The Yeast Two-hybrid System: Criteria for Detecting Physiologically Significant Protein-Protein Interactions

Erica A. Golemis, Ilya Serebriiskii, and Susan F. Law

Division of Basic Research, Fox Chase Cancer Center, 7701 Burholme Ave
Philadelphia, PA 19111, USA

In vivo transcription-based assays for protein-protein interactions such as the two-hybrid system are powerful methods for identifying novel proteins based on their physical association with known proteins of biological interest, or for characterizing the degree and nature of interactions between sets of proteins. Because of the complexity inherent in assays taking place within a living organism, a key issue for the effective use of two-hybrid approaches is the ability to determine whether apparent interactions are likely to be physiologically relevant.

In this article, a number of the different two-hybrid systems currently available for use will be reviewed. Then, taking as a model one such system, the Interaction Trap, examples of different reagents for use in varying the affinity range of detectable interactions will be outlined. Also set forth are a number of protocols to establish an appropriate set of conditions for either screening a library or analysing the interaction phenotype between protein sets. Finally, a number of general guidelines are suggested for trouble-shooting two-hybrid results, and for eliminating falsely positive interactions.

Introduction

In the last five years, two-hybrid systems have emerged as a ubiquitous tool in evaluation of the interaction between defined proteins, and as a screening method to identify novel partners for proteins of biological interest. One reason for this ascendence has proven to be the surprising utility of two-hybrid approaches for analysis of many classes of cellular proteins. A second reason is the relative convenience and speed of two-hybrid system based screens compared to biochemical methods for identifying protein partners. In contrast to other methods, no protein purification steps or development of antibodies are involved, the required cloning manipulations are minimal, and the interacting partner is obtained as a partial or complete cDNA. As a result of these advantages, a number of different two-hybrid systems have been developed, which, although they are all based on a similar theoretical paradigm, utilize somewhat differing components and screening strategies. An ever-increasing number of libraries are being generated with cDNAs derived from many different species and tissues, providing great power and flexibility to these two-hybrid systems. The goal of this article is to provide a general introduction to key elements common to all such two-hybrid systems, and then to focus on one variant, the Interaction Trap, discussing in some detail the steps one would take to establish optimal conditions to use this system with a protein of interest.

Paradigm

Experiments performed by a number of groups through the 1980s led to the realization that many proteins were comprised of multiple sub-domains required for specific functions such as DNA binding, dimerization, transcriptional activation, and other properties. Based on this realization, in 1985, Brent and Ptashne demonstrated that it was possible to take individual domains with particular functions, such as the DNA-binding domain (DBD) from protein 1, and the transcriptional activation domain function from protein 2, and splice the two together. The novel chimaeric protein thus exhibits a DNA-binding specificity similar to protein 1, and a protein 2-derived potential for activating transcription (1)(Figure 1A). In related work, studies on mechanisms of transcriptional control led to the finding that in some cases, proteins such as GAL80 which functioned as transcriptional regulators did not directly contact DNA or possess a covalently linked DNA-binding-domain. Instead, these regulatory proteins were brought to promoter sites via interaction with non-activating partner proteins such as GAL4 that possessed a DNA-binding-domain (2, 3). Exploiting these findings in 1988, Ma and Ptashne generated a synthetic transcriptional activator, which fused a heterologous activation domain to GAL80, allowing both interaction with GAL4 and activation of a GAL4-dependent transcriptional reporter (Figure 1B)(4).

Merging elements of these findings in 1989, Fields and Song described a scheme that established the functional paradigm for the two-hybrid system (5). In this scheme (Figure 1C), a first protein of interest, "X", is synthesized as a fusion to a DNA-binding domain [DBD-X], while a second protein, "Y", is synthesized as a fusion to an activation domain [AD-Y]. DBD-X and AD-Y are coexpressed in a host organism such as yeast which contains reporter genes under the transcriptional control of consensus binding sites for the DBD. If X and Y physically interact, this interaction brings the activation domain to the promoter of the reporter gene, activating its transcription. Although some other variables apply, in general, the strength of the activation provides an indirect measure of the strength of the interaction. This strategy has been widely applied to assess interactions between predefined X and Y protein sets, and has become a standard and powerful mechanism for identification of novel protein partners which interact with X proteins of interest via the use of activation domain-fused cDNA libraries as the AD-Y element.

Variants

Since the original proposal of a two-hybrid strategy to analyse protein-protein interactions, a number of groups have developed reagent sets suitable for performing such studies (Table 1). The organism of choice as a host for two-hybrid applications has been *Saccharomyces cerevisiae* (6, 7, 8, 9). Although some systems have been developed on similar principles for use in mammalian cells with targeted protein pairs (10, 11), these have not attained broad use for such purposes as library screening, and will not be discussed here.

In the yeast systems, the *DNA binding domain* (DBD) function has standardly been supplied by the bacterial repressor protein LexA (12, 8, 9), or by the yeast protein GAL4 (13, 6, 7), although some other variants have been tried (14, 15). *Activation domains* (ADs) utilized have included those of GAL4 (6); an "acid blob", B42 (16, 8); and the viral protein VP16 (17, 9). To improve the accuracy and ease of library screening, two hybrid systems utilize *two independent DBD-dependent reporter constructs* in parallel. As primary reporters, two-hybrid systems utilize genes required for specific amino acid synthetic pathways, rendering growth on particular selective medium dependent on activation of the gene, thereby providing a very simple initial screening mechanism for positive interactors. The two genes of choice for this purpose have been *LEU2* required for growth on medium lacking leucine (8), and *HIS3,* required for growth on medium lacking histidine (6, 7, 9). All current systems include *lacZ* as a secondary reporter (18), allowing rapid blue-white
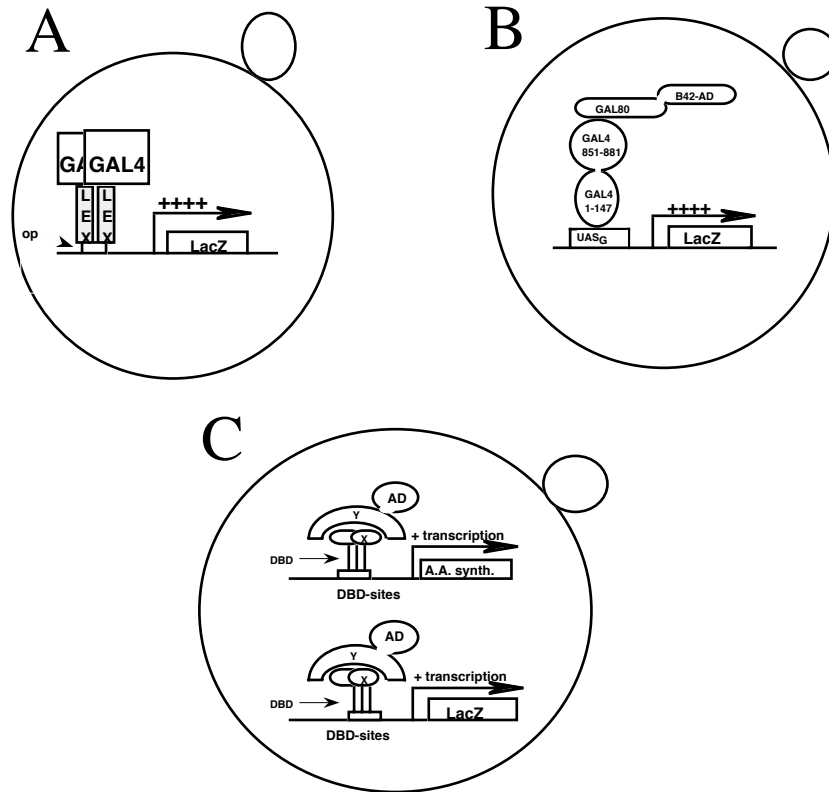
Figure 1. Development of the two-hybrid approach. All events are portrayed as taking place inside the host organism *S. cerevisiae*, budding yeast. A) Construction of a synthetic transcriptional activator with a defined DNA-binding specificity by fusion of the DNA-binding-domain from LexA with the activation domain from GAL4 (1). B) Construction of a synthetic transcriptional activator by fusion of the activation domain B42 (16) to the transcriptional repressor GAL80; this chimera interacts with the C terminal 80 amino acids of GAL4 (851-881) and activates transcription through a GAL4 binding site (UAS$_G$) (4). C) Paradigm for two-hybrid system, in which interaction between activation-domain-fused protein Y and DNA-binding-domain-fused protein X causes activation of a reporter gene under transcriptional control of binding sites for the DNA-binding-domain (5).

confirmation of positives on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as well as approximate quantitation of interaction affinity using a β-galactosidase assay. Detailed protocols have been published to provide step-by-step instructions for performance of two-hybrid library screens, for both GAL4 (19) and and LexA (20) based screening systems.

Interactions Detected by Two-Hybrid Systems

While no extensive database of biologically meaningful interactors isolated by two-hybrid screens as yet exists, a survey of some of the published literature is instructive as to what classes of protein-protein interactions have been successfully studied by two-hybrid screening mechanisms. A number of these studies are summarized in Table 2, where the interacting proteins are grouped based on the nature of the DBD-fused bait protein used. Certain protein classes have been used extensively in two-hybrid screens and have yielded information about interactors that appears to be

Table 1. List of two-hybrid system variants. A number of groups have developed different sets of reagents based on the two-hybrid paradigm. Literature references are described in the text.

Lab. of Origin	System	Host	DNA Binding Domain	Activation Domain	Selection
S.Fields	Two-Hybrid *Matchmaker*	Yeast	GAL4	GAL4	Activation of *lacZ, HIS3*
R.Brent	Interaction Trap	Yeast	LexA	"Acid Blob" B42	Activation of *LEU2, lacZ*
S.Elledge	"Improved Two-Hybrid"	Yeast	GAL4	GAL4	Activation of *HIS3, lacZ*
S.Hollenberg	"Modified Two-Hybrid"	Yeast	LexA	VP16	Activation of *HIS3, lacZ*
E.Fearson/ C.Dang	KISS	Mammalian Cells	GAL4	VP16	Activation of *CAT, hyg*R
S.Weissman	Contingent Replication	Mammalian Cells	GAL4	VP16	Activation of T-Ag, Replication of plasmids

physiologically significant. Many cell cycle regulatory proteins have been utilized, including cyclin dependent kinases, cyclins, and negative regulatory factors. Small GTPases and other cytoplasmic or loosely membrane associated signaling molecules have also been used to identify their interacting regulatory molecules. Aditionally, serine threonine-targeted protein kinases, and in some cases tyrosine-targeted protein kinases have been used (although in some cases the latter are known to be partially toxic to yeast). In other studies, DNA repair molecules such as *RAD* genes, splicing factors, and a large number of genes associated with transcriptional activation, transcriptional silencing, and transcriptional repression have been productively employed to identify relevant partner proteins. Finally, a diverse group of other proteins have been examined using two-hybrid technology, including the tumor suppressor Rb, DNA recombination proteins, viral proteins, apoptotic regulators and others.

What affinity range of interactions are detected by two-hybrid approaches? In recent work by Estojak *et al*., protein sets for which the affinity of association had previously been biochemically determined were fused in all possible combinations to LexA or to an activation domain. These were used with a series of *LexA*-operator-*LacZ* or *LexA*-operator-*LEU2* reporters to establish the degree to which apparent affinity of protein-protein interaction determined by the two-hybrid approach paralleled association affinity determined *in vitro* (21). Results of this study suggested that interactions thought to occur with an affinity of less than 1-5 µM (approx.), which are believed to be non-physiological, were poorly or not at all detected in the Interaction Trap using any of the *LacZ* or *LEU2* reporters. In contrast, interactions that occur with affinities of lower than 20 nM, such as the heterodimerization between the oncoprotein Myc and its partner Max, or the homodimerization between lambda cI repressor molecules, were detected strongly. Additionally, protein affinities ranging from 1 nM to 1 µM could generally be discriminated, with rank order similar to that determined *in vitro*. However, in quantitation of affinity, increase in ß-galactosidase activity (reflecting activation of the *lacZ* gene) was not precisely colinear with fold increase in affinity, suggesting that caution should be used in extrapolating exact affinities from two-hybrid phenotype. Finally, different reporter constructs apparently had different thresholds of affinity for detecting interactions. Those with transcription dependent on fewer *LexA*-operators (such as EGY191 yeast, or pRB1840) failed to identify as positive some of the lowest affinity interactions. However, these less sensitive reporters had the advantage in some cases of suppressing "background" transcriptional activation by the DBD-fused component, making it sometimes possible to discriminate interactions that would be obscured using more sensitive reporters. Cumulatively, these results together with the record of known two-hybrid

applications (Table 2) indicate that two-hybrid approaches can be used flexibly with many classes of biologically interesting proteins.

Issues in Planning Two-Hybrid Screens

Although many proteins have been studied successfully with two-hybrid technology, not all proteins are appropriate for use in a two-hybrid system. Because of the significant effort required in performing and analyzing the results of a two-hybrid screen, before initiating such a screen with a protein of interest (termed a "bait"), it is worth carefully considering some properties of the bait that may specifically indicate that a given protein is *not* likely to be used productively. The following describes problems that have occurred with *some* proteins used in two-hybrid systems, and represent the types of issues an investigator should consider before launching such a screen.

As currently construed, two-hybrid systems depend on selective transcriptional activation of a reporter mediated by interaction of a bait with a library encoded, AD-fused partner protein. For this approach to work, the potential bait must be capable of localizing to the cell nucleus. It must not possess intrinsic ability to strongly activate transcription of the reporter constructs. It must also be able to bind to DBD consensus sites upstream of the reporter constructs. For these reasons, proteins known to be integral membrane components or otherwise known to localize to plasma or organellar membranes may cause difficulties if fused to DBD domains in their native states, as a large proportion of these proteins may be sequestered from the binding sites. Transmembrane and membrane targetting sequences should generally be truncated.

Proteins that are strong transcriptional activators are also unlikely to be successfully employed, because reporter transcripts will be abundantly produced in the absence of a library component. It should be noted that some proteins that normally do not function as transcriptional activators (for example, some cytoplasmic protein kinases) will, when fused to a DBD and expressed in yeast, in fact activate transcription quite strongly. Hence, any protein which is to be used as a bait should first be tested for spurious activation of two-hybrid reporters.

If a potential bait possesses its own DBD (i.e. is normally a DNA-binding protein), it is possible that this DBD will contribute to sequestration of the bait to its preferred consensus sites, occurring at random in yeast DNA, thereby diminishing the quantity of DBD-bait capable of binding upstream of the reporter gene constructs. In some cases, removal of the native DNA-binding domain from the bait results in improved behavior in a two-hybrid system (see reference 22).

If the intended bait is a higher-eukaryotic protein which is normally post-translationally processed, and this processing is thought to be important for interaction with partner proteins, a major concern would be that the appropriate modification may not occur in yeast. Examples of such modification might include glycosylation, or phosphorylation by a specific serine/threonine or tyrosine kinase. "Tri-hybrid" systems, in which an additional kinase is co-expressed with the bait to provide the appropriate modification have been developed to help circumvent some of these problems (23). While some claims of successful two-hybrid use have been made for proteins normally expressed extracellularly (24), it is not clear how common such success is likely to be.

While most proteins can be stably expressed as fusion proteins to a LexA or GAL4 DBD in yeast, some proteins are either poorly expressed, or are clipped by yeast proteases so that the predominant population of DBD-fusion in a cell is significantly less than full length. These problems are more common for large fusion domains (i.e. > 50 kD). Prior to commencing a screen, it is thus generally advisable to use antibody to the DBD in a western blotting analysis of yeast lysates containing fusion proteins to ensure that detectable quantities of an appropriately sized bait are produced.

Table 2. List of protein-protein interactions identified or analyzed by two-hybrid techniques.

Bait Type	DBD Fused Protein	AD Fused Protein	Prey Type	Lib./ set
a	Grb2	sos	gef	s
ag	CREB/ATF	Ig var.domain	ab	s
apopt	Bcl-2	R-Ras	g	l
ca	Calmodulin	Spc110	o	s, l
ca	Calmodulin	Spc110p	o	l
cdc	Cdc23p	Cdc27p	cdc	s
cdk	Cdk2	Rbr-2	Rb	l
cdk	CDK4	p16ink4	o	l
cdk	Cdk6	p16	o	l
cdk	pho2	pho4	tr	s
cdk	HsCdc2, HsCdk2	Cdi2	o	s
cdk	DmCdc2, ScCdc28	Cdi2	o	s
cdk	DmCdc2c, HsCdk3	Cdi2, 4	o	s
cdk	DmCdc2c, ScCdc28	Cdi3, 5	cyclin	s
cdk	DmCdc2	Cdi3, 7	cyclin	s
cdk	HsCdk3, 4	Cdi3	cyclin	s
cyclin	cyclinD1	p27	o	s
cyclin	Cdi5	Cdi2	o	s
cyp	cycA	beta-tubulin	o	l
cyp	cycB	CAML	ca	l
dbp	CENP-B	CENP-B	dbp	s
dbp	Max	Myc	o	s
dbp	RBP3	prot H	dbp	l
g	R-ras	RalGDS	gds	l
g	Rab3A	Rabin	o	l
g	Ras	RalGDS	gd	l
g	ras	cdc25	gef	s
g	Ras	Raf	pk	s
g	ras p21	RGL	gds	l
g	ras1, ras2	byr2	pk	l,s
g	ras1	scd1	o	l,s
g	Ras2	CDC25	cdc	s
g	H-,K-ras, Rap1	RalGDS	gds	s
g	cdc42sp	scd1, scd2	o	s
g-p	Ste4	Ste18	g-p	s
pk	byr2	H-ras	g	s
pk	byr1	spk1	o	s
pk	Bruton's ptk	Fyn	pk	l
pk	cdc7	DBF4	cyclin	s
pk	Fus	Ste5	st	s
pk	MO15	cyclinH	cyclin	l
pk	p52shc	PTP-PEST	pp	s
pk	p110 of PI 3-kinase	p85 of PI 3-kinase	pk	s
pk	PKC	PICK1	o	l
pk	Raf	Ras	g	s
pk	Raf	Rap1	g	s
pk	Raf	MEK	pk	s
pk	MEK	Raf	pk	s
pk	Raf-1	HS1	14-3-3	l
pk	Raf-1	PLA2	14-3-3	l
pk	Raf-1	14-3-3 beta isoform	14-3-3	l
pk	SNF1	MSN3 (STD1)	o	l
pk	SNF1	SIP1, SIP2, SIP3, SIP4	o	l
pk	c-abl	Crk	a	l
pk	Fyn	p62	a	l
pk	ANP	novel	pk	l
pp	Cap1 delta	Cap1 AID	pp	s
pp	Cap1 delta	Cmd1	Ca	s
Rb	p110RB	PP-1 alpha 2	pp	l
Rb	p110RB	T-antigen SV-40	pp	l
Rb	p110RB	p110RB	Rb	s
Rb	p110RB	p84	o	l
Rb	p110RB	CycD1	cyclin	s
Rb	p110RB	BRG1	tr	l, s
rec	RAG1	SRP1	o	l
recep	ins. rec	IRS-1	o	s
recep	Insulin rec.	LIM cont. prot	o	l
recep	mPPAR	hRXR	recep	s
recep	TGF-beta R-I	TGF-beta R-II	recep	l
recep	TNFR2	TRAF1, TRAF2	o	s
recep	TNFR-1	TNFR-1	recep	s, l
recep	TNFR-1IC	TRAP-1	hsp	l
rep	Rad51	Rad51	rep	s
rep	Rad51	Rad52	rep	s
rep	Rad7	SIR3	dbp	s
spl	HRH1	SR protein family	dbp	s
spl	SC35	SC35, U2AF35, U1-70K	spl	l,s
spl	SC35	SF2/ASF, Tra, Tra-2	spl	s
spl	SF2/ASF	SC35, SF2/ASF	spl	s
spl	SF2/ASF	U2AF35, U1-70K	spl	s
spl	SPP91	PRP11	spl	s

Table 2 continued

Bait Type	DBD Fused Protein	AD Fused Protein	Prey Type	Lib./ set
spl	U2AF35	SC35, SF2/ASF, Tra	spl	s
spl	U2AF35	U2AF35, U2AF65, Tra-2	spl	s
st	Vav	hnRNP K	o	l
st	STE11	STE5, STE7, FUS3	st	s
st	STE5	STE7, STE11, FUS3	st	s
st	STE7	STE5, STE7, STE11, FUS3	st	s
st	FUS3	STE7	st	s
t.ad	GCN5	ADA2	t.ad	s
tr	B (maize)	C1	tr	s
tr	Dr1	TBP	tr.f	s
tr	Not1	Not2, Not4	tr	s
tr	Not3	Not2, Not4	tr	s
tr	pho80	Pho4	tr	s
tr	SIR4	SIR4	tr	s
tr	RAP1	RIF	o	l
tr.f	Fos	Jun	tr.f	s
tr.f	RBTN2	TAL1	tr.f	s
tr.f	TAL1, TAL2	RBTN1, RBTN2	tr.f	s
tr.f	LYL1	RBTN1, RBTN2	tr.f	s
tr.f	E47	RBTN1, TAL1	tr.f	s
tr.f	TAL1	E47	tr.f	s
tr.f	E12	Id-2	tr.f	s
tr.f	E12	MyoD	tr.f	s
tr.f	MyoD	E12, E47	tr.f	s
tr.f	hTAF	hTBP	tr.f	s
tr.f	TFB1	SSL1	rep	l
tr.f	HSF2	HSF2	tr.f	s
transl.a	PET54,	PET122, 494	transl.a	s
tta	protX	XAP-1	rep	l
viral	Pr55gag	Pr55gag	viral (gag)	s
viral	M-MuLV	M-MuLV	viral (gag)	s
viral	EBNA-2	EBNA-2	viral	s
viral	C (hepadnavirus)	C (hepadnavirus)	viral	s
viral	E4 ORF6/7	E2F-1	tr.f	s
viral	Nef	beta-COP	o	l
o	SNF4	SNF1	pk	s
o	cdi11	Cdi3	cyclin	s
o	scd2	scd1	o	s
o	myc	myc	o	s
o	FKBP12-rapamycin	RAPT1	o	l
o	FKBP12-rapamycin	TOR2	o	s
o	period	period	o	s
o	TRAF1	TRAF1, TRAF2	o	s
o	TRAF2	TRAF1, TRAF2	o	s
o	BCY1	BCY1	o	s
o	p53	p53	o	l

 Interacting pairs listed are taken from published articles (references available upon request), and were obtained using either GAL4 or LexA-based yeast two-hybrid systems. Abbreviations for classifications of interacting proteins are, *a*, adaptor protein; *ab*, antibody; *ag*, antigen; *apopt*, apoptosis-related protein; *ca*, calcium regulated protein; *cdc*, cell division control; *cdk*, cyclin dependent kinase; *cyp*, cyclophilin protein; *dbp*, DNA binding protein; *g*, GTPase; *g-p*, G-protein; *gds*, GDP-dissociation stimulator; *gef*, GTP-GDP exchange factor; *o*, other; *pk*, protein kinase; *pp*, protein phosphatase; *rec*, recombination protein; *recep*, receptor protein; *rep*, DNA repair factor; *spl*, splicing factor; *st*, signal transduction related; *t. ad.*, transcription adaptor; *tr*, transcriptional regulator; *tr. f.*, transcription factor; *transl.a.*, translational activator; *tta*, transcription transactivator. Abbreviations for the methods used to detect the interaction are *s*, predefined Set of proteins; *l*, library screen.

A small number of higher eukaryotic proteins appear to be toxic to greater or lesser degrees when expressed in yeast. Such toxic properties can generally be detected while performing initial transformations to characterize DBD-baits, since plasmids expressing such proteins will yield significantly fewer colonies than control vector. Alternatively, yeast expressing the toxic DBD-fusion will generally grow at a much slower rate and transform more poorly than yeast expressing non-toxic proteins. While such toxicity can sometimes be exploited to useful purpose (25), it frequently results in screening difficulties, and steps should thus be taken to minimize toxic effects, as outlined below.

One possible variable of unknown importance is the interaction of heterologously expressed DBD-fused proteins with endogenous yeast proteins. Because of evolutionary conservation, an increasingly large number of mammalian proteins
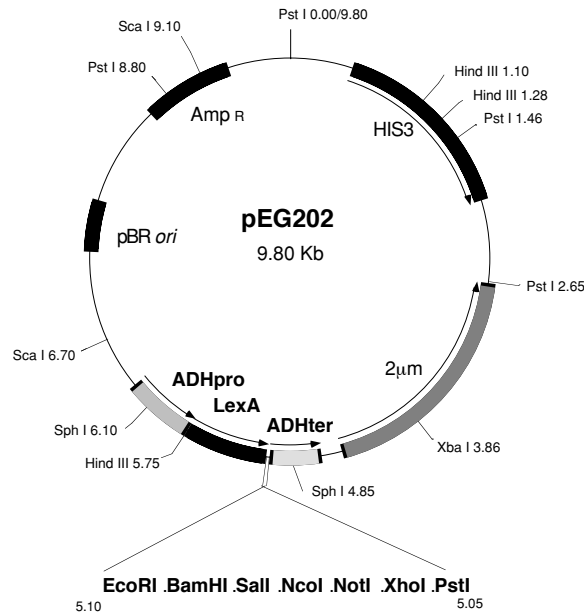
Figure 2. LexA-fusion vector. pEG202, a derivative of Lex202 + PL (16), uses the strong alcohol dehydrogenase promoter (*ADH*pro) to express bait proteins as fusions to the DNA-binding protein LexA. A number of restriction sites immediately upstream of ADHter are available for insertion of coding sequences. The plasmid contains the *HIS3* selectable marker and the 2 μm origin of replication to allow propagation in yeast, and the ampicillin resistance gene (amp[R]) and the pBR origin (*ori*) of replication to allow propagation in *Escherichia coli*. Numbers indicate relative map positions.

have been shown to be capable of complementing mutations in their yeast homolog, and, by inference, to be capable of interacting with yeast partner proteins. Hence, a formal possibility is that some apparent protein-protein interactions may in fact reflect ternary or higher order complexes involving yeast components. Similarly, the detection of some interactions between mammalian proteins may be inhibited or blocked because one component of the complex is sequestered by interaction with an abundant yeast protein.

Finally, it is often desirable or necessary to attempt two-hybrid screens with truncated forms of proteins. Wherever possible, these truncations should be guided by preexisting knowledge of protein domain structure, since inappropriate truncations may lead to synthesis of partially or largely unfolded proteins that isolate false positive proteins such as Hsp70 from library screens.

Establishing Two-Hybrid Assay Conditions for a Protein of Interest Using the Interaction Trap

While proteins that strongly activate transcription are generally not appropriate for two hybrid screening, many proteins which are weak or moderate activators of transcription can be productively utilized, given some initial adjustment of screening parameters. This is most commonly accomplished by varying the sensitivity of the reporter genes utilized, although in some cases it can be addressed by altering the expression of the bait and library proteins. The exact sequence of events to establish screening conditions will vary from system to system. As an example of the general approach, we will here use the Interaction Trap as a model to demonstrate the steps
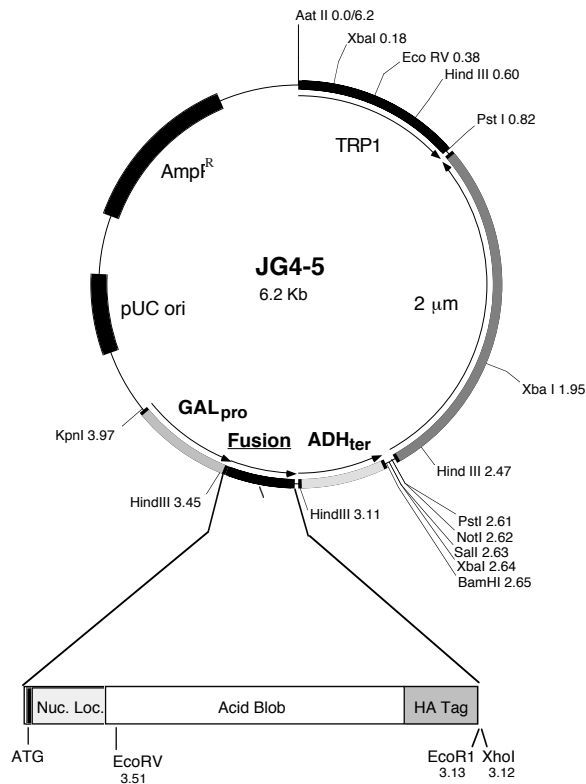
Figure 3. Activation domain-fusion vector. pJG4-5 (8) expresses cDNAs or other coding sequences inserted into the unique *Eco*R I and *Xho* I sites as a translational fusion to a cassette consisting of the SV40 nuclear localization (nuc. loc.) sequence (PPKKKRKVA), the acid blob B42, and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of sequences is under the control of the *GAL1* inducible promoter. The plasmid contains the TRP1 selectable marker and the 2 μm origin to allow propagation in yeast, and the ampicillin resistance (amp^R) gene and the pUC origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions.

one would use to establish screening conditions for a novel DBD-bait protein. Detailed descriptions of how one would perform such a screen are presented in other forums, most notably Current Protocols in Molecular Biology (see reference 20).

Reagents

The Interaction Trap two-hybrid system was initially developed using a single core set of reagents. The plasmid pEG202 uses the strong alcohol dehydrogenase promoter to express baits as fusions to the DBD function of LexA (Figure 2). The plasmid pJG4-5 uses the galactose-inducible *GAL1* promoter to express predefined interacting proteins or cDNA libraries as fusions to a cassette including a nuclear localization sequence, an activation domain, and a hemagglutinin epitope tag (Figure 3). The plasmid pSH18-34 contains 8 operator sites for LexA binding upstream of the *lacZ* reporter gene (Figure 4). Finally, in the yeast strain EGY48, homologous recombination has been used to replace upstream activating sequences for the *LEU2* gene with 6 operators for *LexA*, rendering growth on medium without leucine
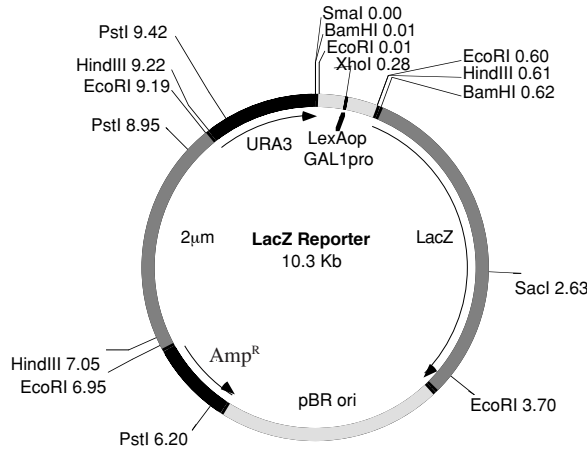
Figure 4. LacZ-Reporter. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1del1 (28) containing 8, 2, or 1 operator for LexA binding inserted into a unique *Xho*I site located in the minimal *GAL1* promoter (0.28 on map). The plasmid contains the URA3 selectable marker and the 2 µm origin to allow propagation in yeast, and the ampicillin resistance (amp[R]) gene and the pBR322 origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions.

dependent on activation through a LexA binding site. Screening for positive interactions is done by transforming a pJG4-5-based library into EGY48 yeast containing a pEG202 fusion and pSH18-34, and assaying for simultaneous activation of *Leu2* and *lacZ* reporters in a galactose-dependent manner.

As large numbers of researchers began to utilize this two-hybrid system, individuals modified the original vector and strain components to increase the general flexibility of the Interaction Trap and render it usable with a greater range of proteins of interest. A number of these additional reagents are listed in Table 3. Reporter sensitivity levels were varied by the simple device of altering the number of *LexA* operators upstream of either the *LEU2* (EGY191, EGY195) or the *lacZ* (pJK103, pRB1840) reporter genes. As detailed above, these reporters have been roughly "calibrated" by the systematic comparison of transcriptional activation by interacting proteins with known affinities of association (21), and have been shown to primarily detect interactions occurring at K_d lower than 10^{-6}M. In contrast, some of the additional reagents allow modulation of the expression of the DBD-fusion. For example, pGilda allows inducible expression of LexA-fused baits, making it possible to only transiently express proteins whose continuous overexpression is toxic to yeast. pEE202I is an integrating form of the pEG202 plasmid; use of an integrating rather than a replicating form of this vector results in much lower levels of the DBD-fused protein being expressed, which can be useful for studies of proteins that are normally present at extremely low levels in cells. pJK202 adds nuclear localization sequences to the DBD-fusion, increasing the nuclear concentration of proteins expressed at very low levels or proteins from which it has been impossible to remove all membrane-targeting sequences. These and other vectors possess similar polylinker cloning sites (primarily based on *Eco*R I-*Xho* I directional cloning cassettes), facilitating rapid recloning of inserts between different vectors.

Table 3. List of Interaction Trap strains and plasmids.

LexA Fusion Plasmids

pEG202 [1*]   *ADH* promoter expresses *LexA* followed by polylinker: basic plasmid used for cloning bait.

pJK202 [2*]   Like pEG202, but incorporates nuclear localization sequences between L*ex*A and polylinker: used to enhance translocation of bait to nucleus.

pNLexA [3]   *ADH* promoter expresses polylinker followed by *LexA* : for use with baits where amino-terminal residues must remain unblocked.

pGilda [4]   *GAL1* promoter expresses same *LexA* and polylinker cassette as pEG202: for use with baits whose continuous presence is toxic to yeast.

pEE202I [5]   An integrating form of pEG202 that can be targeted into *HIS3* following digestion with *Kpn* I: for use where physiological screen requires lower levels of bait to be expressed.

Activation Domain Fusion Plasmids

pJG4-5 [6*]   *GAL1* promoter expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites: used to express cDNA libraries.

pJG4-5I [5]   An integrating form of pJG4-5 that can be targeted into *TRP1* by digestion with *Bsu* 36I (NEB), to be used with pEE202I to study interactions that occur physiologically at low protein concentrations.

LacZ Reporter Plasmids

pSH18-34 [7*]   8 *LexA* operators direct transcription of the *lacZ* gene: most sensitive reporter plasmid for transcriptional activation.

pJK103 [2*]   2 *LexA* operators direct transcription of the *lacZ* gene: intermediate reporter for transcriptional activation.

pRB1840 [8*]   1 *LexA* operator directs transcription of the *lacZ* gene: stringent reporter for transcriptional activation.

pJK101 [2*]   *GAL1* upstream activating sequences followed by 2 *LexA* operators followed by *lacZ* gene: used in repression assay to assess bait binding to operator sequences.

Positive and Negative Controls

pRFHM1 [9*]   *ADH* promoter expresses *LexA* fused to the homeodomain of bicoid to produce non-activating fusion. Use as positive control for repression assay, negative control for activation and interaction assays.

pSH17-4 [7*]   *ADH* promoter expresses *LexA* fused to *GAL4* activation domain. Use as a positive control for transcriptional activation.

LEU2 Selection Strains

EGY48 [1*]   Basic strain used to select for interacting clones from a cDNA library: 6 *LexA* operators direct transcription from the *LEU2* gene.

EGY191 [1]   Like EGY48, but with 2 *LexA* operators rather than 6: a more stringent selection, and producing lower background with baits with intrinsic ability to activate transcription.

Protocol

Control Experiments for DBD Fusions

1. To begin to establish conditions for a two-hybrid screen using the interaction trap, clone the DNA encoding the protein of interest into the vector pEG202. In designing this construction, eliminate any sequences encoding a membrane spanning domain or encoding known transcriptional activation motifs.

2. Co-transform the pEG202-bait construct and the pSH18-34 reporter plasmid into EGY48 yeast. In parallel, transform positive and negative control LexA-fusion proteins with pSH18-34 (positive and negative controls for transcriptional activation vary: see reference 20).

3. When colonies have appeared, for each transformation, re-streak approximately 10 colonies onto (i) medium containing X-Gal, (ii) onto growth medium lacking leucine, and (iii) onto growth medium containing leucine; glucose should be used as a carbon source in all media. Incubate the plates for 3 days, inspecting on a daily basis.

4. The positive control (for example, LexA-GAL4) should be medium-dark blue on plates containing X-Gal within 1-2 days, and have growth on leucine-minus plates approaching that on leucine-plus plates within 3 days. The negative control should be very pale blue or white on X-Gal plates, and have demonstrated no growth on leucine-minus plates at 3 days. If the positive and negative controls do not behave in this way, something may be wrong with the medium.

The DBD-fusion to the protein of interest being tested may behave in a number of different ways on these media. This behavior will predict the steps to use in preparing it for a screen or other two hybrid study. First, it may demonstrate a *lacZ* and *LEU2* profile similar to the negative control, activating neither reporter. For proteins not known in advance to be transcriptional activators, this is the most likely possibility. If so, the only steps recommended prior to commencing a screen are (i) to perform western blotting analysis of exponentially growing yeast lysates from at least 3 independent colonies to confirm that the protein is being made and that it is the correct size and (ii) to perform a repression assay (26, 20) to confirm that the DBD-fusion is effectively binding operator sequences. These tests should take approximately 1 week. If the repression assay does not work, the investigator may wish to reclone the bait protein into pJK202, which contains a nuclear localization signal.

A second possible outcome is that the DBD-protein may activate the reporters very strongly, comparably to the positive control. For proteins not known in advance to be transcriptional activators, this seems to occur approximately 5-10% of the time. For such a protein to be usable in the Interaction Trap or any comparable two-hybrid system, it must be truncated to remove the transactivating sequences. Following the truncation, it may be usable in this or another reporter strain/plasmid combination.

A third possibility is that the DBD-protein may activate the reporters moderately - i.e. colonies showing minor growth on leucine-minus medium by the third day after streaking, or becoming light blue on X-Gal medium. These proteins may be usable in a screen with an EGY48/JK103 reporter combination; or they may yield a high background of "false positive" colonies following library transformation. One option is to restart the tests outlined above using a less sensitive set of *LEU2*/*lacZ* reporters, such as EGY191 with pJK103 or pRB1840. Alternatively, the protein can be truncated.

Finally, the DBD-protein may grow noticeably less well than the positive or negative control on *leucine-plus* medium. This outcome may indicate that the DBD-protein is somewhat toxic to the yeast. As this can cause difficulties in performing library screening, it may be desirable to reclone DNA encoding the protein of interest

into pGilda, which allows inducible expression of the protein from the *GAL1* promoter. Note, however, that tests with a pGilda construct should be performed on media containing galactose as a carbon source.

Two-Hybrid Screens

Depending on the research goals, once a bait with a suitably low transcriptional activation background has been shown to synthesize protein, the next step is either to proceed to screen a library, or to test targeted interactions with other proteins. Library screening usually takes approximately 2 to 3 weeks from start to confirmation of positive clones as specific for a given DBD-protein of interest; these procedures are detailed elsewhere (20). The study of targeted interactions can be accomplished either by individual co-transformation of test pairs, or by an interaction mating approach (27). In this latter approach, panels of LexA-fused proteins are maintained in MATa strains, while additional panels of activation-domain fused proteins are maintained in MATα strains: cross-plating of the two panels rapidly mates all LexA-fused versus all AD-fused combinations, allowing rapid assessment of a large number of interactions. Because of the pseudo-genetic robustness of the two-hybrid approach, simple screening for interaction generally takes a very short period of time. Based on the identity of the interactor isolated, subsequent characterization of the biological significance of the interactor can then take anywhere from weeks to years of effort.

Notes and Tips

What allows one to gauge whether an interactor is likely to be a real or a spurious partner of the protein of interest? While the final answer will generally depend on extensive functional tests in vivo, there are some general indications.

1. Depending on what is used as DBD-fusion, library screens may yield many, few, or no putative positives. In the cases where many apparent interactors are obtained and where transcriptional background on the part of the DBD-fusion itself has been excluded, it is informative to perform diagnostic restriction digests on a reasonably large number of positives (for example 30-60). If within this number, it is apparent that certain cDNAs are being repeatedly isolated, this suggests that the screen is proceeding with reasonable specificity. Under these conditions, both the frequently and rarely occurring cDNAs may be physiological (with the latter possibly representing interactors occurring at low frequency in the library). Alternatively, analysis of 30-60 positives may suggest that many different cDNAs are being isolated, with no individual cDNA being obtained more than once or twice. If so, this argues that the DBD-fused protein is possibly interacting non-specifically with false positives, and the presumed interactors may be artifactual.

2. Whatever the pattern of cDNA recurrence obtained, plasmids containing putative positives should invariably be retransformed with the original DBD-fusion versus a reasonable number (3-10) of non-related DBD-fusions, to confirm specificity of the interaction with the individual bait.

3. Some proteins appear to be common false positives, being isolated with a large number of proteins. These include HSP70, ribosomal subunits, components of the cellular oxidative phosphorylation pathway, and in some cases, novel proteins of unknown function that sequence analysis suggests might be isolated because of particular sequence traits (such as large hydrophobic patches or long stretches of potential coiled-coil). Formal lists of false positives are only now beginning to be compiled; one source of information can be found on the World Wide Web, at http://www.fccc.edu:80/research/labs/golemis/

4. However, even for proteins which do not fall into the category of common false positives, questions of obvious importance remain. Firstly, it is important to ask if the protein has any conceivable biological relevance to the DBD-fusion used to isolate it. Secondly, one must determine if the protein is known to be

expressed in an appropriate cell type and intracellular compartment to function in conjunction with the DBD-fusion.

Summary

In sum, two-hybrid technologies are a very powerful means of applying genetic approaches to the study of protein-protein interactions. Because of the relative ease of working with yeast, these approaches can usually be mastered by any laboratory with basic cloning and molecular biology experience. The majority of proteins tested appear to be amenable to two-hybrid applications. While some are not at this time suitable, efforts are under way in a number of laboratories to address issues such as development of two-hybrid approaches with selection not dependent on nuclear localization or selective activation of transcription. Also, systems are being investigated which would permit more complex screens for interaction between proteins with multiple associating partners, or between proteins and peptides, ligands, or drugs. With the evolution of two-hybrid techniques to allow greater flexibility in the proteins that can be analyzed, and with the ever-increasing diversity of libraries available for screening, the rapid analysis of protein-protein interactions will help to elucidate many signaling pathways of great biological importance.

Acknowledgements

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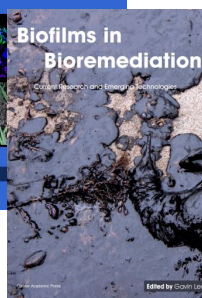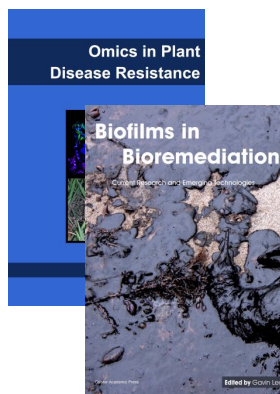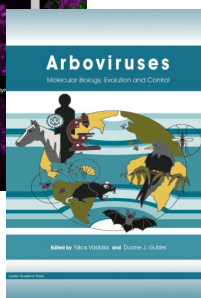
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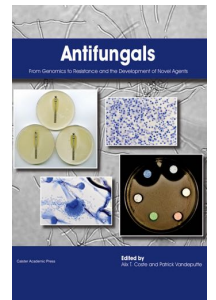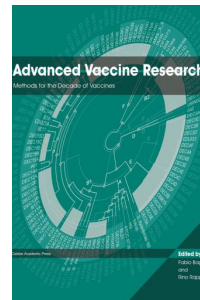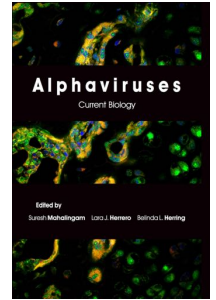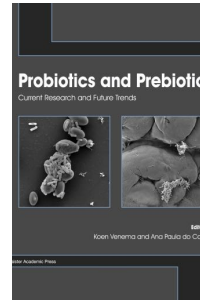
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