA3::GAL1-lacZ, LYS2::GAL1-HIS3, cyh<sup>r</sup></i>  | <i>GAL1-lacZ</i><br><i>GAL1-HIS3</i>                       | 25 mM      | <i>TRP1</i><br><i>LEU2</i> | 20         |
| PJ69-4A            | <i>MATa, ade2, trp1-Δ901, leu2-3, 112, his3-200, gal4Δ, gal80Δ, ura3-52, GAL7-lacZ::metI, GAL2-ADE2::ADE2, GAL1-HIS3::LYS2</i> | <i>GAL7-LacZ</i><br><i>GAL2-ADE2</i><br><i>GAL1-HIS3</i>   | 1 mM       | <i>TRP1</i><br><i>LEU2</i> | P. James   |
| KGY94 <sup>1</sup> | <i>MATa, ade2, gal4Δ, gal80Δ, his3-200, leu2-3, 112, trp1-Δ901, URA3::lexAop-lacZ, lys2-inv::lexA-GAL1-HIS3</i>                | <i>lexAop-lacZ</i><br><i>lexA-GAL1-HIS3</i><br><i>HIS3</i> | 1 mM       | <i>TRP1</i><br><i>LEU2</i> | R.D. Gietz |
| DGY632             | <i>MATa, ade2, gal4Δ, gal80Δ, his3-200, leu2-3, 112, trp1-Δ901, URA::GAL1-lacZ</i>   | <i>GAL1-lacZ</i>   | NA         | <i>TRP1</i><br><i>LEU2</i> | R.D. Gietz |

<sup>1</sup>Derived from CTY10-5D [11] by integration of *lys2-inv::lexA-GAL1-HIS3*.

<sup>2</sup>Derived from CTY10-5D by replacement of *URA3::lexAop-lacZ* with *URA3::GAL1-lacZ*.

Table 2. Characteristics of two-hybrid plasmid vectors

| Plasmid | Binding sequence*        | Restriction sites  | Selected marker | Hemagglutinin tag | References |
|---------|--------------------------|--|-----------------|-------------------|------------|
| pAS1    | <i>GAL4<sub>BD</sub></i> | <i>Sal1, BamHI, Sma1, Nco1, Sfi1, Nde1, EcoR1</i>        | <i>TRP1</i>     | Yes               | 3          |
| pAS2    | <i>GAL4<sub>BD</sub></i> | <i>Nde1, Nco1, Sfi1, Sma1, BamHI, Sal1</i>               | <i>TRP1</i>     | Yes               | 20         |
| pGBT9   | <i>GAL4<sub>BD</sub></i> | <i>EcoR1, Sma1, BamHI, Sal1, Pst1</i>                    | <i>TRP</i>      | No                | 21         |
| pBTM116 | <i>lexAop</i>            | <i>EcoR1, Sma1, BamHI, Sal1, Pst1</i>                    | <i>TRP1</i>     | No                | 21         |
| pACT1   | <i>GAL4<sub>AD</sub></i> | <i>BglII, EcoR1, BamHI, Xho1, BglIII</i>                 | <i>LEU2</i>     | Yes               | 3          |
| pACT2   | <i>GAL4<sub>AD</sub></i> | <i>Nde1, Nco1, Sfi1, Sma1, BamHI, Sac1, Xho1, BglIII</i> | <i>LEU2</i>     | Yes               | 20         |
| pGAD10  | <i>GAL4<sub>AD</sub></i> | <i>BglII, Xho1, BamHI, EcoR1, BglIII</i>                 | <i>LEU2</i>     | No                | 21         |
| pGAD424 | <i>GAL4<sub>AD</sub></i> | <i>EcoR1, Sma1, BamHI, Sal1, Pst1, BglIII</i>            | <i>LEU2</i>     | No                | 21         |

\*The *GAL4<sub>BD</sub>* codes for amino acids 1–147 of Gal4p and the *GAL4<sub>AD</sub>* codes for amino acids 768–881 of Gal4p.

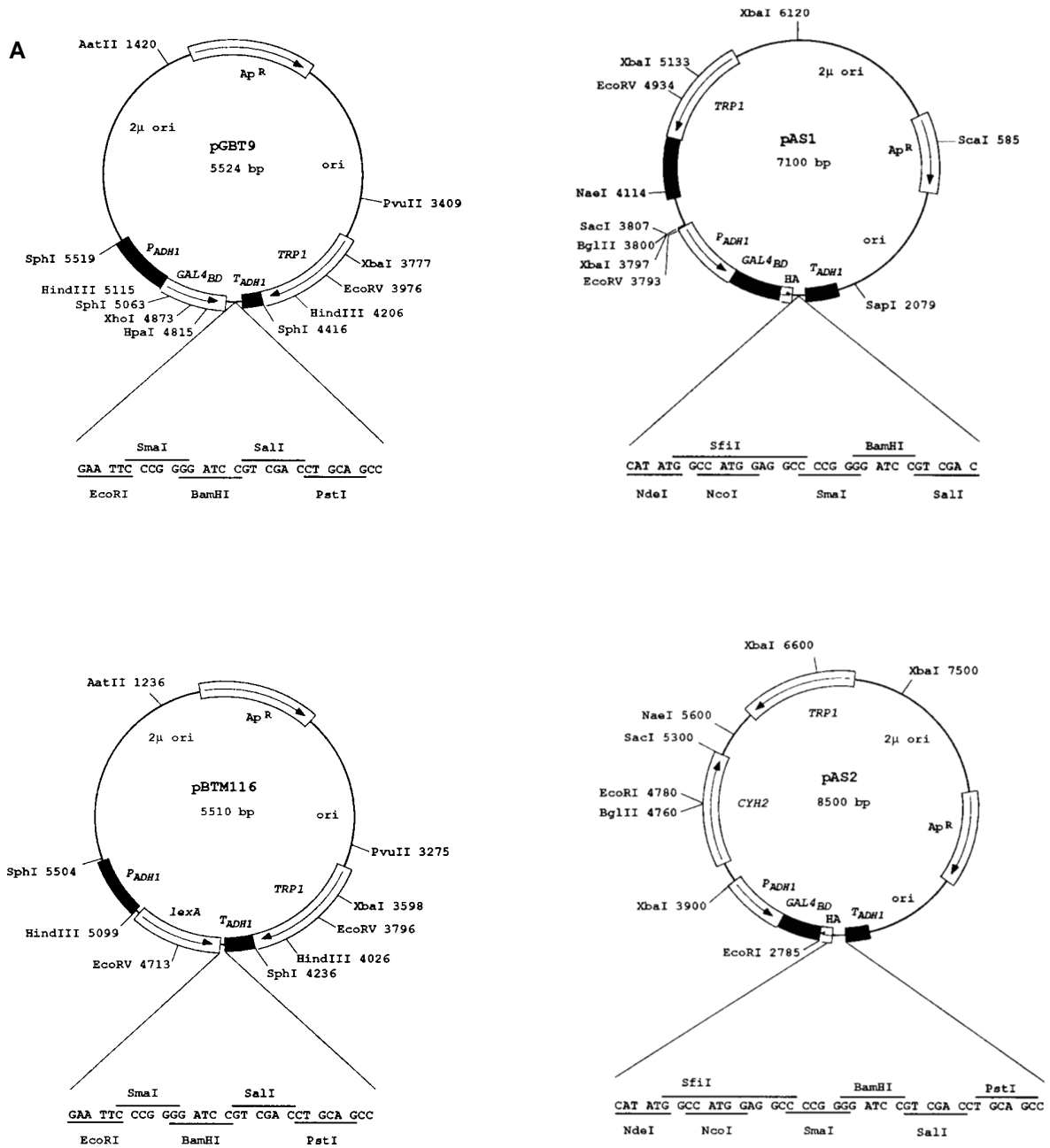
plasmid library. (6) Transformation of the *AD:cDNA* fusion plasmid library into the yeast strain carrying the *BD:YFG* fusion plasmid and selection for the activation of *GAL1-HIS3*. (7) Screening putative positives for the activation of other reporter genes. (8) Identification of Type I false positives. (9) Reconstruction of two-hybrid positives. (10) Identification of Type II and III false positives. (11) Analysis of true positives. (12) DNA sequence analysis of *AD:cDNA* fusions. (13) Deletion mapping of interacting domains.

These procedures require some expertise in DNA manipulation and it helps to have access to an individual familiar with yeast. All of the following operations are necessary to ensure that the outcome is a collection of clones with the desired properties.

#### Construction of the *BD:YFG* fusion plasmid

The plasmids that can be utilized for construction of the *BD:YFG* fusion are diagrammed in Fig. 2. The coding region

of *YFG*, or the portion that specifies a polypeptide known or suspected to interact with other proteins, must be inserted into one of the *BD* plasmids in the correct translational reading frame (see Fig. 2A). This can be accomplished in several ways. (a) *YFG* may contain a restriction site close to the 5' end of the gene that is compatible and in frame with a restriction site in the multicloning site (MCS) of the *BD* plasmid; (b) DNA fragments with compatible in-frame restriction sites at both ends can be generated using the polymerase chain reaction (PCR) with oligonucleotide primers containing specific restriction sites. PCR-generated gene fragments should be cloned and sequenced *before* construction of the *BD:YFG* fusion to ensure that mutations that can occur during DNA amplification have not been introduced; (c) Linkers or adapters can be used to generate restriction sites compatible and in-frame with the MCS of the *BD* plasmid. The linker or adapter should fuse the *YFG* sequence in-frame with the *BD* in the plasmid; (d) The multicloning site of each *BD* plasmid contains restriction sites that can be blunt-end ligated into each reading frame. *YFG* fragments can be blunt-end ligated



*Fig. 2.* Maps of Plasmids used in the Two-Hybrid System. (A) DNA binding domain plasmids: The plasmid maps of pGBT9 [21], pBTM116 [21], pAS1 [3], and pAS2 [20], are shown. Each plasmid contains the *GAL4* DNA binding domain (*GAL4<sub>BD</sub>*) (1–147 aa) or the *lexA* gene (1–202 aa) expressed constitutively between the yeast alcohol dehydrogenase 1 promoter (*P<sub>ADH1</sub>*) and terminator (*T<sub>ADH1</sub>*). All of these plasmids contain the yeast 2 micron circle origin of replication (2 μ, ori), the *E. coli* DNA replication origin (ori), the β-lactamase gene responsible for ampicillin resistance (Ap<sup>R</sup>), and the yeast *TRP1* gene for selection. The plasmids pAS1 and pAS2 both contain the DNA sequence encoding the hemagglutinin tag (HA) fused to the *GAL4<sub>BD</sub>*. The gene *CYH2* in plasmid pAS2 confers sensitivity to cycloheximide in strain Y190 (See Table 1). The direction of transcription of each gene is indicated by an arrow. A DNA fragment containing *YFG* can be inserted into the multicloning site shown below each plasmid. The restriction sites and the translation frames are illustrated. Other useful restriction sites for each plasmid are also shown; (B) Activation domain plasmids: The plasmid maps of pGAD10 [21], pGAD424 [21], pACT [3], and pACT2 [20] are shown. Each plasmid contains the *GAL4* transcription activating domain (*GAL4<sub>AD</sub>*) (768–881 aa) expressed constitutively between the yeast alcohol dehydrogenase 1 promoter (*P<sub>ADH1</sub>*) and terminator (*T<sub>ADH1</sub>*). All of these plasmids contain the yeast 2 micron circle origin of replication (2 μ ori), the *E. coli* DNA replication origin (ori), the β-lactamase gene responsible for ampicillin resistance (Ap<sup>R</sup>), and the yeast *LEU2* gene for selection. The plasmid pACT2 contains the DNA sequence encoding the hemagglutinin tag (HA) fused to the *GAL4<sub>AD</sub>*. The direction of transcription is indicated by an arrow within each gene. DNA fragments can be inserted into the multicloning site shown below each plasmid. The restriction sites and the translation frames are illustrated. Other restriction sites for each plasmid are also shown.

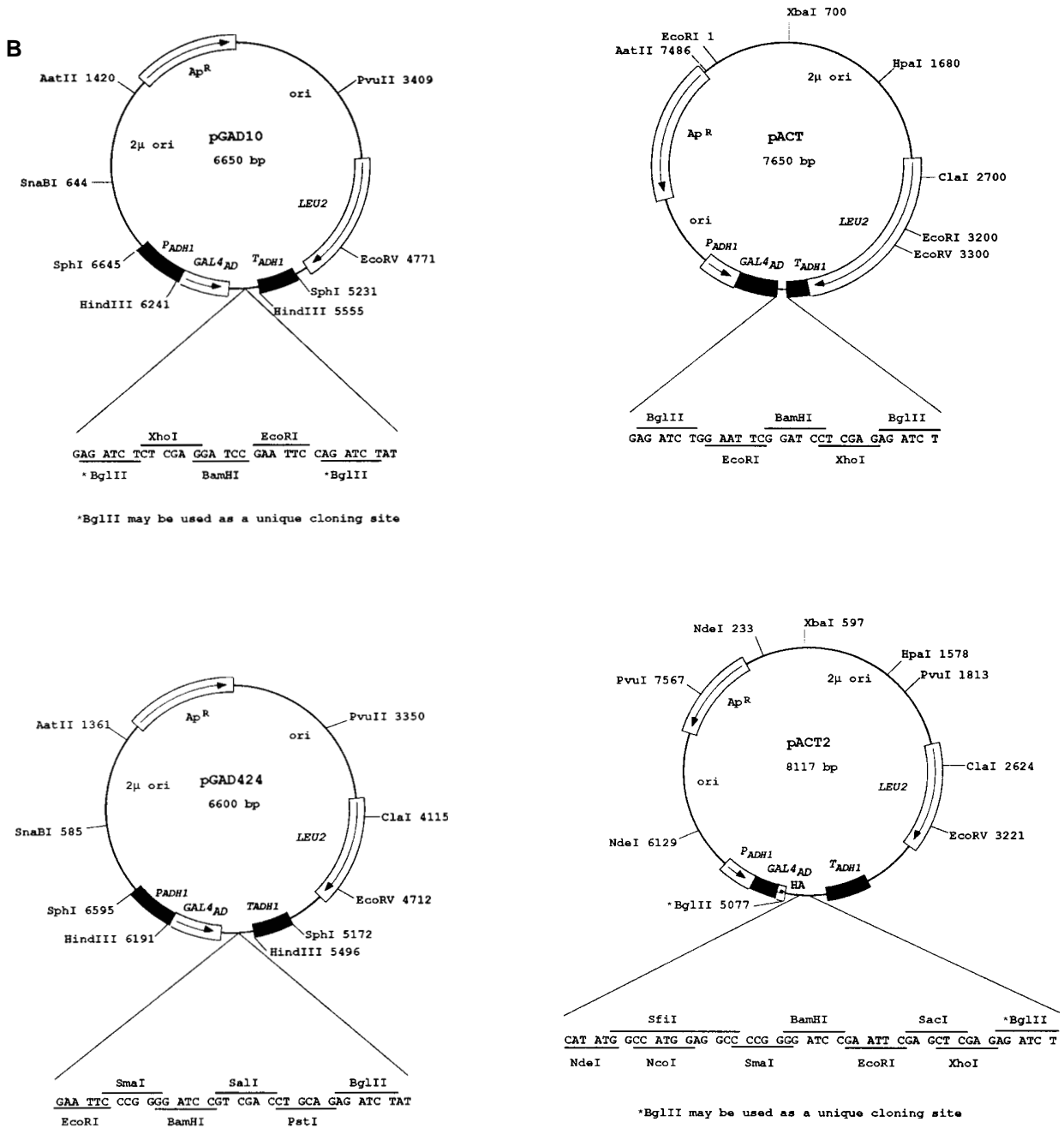


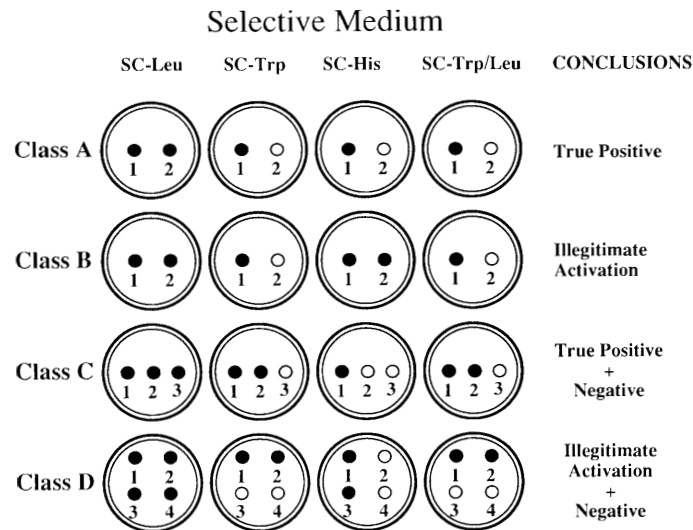
Fig. 2. Continued.

into these sites to produce the correct reading frame if suitable restriction sites with blunt ends or 5' overhangs are present in the 5' end of *YFG*.

The fourth approach (d) is the one we employ most frequently. For example, the MCS of the *GAL4<sub>BD</sub>* vector pGBT9 contains: (1) *EcoRI*, *SmaI*, and *SalI* sites that give a '-1' frame (they require the addition of 1 nucleotide to maintain the *GAL4<sub>BD</sub>* reading frame) when digested and filled with Klenow polymerase; (2) a *BamHI* site that gives an 'even' or '0' frame

(this requires no additional nucleotides to maintain the *GAL4<sub>BD</sub>* reading frame); (3) and an *XmaI* site that gives a '-2' frame (this requires the addition of 2 nucleotides to maintain the *GAL4<sub>BD</sub>* reading frame).

The *lexA* plasmid, pBTM116, can also be used to construct *BD* fusion genes. It can be used with any *AD* plasmid to screen for interactions but must be transformed into a yeast strain designed for this plasmid. The *E. coli lexA* protein binds to the *lexA* operator sequences that have been introduced into



**Fig. 3.** Two hybrid system plasmid segregation analysis. Growth of transformants in non-selective medium (YPAD) followed by selection for *LEU2* results in the retention of the *GAL4* transcription activating domain plasmid *pAD:cDNA (LEU2)* and loss of the DNA binding domain plasmid *pBD:YFG (TRP1)* from some yeast cells. Replica-plating the yeast colonies formed on SC-Leu onto SC-Trp, SC-His, and SC-Trp/Leu facilitates the identification of colonies containing true positive plasmids (*pAD:cDNA-TP*) and of plasmids which show illegitimate activation (*pAD:cDNA-IA*) of the reporter gene in the absence of *pBD:YFG*. Yeast cells may also contain a second *LEU2* plasmid (*pAD:cDNA-Neg*), which does not activate the reporter genes. The double circles represent petri plates. The filled and open circles represent growing and non-growing yeast colony replicas, respectively. Listed below are the 4 different classes of two-hybrid system putative positives you are likely to encounter. Class A: The phenotypes of yeast colonies which contain a single *LEU2* plasmid, *pAD:cDNA-TP* that encodes a true interacting protein, after the segregational loss of the *pBD:YFG-TRP1* plasmid from some yeast cells (A 2). The plasmids found in each yeast colony are: A 1 – *pBD:YFG (TRP1) + pAD:cDNA-TP (LEU2)*; A 2 – *pAD:cDNA-TP (LEU2)*. Class B: The phenotypes of yeast colonies which contain a single *LEU2* plasmid, *pAD:cDNA-IA* encoding an illegitimate activating protein, which stimulates reporter gene activity in the absence of the *pBD:YFG TRP1* plasmid (B 2). The plasmids found in each yeast colony are: B 1 – *pBD:YFG (TRP1) + pAD:cDNA-IA (LEU2)*; B 2 – *pAD:cDNA-IA (LEU2)*. Class C: The phenotypes of yeast colonies which contain a *LEU2* plasmid, *pAD:cDNA-TP* encoding a true interacting protein, and a second *LEU2* library plasmid *pAD:cDNA-Neg* which does not activate the reporter genes. The plasmids found in each yeast colony are: C1 – *pBD:YFG (TRP1) + pAD:cDNA-TP (LEU2) + pAD:cDNA-Neg (LEU2)* or *pBD:YFG (TRP1) + pAD:cDNA-TP (LEU2)*; C2 – *pBD:YFG (TRP1) + pAD:cDNA-Neg (LEU2)*; C3 – *pAD:cDNA-TP (LEU2)* or *pAD:cDNA-Neg (LEU2)*. Class D: The phenotypes of yeast colonies which contain a *LEU2* plasmid, *pAD:cDNA-IA* encoding an illegitimate activating protein, which stimulates reporter gene activity in the absence of the *pBD:YFG TRP1* plasmid, and a second *LEU2* library plasmid *pAD:cDNA-Neg* which does not activate the reporter genes. The plasmids found in each yeast colony are: D1 – *pBD:YFG (TRP1) + pAD:cDNA-IA (LEU2) + pAD:cDNA-Neg (LEU2)* or *pBD:YFG (TRP1) + pAD:cDNA-IA (LEU2)*; D2 – *pBD:YFG (TRP1) + pAD:cDNA-Neg (LEU2)*; D3 – *pAD:cDNA-IA (LEU2)*; D4 – *pAD:cDNA-Neg (LEU2)*.

the yeast *GAL1* promoters of the strain KGY94. In our hands pBTM116 usually results in higher levels of reporter gene activity compared to a *GAL4<sub>BD</sub>* plasmid carrying the same *YFG* fusion.

It may not be essential to use the entire *YFG* gene in your BD fusion construct; however, unless you know the protein interaction motifs of *YFGp* it is best to use as much of the gene as possible. After cloning each binding domain construct should be verified by DNA sequence analysis using the appropriate primer. The *GAL4<sub>BD</sub>* primer, 5'-TCA TCG GAA GAG AGT AG-3', should be used for *GAL4<sub>BD</sub>* plasmids such as pGBT9, pAS1, and pAS2; the *lexA* primer, 5'-CTT CGT CAG CAG AGC TTC-3', for *lexA* plasmids such as pBTM116.

#### *Transformation of the BD:YFG fusion plasmid into yeast*

The *BD:YFG* fusion plasmid must be tested in yeast to ensure that the construct does not autoactivate the reporter

gene(s). This is carried out by transformation of the plasmid into an appropriate yeast strain. Each of the yeast strains listed in Table 1 contains reporter gene constructs that are matched to a specific binding domain plasmid. Ensure that the yeast strain is compatible with your *BD:YFG* fusion plasmid.

There are several protocols for introducing plasmid DNA into yeast. The simplest and most efficient is the lithium acetate method, developed by Ito *et al.* [5] and improved by Gietz and Woods [6]. Yeast cells grown in rich medium are harvested and treated with lithium acetate (LiAc), polyethylene glycol (PEG) and single stranded carrier DNA (SS-DNA) to induce plasmid DNA uptake. The yeast cells that contain the plasmid are selected by growth on synthetic complete (SC) medium lacking specific nutrients. These transformants can then be tested for reporter gene activity. The yeast media and reagents required for the transformation protocol are listed in the Appendix.

The following simple protocol yields up to 5,000 transformants/ $\mu$ g of plasmid DNA; sufficient for the isolation of

single plasmid transformants [6]. Actively growing yeast cells give the highest efficiency in this protocol. Cultures that are several days or even weeks old can be used although transformation will be less efficient. Set up separate transformations for each *BD:YFG* plasmid construct you have prepared.

*Protocol 1. Transformation of yeast with the BD:YFG fusion plasmid*

(1) Subculture the yeast strain onto a YPAD plate (Appendix 1) and incubate overnight at 30°C. (2) Scrape a 20 µl blob of yeast from the plate and resuspend the cells in 1.0 ml of sterile double distilled (sdd) water. (3) Pellet the cells by centrifugation for 30 sec at maximum speed in a microcentrifuge. (4) Boil a sample of carrier DNA (2 mg/ml, Appendix) for 5 min and immediately chill in an ice/water bath. (5) Add the following reagents to the cell pellet in the order specified: (a) PEG 3350 (50% w/v) 240 µl, (b) LiAc 1.0 M 36 µl, (c) Carrier DNA (2 mg/ml) 50 µl, (d) sdd water 30 µl, (e) *BD:YFG* plasmid DNA (1 µg) 5 µl. (6) Vortex vigorously to suspend the cell pellet. (7) Incubate for 30 min at 30°C. (8) Heat shock in a water bath at 42°C for 20 min. (9) Pellet the cells at maximum speed in a microcentrifuge for 30 sec. (10) Remove the supernatant, resuspend the cells in 1 ml of sdd water, and plate 200 µl of this suspension onto each of two SC-Trp plates. (11) Incubate the SC-Trp plates for 3–4 days at 30°C.

It is possible that the some *BD:YFG* fusion proteins will be toxic to yeast cells. Therefore, if you are transforming a *BD:YFG* fusion plasmid for the first time, it is a good idea to use the BD plasmid as a positive control. If the *BD:YFG* fusion protein is toxic to yeast a smaller fragment of the *YFG* DNA sequence can be used in an attempt to overcome this problem.

*Testing the BD:YFG fusion plasmid transformants for auto-activation of reporter genes*

The Trp<sup>+</sup> transformants that appear as colonies on the plates of SC-Trp medium contain the *BD:YFG* fusion plasmid. They can now be tested for auto-activation of the *GALI-HIS3* reporter gene construct by testing for growth on SC-His medium. This particular reporter gene is leaky in most strains. However, if the histidine analogue 3-amino-1,2,4-triazole (3-AT) is added to the medium, background growth is reduced. The appropriate levels of 3-AT required for specific yeast strains are listed in Table 1.

Auto-activation of the *GALI-HIS3* gene can be tested by either of the following methods: (1) Streak several transformants containing the *BD:YFG* fusion plasmid onto an SC-His plate containing the appropriate molarity of 3-AT. If the *BD:YFG* fusion plasmid does not auto-activate growth will be negligible after 3–4 days at 30°C and you may proceed to

screen for interacting proteins with this plasmid. Auto-activation of *GALI-HIS3* will result in good growth of all of the cells in the streak. (2) Plate samples of 1000 yeast cells onto petri plates of both SC-His + 3-AT and SC-Trp media. A *BD:YFG* fusion plasmid which does not auto-activate will not form colonies on SC-His + 3-AT plates. Auto-activation of *GALI-HIS3* will allow growth on SC-His + 3-AT medium and will result in similar colony numbers on both types of medium.

*BD:YFG* plasmids which auto-activate the *GALI-HIS3* reporter gene should not be used to screen for interacting proteins.

Auto-activation of the *GALI-lacZ* reporter gene results in expression of β-galactosidase activity. This can be detected either by the filter assay of Breeden and Nasmyth [7], as modified by Bartel and Fields [8] (Protocol 2), or by measuring the hydrolysis of *O*-nitrophenyl-β-D-galacto-pyranoside (ONPG) to the yellow chromophore *O*-nitrophenol (ONP) [9] (Protocol 3). The filter assay is more sensitive but requires liquid nitrogen to freeze-fracture the yeast cells. *Note*: Protocols 2 and 3 are also used to screen putative positives for activation of *GALI-lacZ* and Protocol 3 for *GAL7-lacZ*.

*Protocol 2. Filter assay for β-galactosidase activity*

(1) Carefully place a 75 mm diameter circle of sterile filter paper (Whatman #1) on top of transformant colonies growing on selective medium. Ensure that the filter makes good contact with all of the colonies. (2) Remove the filter from the plate and place it colony side up in a pool of liquid nitrogen for 10–15 sec. (3) Remove the filter from the liquid nitrogen and allow it to thaw at room temperature. Repeat the freezing process twice. (4) Soak another 75 mm circle of sterile Whatman #1 paper in Z buffer + X-Gal (See Appendix). (5) Place the filter, yeast colonies up, onto the filter paper soaked in Z buffer + X-Gal, transfer the filters to a plastic bag and incubate at 37°C.

Strong activation of the *lacZ* gene will give a blue colour in a few hours. If no colour develops or only faint blue coloration occurs following an overnight incubation the *BD:YFG* fusion can be used in a screen for interacting protein partners. The transformants tested by this protocol must be grown on SC-Trp medium since colonies grown on YPAD medium all develop blue colour.

A more quantitative measurement of β-galactosidase activity can be obtained by assaying the hydrolysis of ONPG using the following protocol [9].

*Protocol 3. ONPG assay of β-galactosidase activity in yeast*

(1) Cells can be grown either in liquid, or on plates of, SC medium selecting for the transformant to be assayed; (a) Inoculate a transformant into 2.0 ml of selective medium and incubate overnight with agitation. This will result in a titre of approximately  $1-2 \times 10^7$  cells/ml. Determine the OD<sub>600</sub> of

a 1 in 10 dilution. Transfer a 1 ml sample of the culture to a microcentrifuge tube. Pellet the cells and resuspend them in 500  $\mu$ l of Z buffer (see Appendix); (b) Grow a transformant overnight on an SC-medium plate. Suspend 25–50  $\mu$ l of yeast cells in 1 ml of water in a microfuge tube and measure the OD<sub>600</sub>. Pellet the cells and resuspend them in 500  $\mu$ l of Z buffer. (2) Add 50  $\mu$ l of 0.1% SDS and vortex vigorously for 15 sec. (3) Add 50  $\mu$ l of chloroform and vortex for again for 15 sec. (4) Add 100  $\mu$ l of ONPG (4 mg/ml), vortex, and incubate at 37°C for 2–30 min. (5) Quench the reaction with 500  $\mu$ l of 1.0 M Na<sub>2</sub>CO<sub>3</sub>. (6) Pellet the cells at maximum speed in a microcentrifuge and carefully remove the supernatant to a clean tube. (7) Measure the OD<sub>420</sub> of the supernatant against a blank containing Z buffer, ONPG and Na<sub>2</sub>CO<sub>3</sub>. You may need to use a smaller volume of cells or reduce the incubation time if activity is high and the yellow colour develops rapidly (in less than 5 min). (8) Calculate the units of  $\beta$ -galactosidase activity using the following formula:

$$\text{Units of } \beta\text{-galactosidase activity} = \frac{1000 \times \text{OD}_{420}}{V \times t \times \text{OD}_{600}}$$

where  $V$  = the volume of cells (ml) and  $t$  = the incubation time in min.

If the level of  $\beta$ -galactosidase activity is less than 2–3 units the *BD:YFG* plasmid does not autoactivate and can be used to screen for interacting protein partners.

If your *BD:YFG* fusion plasmid activates either reporter gene the cloned *YFG* sequence must be re-designed. This can be accomplished by using restriction sites to create deletions of *YFG* from the 5' or 3' ends to determine the location of the auto-activating region. A *BD:YFG* plasmid with the auto-activating region deleted can be used to screen for interacting proteins. Alternatively, we have found that some *BD:YFG* fusions that auto-activate in the vectors pAS1 or pAS2 do not when cloned into the vector pGBT9.

If your *BD:YFG* fusion plasmid does not activate either reporter gene you are ready to screen for interacting protein partners.

#### Testing the *BD:YFG* fusion plasmid for expression of *YFGp*

Expression of the cloned *YFG* sequence can be tested by Western blotting with an antibody to *YFGp*. If a *YFGp* antibody is not available you can utilize either the 12CA5 monoclonal antibody (BAbCO, 4131 Lakeside Drive, Richmond, CA, 94806-1965) against the influenza virus hemagglutinin (HA) tag [10] for *GAL4<sub>BD</sub>:YFG* fusions constructed in the vectors pAS1 and pAS2, or the *GAL4<sub>BD</sub>* antibody (Santa Cruz Biotechnology Inc., 2161 Delaware Avenue, Santa Cruz, California 95060).

#### Construction of the *GAL4<sub>AD</sub>:cDNA* fusion library

The libraries needed to screen for interacting protein partners must be constructed in one of the *GAL4<sub>AD</sub>* vectors (see Table 2). Libraries can be constructed by standard cloning methodologies with cDNA derived from appropriate tissue or cell line mRNA. A list of some of the available *AD:cDNA* libraries can be found in Bartel and Fields [8]. In addition, a selection of libraries is available from ClonTech Laboratories Inc. (4030 Fabian Way, Palo Alto, CA, 94303-4607). We have used a number of their human cDNA libraries with good success.

#### Transformation of the *AD:cDNA* fusion library into the yeast strain carrying the *BD:YFG* fusion plasmid and selection for the activation of *GAL1-HIS3*

The most effective way to identify interacting protein partners is to select for transformants that activate the *HIS3* reporter gene and as a result grow on SC-Trp/Leu/His medium containing 3-AT. The high efficiency yeast transformation protocol [6] yields up to  $5 \times 10^6$  transformants/ $\mu$ g of plasmid DNA and allows complex cDNA plasmid libraries constructed in *GAL4<sub>AD</sub>* vectors to be screened quickly and easily. In most cases it is best to transform the *AD:cDNA* fusion plasmid library into a yeast strain that contains the *BD:YFG* fusion plasmid. However, co-transformation with the *BD:YFG* fusion plasmid and the *AD:cDNA* fusion library is recommended if the *BD:YFG* fusion plasmid adversely affects yeast cell growth. Co-transformation requires careful attention to the relative amounts of *BD:YFG* fusion plasmid and *AD:cDNA* fusion plasmid library DNA in the transformation reaction to ensure maximal efficiency. The protocol given below can be used for both approaches.

A typical two-hybrid screen requires at least 10–12  $\times 10^6$  transformants. To achieve these numbers we have found that 50  $\mu$ g of *AD:cDNA* library plasmid DNA can be used with the culture and reactions volumes listed in Protocol 4. These volumes can be scaled up if required.

#### Protocol 4. High efficiency transformation of a yeast strain containing a plasmid

(1) Inoculate the yeast strain containing the *BD:YFG* fusion plasmid into 25 ml of SC-Trp medium in a 250 ml flask and incubate at 30°C overnight at 200 rpm. (2) Determine the cell titre and calculate the volume of cells that contains  $2.5 \times 10^8$  cells (usually about 10 ml). (3) Pipette this volume into a sterile 50 ml centrifuge tube and pellet the cells at  $3000 \times g$  for 5 min. (4) Resuspend the cell pellet in 50 ml of pre-warmed (30°C) YPAD and transfer to a sterile 250 ml culture flask. This will give a titre of  $5 \times 10^6$  cells/ml. (5) Incubate at 30°C and 200 rpm until the cell titre reaches  $2 \times 10^7$  cells/ml. This

will require 3–4 h. (6) Harvest the cells by centrifugation at  $3000 \times g$  for 5 min. (7) Wash the cells in 10 ml sterile water. (8) Boil a sample of SS-DNA (2 mg/ml) for 5 min and chill immediately in ice/water. (9) Resuspend the cells in 10 ml of sterile 100 mM LiAc and incubate at 30°C for 10 min. (10) Pellet the cells and remove the LiAc supernatant. (11) Add the following reagents, in the order specified, to the cell pellet: (a) PEG 3350 (50% w/v)<sup>a</sup> 2400  $\mu$ l; (b) LiAc 1.0M 360  $\mu$ l; (c) Carrier DNA (2 mg/ml) 500  $\mu$ l; (d) sdd water<sup>b</sup> 300  $\mu$ l; (e) *AD:cDNA* library DNA (50  $\mu$ g) 50  $\mu$ l; (12) Vortex vigorously to suspend the cell pellet. (13) Incubate, without agitation, at 30°C for 30 min. (14) Heat shock the transformation reaction in a water bath at 42°C for 25 min and mix by inversion for 15 sec every 5 min. (15) Centrifuge at  $3000 \times g$  for 5 min and gently resuspend the cells in 10 ml sterile water. (16) Plate all of the suspension onto one hundred  $150 \times 15$  mm SC-Trp/Leu/His + 3-AT (see Table 1 for molarity used with your yeast strain). Plate samples of 1 and 5  $\mu$ l into puddles of 100  $\mu$ l of sdd water on each of two SC-Trp/Leu plates to allow estimation of the total number of transformants screened. (17) Incubate the plates at 30°C for 7 days. Drying of the plates can be prevented by incubating them in loosely sealed plastic bags. <sup>a</sup>Use plastic pipettes to deliver the PEG solution; PEG adheres to glass; <sup>b</sup>The total volume of water + plasmid should not exceed 350  $\mu$ l.

#### Screening putative positives for the activation of other reporter genes

The yeast strains listed in Table 1 all contain one or more of the reporter genes: *GAL1-HIS3*, *GAL1-lacZ*, *GAL7-lacZ* or *GAL2-ADE2*. The transformation protocol above specifies that the cells be plated onto SC-Trp/Leu/His to select for presence of both the *BD:YFG* and *AD:cDNA* fusion plasmids, and to select for activation of the *GAL1-HIS3* reporter gene. The Trp<sup>+</sup>/Leu<sup>+</sup>/His<sup>+</sup> transformants can usually be seen 4 days after transformation. In our experience colonies appearing after this time are mostly false positives, although some *bona fide* positives do take up to 7 days to form large colonies. In some yeast strains, such as Y190, some His<sup>+</sup> transformants are not associated with activation of other reporter genes. Screening for the activation of a second reporter system, *GAL1-lacZ* or *GAL2-ADE2*, is necessary to eliminate this class of false positives.

If you recover more than 1000 putative positives it is advisable to use the filter assay (Protocol 2) to test for  $\beta$ -galactosidase activity directly from the screening plates. If there are fewer than 300 positives it is relatively easy to subculture them in a grid pattern on a plate of fresh SC-Trp/Leu/His medium. After the inocula have grown, which may take several days, they can be assayed by Protocol 2.

The yeast strain PJ69-4a has *GAL2-ADE2* as a reporter

gene. Putative positives can be replica plated onto SC-Trp/Leu/Ade to test for activation of this reporter system. Unfortunately the *GAL7-lacZ* reporter in PJ69-4a shows endogenous expression when tested using the filter assay (Protocol 2); however increased  $\beta$ -galactosidase activity in the ONPG assay (Protocol 3) correlates well with activation of the other reporter genes in this strain.

At this stage you may have isolated a considerable number of putative positives. The next step is to eliminate false positives. We suggest that you carry out the next operation on a sample of not more than 20.

#### Identification of Type 1 false positives

Bartel *et al.* [11] describe a number of different types of two-hybrid system false positives. The most common, which we refer to as Type 1 false positives, are those *AD:cDNA* fusion products that can activate reporter gene transcription without interacting with the *BD:YFG* fusion protein. This type can be identified using the technique of ‘segregation analysis’ (Protocol 5). Since you have already established that the *BD:YFG* fusion plasmid does not autoactivate this analysis focuses on the *AD:cDNA* fusion plasmids. These plasmids carry the selectable yeast gene *LEU2* and support growth on SC-Leu. Putative positives are first grown in YPAD, regrown in SC-Leu and then plated on SC-Leu plates. Colonies that grow must contain the *AD:cDNA* fusion plasmid; they may or may not contain the *BD:YFG* fusion plasmid. The test also allows detection of cells that have been simultaneously transformed by two *AD:cDNA* plasmids, one which results in activation of the reporter gene and the other which does not.

#### Protocol 5. Segregation analysis

(1) Grow Trp<sup>+</sup> Leu<sup>+</sup> His<sup>+</sup> transformants from an initial titre of  $1 \times 10^5$  cells/ml in 2 ml of YPAD at 30°C with shaking overnight. (2) Inoculate 5  $\mu$ l of this culture into 2 ml of SC-Leu medium and grow overnight at 30°C with shaking. The titre should be  $1-2 \times 10^7$  cells/ml. (3) Dilute 1 in 10 in sdd water and plate 100  $\mu$ l onto each of two SC-Leu plates ( $100 \times 15$  mm) and incubate at 30°C for 2–3 days. This should give about 1000 colonies per plate. (4) Replica-plate the Leu<sup>+</sup> colonies onto separate plates of SC-Trp, SC-His, and SC-Trp/Leu and incubate at 30°C for 1–2 days. It is essential to mark the orientation of the plates to facilitate identification of specific colonies. Remember that all colonies grow on SC-Leu. True positives and false positives will have different colony phenotype patterns on each type of medium (Fig. 3). The *AD:cDNA* plasmids that give rise to each type of two-hybrid system positive are as follows: (A) True positive (p*AD:cDNA*-TP): Leu<sup>+</sup> colonies from a true positive can have either of two phenotypes. If both the p*BD:YFG* and p*AD:cDNA*-TP plasmids are retained, *GAL1-HIS3* will be activated and the

colony will grow on all four media (Fig. 3A, 1). If a segregant retains only *pAD:cDNA-TP* it will grow on SC-Leu, but not on any of the other media (Fig. 3A, 2). *Note:* If you obtain a positive of this kind, isolate one of the  $\text{Leu}^+ \text{Trp}^- \text{His}^-$  colonies, which will carry only the *pAD:cDNA-TP* plasmid and proceed to the next operation – reconstruction of two-hybrid positives. (B) False positive resulting from illegitimate activation of the reporter gene by *pAD:cDNA-IA* in the absence of *pBD:YFG*: These positives can also show two phenotypes. Retention of both *pBD:YFG* and *pAD:cDNA-IA* will allow growth on all four media (Fig. 3B, 1). Retention of only *pAD:cDNA-IA* will allow growth on SC-Leu and SC-His but not on SC-Trp or SC-Trp/Leu (Fig. 3B, 2). *Note:* Discard all positives that fall into this class. (C) True Positive (*pAD:cDNA-TP*) also containing a second library plasmid which does not activate the reporter gene (*pAD:cDNA-Neg*): This situation results in three possible colony phenotypes. Retention of *pBD:YFG* and both *pAD:cDNA-TP* and *pAD:cDNA-Neg* or retention of *pBD:YFG* and *pAD:cDNA-TP* plasmid will allow growth on all four media (Fig. 3C, 1). Retention of *pBD:YFG* and *pAD:cDNA-Neg* will allow growth on SC-Leu, SC-Trp, and SC-Trp/Leu, but not on SC-His (Fig. 3C, 2). Retention of either *pAD:cDNA* plasmid and loss of *pBD:YFG* will result in growth on SC-Leu alone (Fig. 3C, 3). *Note:* Further segregation analysis must be carried out on these positives to allow the isolation of *pAD:cDNA-TP*. Subculture at least 4 colonies that grow on all 4 media (Fig. 3C, 1) into YPAD and repeat Protocol 5 until you obtain a yeast strain that displays only the two phenotypes shown in Fig. 3A. This  $\text{Leu}^+ \text{Trp}^- \text{His}^-$  strain will contain only *pAD:cDNA-TP*. Proceed to the next operation – reconstruction of two-hybrid positives. (D) Illegitimate activating false positive (*pAD:cDNA-IA*) also containing a second library plasmid which does not activate the reporter gene (*pAD:cDNA-Neg*): This situation results in four possible colony phenotypes. Retention of all three plasmids, *pBD:YFG*, *pAD:cDNA-IA* and *pAD:cDNA-Neg*, or only *pBD:YFG* and *pAD:cDNA-IA* will allow growth on all four media (Fig. 3D, 1). Retention of *pBD:YFG* and *pAD:cDNA-Neg* will allow growth on all except SC-His (Fig. 3D, 2). *pAD:cDNA-IA* alone will allow growth on SC-Leu and SC-His (Fig. 3D, 3). *pAD:cDNA-Neg* alone will allow growth only on SC-Leu (Fig. 3D, 4). *Note:* Discard all two-hybrid positives that fall into this class.

If no  $\text{Leu}^+ \text{Trp}^-$  colonies are obtained, it is not possible to differentiate between true and false positives. Should this happen, repeat Protocol 5, but carry out Step 1, growth in YPAD, twice and then select in SC-Leu. If you still obtain no  $\text{Leu}^+ \text{Trp}^-$  colonies, the *pAD:cDNA* plasmids can be isolated by the extraction of plasmid DNA from the yeast cells (see Protocol 6) and transformation or electroporation into a suitable strain of *E. coli*. The *pAD:cDNA* plasmid can be isolated from *E. coli* and transformed back into (1) a two-hybrid yeast strain lacking the *pBD:YFG* fusion plasmid to test for il-

legitimate activation of the *GALI-HIS3* reporter gene, (2) a two-hybrid yeast strain containing the *pBD:YFG* fusion plasmid to test for true activation of the *GALI-HIS3* reporter gene.

### Reconstruction of two-hybrid positives

The reconstruction of yeast two-hybrid system positives requires the isolation of the *pAD:cDNA* LEU2 plasmid responsible for the activation of the reporter gene(s). The protocol below [12] is employed to isolate nucleic acids from yeast that can then be used to transform an appropriate *E. coli* strain. We use electroporation [13] to transform plasmids into *E. coli*. This procedure yields between 500–1000 transformants on LB+ ampicillin plates [14]. The high efficiency transformation protocol of Hanahan [15] may also be used.

### Protocol 6. Isolation of yeast DNA

This protocol can be applied to recover the *pAD:cDNA* plasmid from (a) true positives identified by segregation analysis (Fig. 3A), and (b) putative positives in which plasmid segregation did not occur. (1) Inoculate the yeast strain into 2 ml of SC-Leu medium, and incubate overnight at 30°C with shaking. (2) Pellet the cells in a 1.5 ml microcentrifuge tube and remove the supernatant. Add 200  $\mu\text{l}$  of yeast DNA extraction buffer (see Appendix) and gently resuspend the cell pellet with a micropipette tip. Alternatively, colonies from plates can also be transferred directly into lysis buffer. (3) Add 400  $\mu\text{l}$  of glass beads (425–600  $\mu\text{m}$  Sigma G-8772) and 200  $\mu\text{l}$  of buffer saturated phenol:chloroform [13]. (4) Vortex vigorously for 2 min and centrifuge at maximum speed for 1 min. (5) Remove the aqueous phase to a fresh tube and precipitate the nucleic acids by adding 20  $\mu\text{l}$  of 3.0 M sodium acetate (pH 6.0) and 500  $\mu\text{l}$  of 95% ethanol. Collect the precipitate by centrifugation at maximum speed for 5 min and wash the nucleic acid pellet once with 500  $\mu\text{l}$  of 70% ethanol. Air dry the pellet for 5 min at room temperature. (6) Dissolve the pellet in 25  $\mu\text{l}$  of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). (7) The DNA can now be used to transform *E. coli*: (a) Transform 2  $\mu\text{l}$  of DNA isolated from a true positive into your favourite *E. coli* strain (e.g. DH5 $\alpha$ ); (b) Transform DNA isolated from putative positives that failed to segregate into an *E. coli* strain containing a *leuB* mutation (e.g. KC8). (8) Plate transformations on LB+ ampicillin (100  $\mu\text{g}/\text{ml}$ ) medium and incubate overnight at 37°C. (9) Proceed below according to the type of positive used in step 7: (a) The transformants from 7: (a) can be picked, grown in 2.0 ml of LB medium + 50  $\mu\text{g}/\text{ml}$  ampicillin and the plasmid DNA isolated by standard procedures [16]; (b) The Ap<sup>R</sup> transformants from 7 (b) should be replica-plated onto plates of M9 complete medium minus Leu [13] and incubated another 24 h at 37°C. Plasmid DNA can be isolated from Amp<sup>R</sup> Leu<sup>+</sup> transformants as above.

We usually analyze the plasmids from at least 4 transformants from each two-hybrid positive by restriction enzyme digestion and agarose gel electrophoresis. One representative of each identified plasmid type can then be transformed back into the yeast strain containing the *BD:YFG* fusion plasmid (Protocol 1) and plated onto both SC-Trp/Leu and SC-Trp/Leu/His. Growth on the latter medium requires the presence of both the *pBD:YFG* and *pAD:cDNA* plasmids plus the activation of the *GALI-HIS3* reporter gene. This will confirm that the *pAD:cDNA* plasmid codes for an interacting protein necessary for activation of the reporter gene. If you obtain transformants on SC-Trp/Leu, but not on SC-Trp/Leu/His medium the *pAD:cDNA* plasmid does not activate the reporter gene. If this occurs the yeast transformant from which the DNA was isolated may have contained more than one *pAD:cDNA* plasmid (Fig. 3 class C). Characterize several more Ap<sup>R</sup> Leu<sup>+</sup> *E. coli* transformants to screen for the presence of additional *pAD:cDNA* plasmids. If no other plasmids are identified, re-evaluate your segregation analysis data and repeat the segregation if necessary.

#### *Identification of Type II and III false positives*

There are two further types of false positives that you should now test for: (1) Type II false positives which activate the reporter gene in the presence of *any* BD fusion plasmid. They can be detected by transformation of your *pAD:cDNA* fusion plasmid into a yeast strain containing an unrelated BD fusion plasmid and plating on SC-Trp/Leu/His. Growth on this medium is indicative of a Type II false positive. We have used the BD fusion plasmid pDG862 which carries an insert derived from the yeast *RAD7* gene for this purpose. (2) Type III false positives which activate the reporter gene(s) in the presence of an 'empty' BD vector. We have only found this type of false positive when using the pAS vectors and conclude that it is probably the result of an interaction with the HA tag. Transformation of the *pAD:cDNA* fusion plasmid into a yeast strain containing an empty BD vector, followed by selection on SC-Trp/Leu/His will identify false positives of this kind.

#### *Analysis of true positives*

One of the hallmarks of a true two-hybrid positive is multiple representation among the clones isolated from a library screen. From the original 20 putative positives analyzed by segregation analysis you may have retained only a fraction, perhaps 10, that are true positives. Restriction enzyme analysis of each of these will allow you to classify them into groups. You may still have several hundred additional putative positives to analyze. To reduce the amount of work in-

involved it is a good idea to determine which of these show homology to the cDNA sequences in the plasmids that you have already characterized. This can be done by yeast colony hybridization [e.g. [17], pp. 265–267] using the cDNA inserts from the true positives as probes. The uncharacterized putative positives are subcultured onto selective medium, grow overnight, and lifted onto nylon membranes. The insert isolated from a *pAD:cDNA* fusion plasmid is labeled and used to probe the colonies on the filter(s) for complementary DNA sequences. Colonies that hybridize to a specific probe contain *pAD:cDNA* fusion plasmids that carry sequences representing the same or related genes. Further analysis of these plasmids, after rescue from yeast, will allow you to allocate the cloned sequences to a number of discrete classes.

#### *DNA sequence analysis of true positive AD:cDNA fusion clones*

Representative members of each class of true positive *pAD:cDNA* plasmids can now be sequenced to identify those containing large open reading frames (ORFs) fused to *GAL4<sub>AD</sub>*. Double-stranded plasmid DNA can be sequenced by standard methods using the *GAL4<sub>AD</sub>* sequencing primer 5'-TAC CAC TAC AAT GGA TG-3'. Long ORFs in-frame with the *GAL4<sub>AD</sub>* can be identified using your favourite DNA analysis software package. These sequences should be compared to those in the GenBank, EMBL, and other databases using the BLAST Notebook on the World Wide Web site <http://www.ncbi.nlm.nih.gov/BLAST/>, or the Blast E-mail server (send a single word 'Help' in the body of the message to [blast@nchi.nlm.nih.gov](mailto:blast@nchi.nlm.nih.gov) for instructions). This will identify those two-hybrid clones that correspond to known genes. If a two-hybrid clone does not show homology to any known gene, the putative amino acid sequence can be analyzed for homology to known protein motifs using either the Blast program or the Blitz program on the World Wide Web ([http://www.ebi.ac.uk/searches/blitz\\_input.html](http://www.ebi.ac.uk/searches/blitz_input.html)). This analysis should enable you to determine which of the two-hybrid system positives to pursue further.

#### *Deletion mapping of interacting domains*

The domains responsible for the interaction of the *BD:YFG* fusion protein with the *AD:cDNA* fusion protein can be mapped using the two-hybrid system. Strategically located restriction sites can be used to generate in-frame deletions of both genes. These deleted DNA fragments can be cloned into an appropriate two-hybrid vector and tested for interaction. It is best to begin your deletion analysis with a 5' fusion junction that is known to work and to delete from the 3' end of the gene fragment. Specific 5' deletions can be generated and

tested after identification of the 3' boundary of the interacting domain. However, different fusion junctions between either the *BD:YFG* or the *AD:cDNA* sequences can affect the level of reporter gene activity and one must interpret these results cautiously. Fine structure mapping of the interacting domains can be carried out using other techniques [18].

## Conclusion

The two-hybrid system is a powerful and sensitive technique for the identification of genes that code for proteins which interact in a biologically significant fashion with a protein of interest. The technique has been utilized by many different laboratories for the identification of countless new interacting protein partners. Unfortunately, the technique cannot be applied to all proteins; some require modification, such as glycosylation, and others are only active in specific cellular compartments other than the nucleus. Further developments of the two-hybrid system, such as the recently reported yeast tri-brid system [19], should allow the investigation of more complex protein:protein interactions.

## Appendix

### Media

- (i) Yeast Extract – Peptone – Dextrose Medium plus Adenine (YPAD)

|                      |                              |
|----------------------|------------------------------|
| yeast extract        | 10 g                         |
| peptone              | 10 g                         |
| glucose              | 20 g                         |
| adenine hemisulphate | 100 mg                       |
| distilled water      | 1000 ml                      |
| Bacto-agar (Difco)   | 15 g (added for agar medium) |

Sterilise by autoclaving at 121°C for 15 min.

The basic YPD medium is commercially available, with and without added agar, from BBL.

- (ii) Synthetic Complete Medium (SC)

|  |                                  |
|--|----------------------------------|
| Difco Yeast Nitrogen Base<br>(w/o amino acids) | 4.0 gm                           |
| glucose  | 12.0 gm                          |
| Synthetic Complete Selection<br>Medium Mix     | 0.4 gm                           |
| distilled water                                | 600.0 ml                         |
| Ditco Bacto Agar                               | 10.0 gm<br>(add for agar medium) |

Add the ingredients to the water and mix and adjust the pH to 5.6 with NaOH. Autoclave at 121°C for 15 min.

- (iii) Synthetic Complete Selection Medium Mix (SC medium)<sup>1</sup>

| <i>Supplement</i>          | <i>Amount</i> | <i>Final Concentration</i> |
|----------------------------|---------------|----------------------------|
| <b>Adenine hemisulfate</b> | <b>1.8 g</b>  | <b>30 mg/L</b>             |
| Arginine HCl               | 1.2 g         | 20 mg/L                    |
| Glutamic Acid              | 6.0 g         | 100 mg/L                   |
| <b>Histidine HCl</b>       | <b>1.2 g</b>  | <b>20 mg/L</b>             |
| Inositol                   | 2.0 g         | 33 mg/L                    |
| Isoleucine                 | 1.8 g         | 30 mg/L                    |
| <b>Leucine</b>             | <b>1.8 g</b>  | <b>30 mg/L</b>             |
| Lysine HCl                 | 1.8 g         | 30 mg/L                    |
| Methionine                 | 1.2 g         | 20 mg/L                    |
| p-aminobenzoic acid        | 0.2 g         | 3 mg/L                     |
| Phenylalanine              | 3.0 g         | 50 mg/L                    |
| Homoserine                 | 6.0 g         | 100 mg/L                   |
| <b>Tryptophan</b>          | <b>2.4 g</b>  | <b>40 mg/L</b>             |
| Tyrosine                   | 1.8 g         | 30 mg/L                    |
| Uracil                     | 1.2 g         | 20 mg/L                    |
| Valine                     | 9.0 g         | 150 mg/L                   |

<sup>1</sup>Omit the appropriate component(s), indicated in bold type, to prepare SC-Ade, SC-His, SC-Leu, SC-Trp, SC-Leu/Trp, and SC-His/Leu/Trp.

Combine the ingredients in a clean 250 ml plastic bottle. Add three or four clean glass marbles and shake vigorously to mix. This quantity will be sufficient for approximately 60 litres of SC medium.

### Solutions

- (i) Polyethylene glycol (PEG) 50% w/v.

Place a magnetic stirring bar in 35 ml of distilled de-ionized water in a 150 ml glass beaker on a magnetic stirrer and add 50 gm of polyethylene glycol, MW 3350 (Sigma P-3640). Stir until dissolved, this will take at least 30 min. Pour the solution into a 100 ml graduated cylinder, rinse out the beaker with water and add to the solution in the cylinder. Bring the volume to exactly 100 ml, cap the cylinder with Parafilm™ and mix thoroughly by inversion. Pour into a glass bottle and sterilize by autoclaving at 121°C for 15 min. It is important to store the PEG in a securely capped bottle to prevent evaporation as an increase in PEG concentration reduces transformation efficiency.

- (ii) Lithium acetate (LiAc) 1.0 M

Dissolve 10.2 g of lithium acetate in 90 ml of distilled de-ionized water and adjust the final volume to 100 ml. This solution will have a pH of about 8.9; it is not necessary to titrate this solution as previously described by Schiestl and Gietz (1989). Sterilize the solution by autoclaving at 121°C for 15 min. The 100 mM LiAc used to pre-treat cells can be made in 100 ml aliquots from the 1.0 M stock solution.

- (iii) Single-stranded carrier DNA (SS-DNA) 2 mg/ml  
Dissolve 200 mg of high molecular weight DNA (e.g. deoxyribonucleic acid sodium salt from salmon testes, Sigma D-1626) in 100 ml of sterile TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) by mixing vigorously on a magnetic stirrer overnight in a cold room. Dispense the carrier DNA solution into 10 ml or 1 ml aliquots and store at  $-20^{\circ}\text{C}$ . Before use the 1 ml carrier DNA aliquots should be boiled for 5 min and quick cooled in an ice water bath. SS-DNA can be boiled three or four times without reduction of transformation efficiency.
- (iv) Z Buffer (1.0 litre) pH 7.0
- |   |        |
|---|--------|
| $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ | 16.1 g |
| $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  | 5.5 g  |
| KCl   | 750 mg |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$           | 246 mg |
- (v) X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside), 20 mg/ml  
Dissolve 1.0 gm of X-Gal in 50 ml of N,N-dimethylformamide and store at  $-20^{\circ}\text{C}$ .
- (vi) Z buffer + X-Gal  
This should be made fresh by adding 270  $\mu\text{l}$  of 2-mercaptoethanol and 1.67 ml of X-Gal solution to 100 ml of Z buffer.
- (vii) ONPG (o-nitrophenyl- $\beta$ -D-galacto-pyranoside), 4 mg/ml  
Dissolve 200 mg of ONPG in sdd water. Store at  $-20^{\circ}\text{C}$  in aliquots.
- (viii) Yeast Lysis Buffer
- |                       |
|-----------------------|
| 10 mM Tris-Cl pH 8.0  |
| 100 mM NaCl           |
| 1 mM EDTA             |
| 2% (v/v) Triton X-100 |
| 1% (w/v) SDS          |

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