Applications of the Yeast Two-Hybrid System

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In recent years, the yeast two-hybrid system has become the method of choice for detection and analysis of protein–protein interactions in an in vivo context. This system, which capitalizes on the significant genetic history and ease of protocols for manipulation of *Saccharomyces cerevisiae*, is accessible to most laboratories and is applicable to the pursuit of a large variety of experimental goals. To date, the two-hybrid system has seen widespread application for identification of interaction partners by screening methods using a particular protein of interest as a “bait.” Large-scale ventures are also in progress, for example, a cataloging of interactions among the cellular proteins in yeast. However, this method also has tremendous potential for more focused analyses of specific proteins and should become more routine as an alternative or adjunct approach for many structure–function investigations.

Since its initial description almost a decade ago (1, 2), the yeast two-hybrid system has proven to be a revolutionary method for identification and analysis of protein interactions in vivo. The success and widespread application of this procedure have recently been reviewed (3, 4) and there are several excellent descriptions of current practical approaches (5–7). In addition, the two-hybrid system has provided a conceptual framework that has led to promising new alternative systems (8, 9) for analysis of interactions in cellular compartments other than the nucleus. Applications, evaluations, and refinements of these systems are described in publications appearing at such a rate as to make any review rapidly obsolete. Thus, rather than attempting a comprehensive review, this article takes a more narrow perspective toward some practical applications of two-hybrid methods.

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vantage of the LexA system is a galactose-inducible promoter in the activation domain plasmid used for library construction which permits controlled expression of chimeric proteins that might otherwise be toxic to host yeast strains. This feature has been termed an “interaction trap” (12).

Both two-hybrid systems provide exquisitely sensitive assays (13, 14) for detection of protein interactions in an in vivo context and internal controls that are important for validation of these interactions. Among the controls to eliminate false positives are multiple reporter genes with promoters that have been engineered to contain upstream activation sequences recognized by the specific DNA binding domain of the system. Commonly, these include a HIS3 or a LEU2 gene, which allows a positive selection for prototrophic growth, and a lacZ gene, which permits a colorimetric colony screen (Table 1). The LEU2 reporter provides a tight nutritional selection, whereas the HIS3 reporter system is leaky. The latter requires addition of 3-aminotriazole, a histidine analogue inhibitor of the HIS3 gene product, imidazolglycerol-phosphate dehydratase, to reduce background colony growth during screening (13). Another important control is demonstration that independent expression of the activation domain chimera or of the DNA binding domain chimera is insufficient to activate transcription. This is established prior to screens with the binding domain/bait plasmid. Following a screen, testing of putative activation domain/positive chimeras is facilitated by

### Table 1

Reporters in Two-Hybrid Systems

<table>
<thead>
<tr>
<th>Fusion proteins</th>
<th>Genomic reporters</th>
<th>Phenotypes AB interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Gal4 systema</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal4 BD</td>
<td>protein A</td>
<td>GAL promoter</td>
</tr>
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<td>GAL promoter</td>
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<tr>
<td>LexA systemb</td>
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<td>LexA BD</td>
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<td>lexA operator</td>
</tr>
<tr>
<td>VP16 AD</td>
<td>protein B</td>
<td>lexA operator</td>
</tr>
<tr>
<td>Reverse Gal4 systemsc</td>
<td></td>
<td></td>
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<tr>
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<td>protein A</td>
<td>GAL promoter</td>
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<td>Gal4 AD</td>
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<td>GAL promoter</td>
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<tr>
<td>Reverse LexA systemd</td>
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<tr>
<td>LexA BD</td>
<td>protein A</td>
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<tr>
<td>VP16 AD</td>
<td>protein B</td>
<td>tetR operator</td>
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<table>
<thead>
<tr>
<th>Fusion proteins</th>
<th>Reporters in alternative systems</th>
<th>Phenotypes AB interaction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Split-ubiquitin systeme</td>
<td>lexA operator</td>
<td>lacZ</td>
</tr>
<tr>
<td>Cyto-trap systemf</td>
<td>human Sos</td>
<td>protein A</td>
</tr>
</tbody>
</table>

a The original Gal4 system (1, 2) used upstream activation sequences from GAL1 to drive expression of a lacZ reporter gene. Durfee et al. (13) introduced HIS3 as a nutritional reporter also controlled by GAL1 promoter elements. James et al. (18) added an ADE2 reporter with upstream activation sequences from GAL2 and placed lacZ under control of the GAL7 promoter.
b The LexA system is described in Ref. (12).
c Reverse Gal4 systems are described in Refs. (35, 36).
d The reverse LexA system is described in Ref. (38).
e The split-ubiquitin system (8) originally used DHFR with a hemagglutinin tag as the reporter attached to the carboxyl-terminal portion of ubiquitin and immunoblot analysis to detect interactions. In subsequent applications (29), that reporter was replaced with a chimeric transcription factor containing LexA, VP16, and a region of Staphylococcus aureus protein A with two IgG-binding domains to provide both colorimetric and immunochemical tests for interactions.
f The cyto-trap system is described in Refs. (9, 30).
the presence of a CYH2 gene on the binding domain/bait plasmid. This gene, which encodes a ribosomal protein, confers cycloheximide sensitivity to a cyh2 host yeast strain (15), allowing selection of cells that have lost the bait plasmid as cycloheximide-resistant colonies. A final recommended control is a test of the putative activation domain/positive chimera with an unrelated binding domain fusion protein (7, 16).

While two-hybrid library screens are conceptually and practically straightforward, the screening procedure and control assays involved in successful identification of meaningful interactions are labor intensive. Therefore, before embarking on a screen, it is important to verify, as much as possible, that the bait protein of interest is expressed, stable, and properly folded in yeast transformants. Expression may sometimes be verified with immunoblot analysis of extracts from transformants using an antisera specific for the protein of interest or an antisera that recognizes the Gal4 binding domain. Many binding domain plasmids also contain codons for a hemagglutinin tag (17) that is attached to the carboxyl terminus of the bait protein, permitting use of commercially available antisera that recognize the tag. However, it has been our experience that a functional assay is often more sensitive than these immunochemical tests for expression. Functional assays may involve two-hybrid tests with a known interaction partner or, for a multimeric protein, direct tests for subunit interactions. A positive functional assay can satisfy all criteria for expression, stability, and folding in the two-hybrid system. A positive functional assay also provides an excellent internal control to assess the complexity and representation of genomic or cDNA libraries during subsequent mass screens.

Another crucial variable deserving attention prior to a screen is the activation domain library to be used in the screen. Although most newly constructed or commercially available libraries are generally subjected to some standard assays for quality control, some simple preliminary tests can provide some reassurance that the library is sufficiently complete to be representative of the population to be tested. First, restriction digests of library DNA can give an estimate of the average size and range of sizes of DNA fragment inserts. Second, in most cases, the library would be expected to contain the gene or cDNA encoding the protein to be used as bait. This can be ascertained by Southern blot analysis or by polymerase chain reaction (PCR) designed to identify a discrete restriction fragment or region from the bait protein gene among the library DNA inserts (for cDNA libraries, restriction fragments near the 5’ end of the gene are more likely to be intact). A good control in these analyses is a constitutively and ubiquitously expressed gene, e.g., that encoding actin.

Once putative positive interaction partners have been identified with a two-hybrid screen, it is often important to verify interactions using a second experimental method. A tremendous advantage of using the two-hybrid system as the first approach in identification of interactions is that the genes encoding interaction partners are immediately accessible for designing secondary approaches. Therefore, one common approach for verification is copurification using affinity methods, following introduction of an affinity tag onto the bait or interacting protein. Another accessible method for verification is co-immunoprecipitation using antisera generated for each protein or for internal peptides based on sequence analyses. These techniques are described in more detail in other articles of this issue.

Some Significant Refinements

Despite the substantial success of the original two-hybrid systems, some relatively recent refinements are likely to make the technique even more useful and accessible. For example, host yeast strains have been notably improved. To reduce the frequency and improve on detection of false positives, James et al. (18) constructed a strain with upstream activation sequences derived from different GAL genes for each reporter gene and with an additional ADE2 reporter gene. They and others (7) also introduced use of a mutant allele (his3–200) of the HIS3 locus that is less leaky than the original his3 allele. This improves on selection sensitivity by reducing the levels of 3-aminotriazole that must be used during screening.

Another recent alternative approach for two-hybrid screens that is likely to have significant commercial and practical impact has been termed “interaction mapping” (19, 20). With this approach, a highly complex activation domain library is generated, transformed into yeast, and stored as a collection of yeast transformants. The library is then screened by mating these transformants with a strain of the opposite mating type expressing the binding domain chimera of interest. With a control to assess frequency of mating, diploids expressing interacting proteins can be directly selected for reporter gene expression. This approach eliminates concerns about transformation frequency with library DNA. It dramatically increases the yield of cells expressing both plasmids and, consequently, increases the potential for exhaustive screens. It has become the method of choice for large initiatives like the yeast proteome project (21, 22) and, because of the simplicity and reproducibility of the technique, is likely to become much more widespread.

Directed Two-Hybrid Assays

Thus, yeast two-hybrid screens have become increasingly sophisticated and reliable approaches for defining specific and general protein interaction networks.
As described below, the method also allows subsequent genetic applications for probing structural and functional aspects of specific interactions. However, the utility of two-hybrid assays for other applications, for example, as a sensitive probe for conformational stability, is less widely appreciated. For investigators interested in structural/functional analyses of specific proteins, expression of mutant alleles in two-hybrid assays can provide a sensitive and convincing measure of effects on structural integrity in vivo. It can also be an exceptional probe for defining the pattern and strength of subunit interactions of multimeric proteins. For these applications or for exploration of a hypothetical interaction between two specific proteins, it may be necessary to try more exhaustive initial tests of the two-hybrid assays.

Among considerations for a “fair” test of interaction between two specific proteins or subunits (A and B) of a protein in the Gal4 two-hybrid system is domain specificity. We frequently find that an interaction between A and B may be detected, for example, with A as the binding domain fusion partner but not with A as the activation domain fusion partner. Thus, an initial recommendation is cloning of both coding regions (generally as sequenced PCR products) into each domain fusion plasmid. Also recommended is cloning of both coding regions in a 5’ orientation relative to the Gal4 domain sequences in addition to the normal 3’ orientation. This will help bypass another common obstacle to detection of interactions that require a free amino terminus. Plasmids for 5’ cloning in the LexA system have been described (23, 24) and can be constructed by PCR in the Gal4 system. These subcloning steps provide a starting collection of eight plasmids for subsequent assays. Initial controls in two-hybrid assays normally include the proscribed positive system controls (expression of Gal4 or of known interacting proteins) and negative controls (independent expression of protein A or protein B chimeras). However, more relevant negative controls are coexpression assays of each chimeric plasmid with the opposite “empty” Gal4 plasmid to eliminate the possibility of some inherent affinity of the chimeric protein for the other Gal4 domain. These and subsequent assays to test for homo- or heterochimeric interactions are listed in Table 2. We have found this fairly comprehensive approach to be necessary since, in a limited number of studies, we have encountered virtually all of these interaction permutations. However, this approach can also be quite informative, for example, about the order and orientation of subunit interactions in multimeric enzymes. Clearly, similar considerations would also be necessary for truly ex-

**TABLE 2**

Strategies for Analysis of Interactions between Specific Proteins or Subunits

<table>
<thead>
<tr>
<th>N-terminal Gal4 fusions</th>
<th>C-terminal Gal4 fusions</th>
<th>Cross tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal4 BD</td>
<td>protein A</td>
<td>Protein A</td>
</tr>
<tr>
<td>Gal4 AD</td>
<td>Gal4 AD</td>
<td>Gal4 AD</td>
</tr>
<tr>
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<td>Gal4 AD</td>
<td>Protein A</td>
</tr>
<tr>
<td>Gal4 AD</td>
<td>protein A</td>
<td>Protein A</td>
</tr>
<tr>
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<td>protein B</td>
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</tr>
<tr>
<td>Gal4 AD</td>
<td>Gal4 AD</td>
<td>Gal4 AD</td>
</tr>
<tr>
<td>Gal4 AD</td>
<td>protein B</td>
<td>Protein B</td>
</tr>
<tr>
<td>Homomer interaction pairs</td>
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<td>Protein A</td>
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<tr>
<td>Gal4 AD</td>
<td>protein A</td>
<td>Protein A</td>
</tr>
<tr>
<td>Gal4 BD</td>
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<td>Protein B</td>
</tr>
<tr>
<td>Gal4 AD</td>
<td>protein B</td>
<td>Protein B</td>
</tr>
</tbody>
</table>
haustive library screens to detect interactions involving a specific protein of interest.

Limitations of Two-Hybrid Analyses and Some Alternatives

One limitation of the classic two-hybrid system is simultaneous detection of multiple protein interactions. This is also obviously a serious obstacle to screening for meaningful interactions involving a heteromeric protein. To extend the system for analysis of ternary protein interactions, Zhang and Lautar (25) successfully introduced a third plasmid into yeast transformants containing activation and binding domains of three proteins. Osborne et al. (26) developed a "tribrid" system to express an enzyme catalyzing a modification essential for interaction of two chimeric proteins. These examples suggest that it may at least be fairly straightforward to design similar approaches for library screens to identify interactions involving a protein that is normally a heterodimer or a multimer composed of two different subunits. For more than three interacting polypeptides, current technologies require multiple rounds of screening (21).

Another obvious limitation of two-hybrid methods is the necessity for nuclear localization of interaction partners to obtain reporter gene expression. This may be problematic since many proteins, for example, membrane proteins and proteins with competing organelar targeting sequences, may not be amenable to this localization. Alternatives, since many interactions are dependent on relatively small regions of the proteins involved, are screens using libraries encoding designed or random peptides. This type of approach was successfully used to identify 20-residue peptide aptamer inhibitors of cyclin-dependent kinase 2 (27). Peptide library screens are particularly suitable for investigation of receptor/ligand interactions and may be adapted, for example, for identification of orphan receptors (28).

Also, the nucleus may not be the appropriate organelle for investigation of some interactions and several clever new approaches for detection of interactions in the cytosol have been developed to facilitate such investigations. Johnsson and Varshavsky (8) described a "split-ubiquitin" system based on interacting fusion chimeras that places a mutant form of the amino-terminal portion of the ubiquitin polypeptide (residues 1–34 with a glycine substitution for isoleucine at residue position 13) and the carboxyl terminal portion of the polypeptide (residues 35–76), which otherwise do not associate, into sufficient proximity for recognition by ubiquitin-binding proteins. This binding permits proteolytic cleavage and release of a reporter protein attached to the carboxyl terminal ubiquitin peptide. The split-ubiquitin system with a transcription factor derivative of LexA as the reporter (Table 1) was recently used to examine interactions among proteins of the endoplasmic reticulum membrane (29).

Another novel system (9), now marketed as the "Cyto-trap" two-hybrid system (Stratagene), employs a myristylation signal to obtain plasma membrane association of proteins expressed using cDNA libraries in yeast. Interacting proteins recruit the bait protein, expressed as a fusion chimera with human Sos, to the membrane. In that location, Sos functionally substitutes for a temperature-sensitive mutant form of the yeast guanyl nucleotide exchange factor (cdc25H) in activating the Ras signaling pathway, thus permitting growth of cotransformants at 37°C. This system has been used to examine interactions among transcription factors independent of their transcriptional activation properties (9, 30). A variation of the Cyto-trap method, based on direct recruitment of Ras to the plasma membrane, has recently been described (31).

These new approaches offer promising alternatives for exploring interactions of proteins that are not amenable to nuclear localization or to the selection/reporter schemes in conventional two-hybrid assays. A safe prediction is future development of additional systems to optimize conditions for detection of other specific types of interactions, perhaps in a variety of cellular organelles.

Mutagenesis and Counterselections

Once a positive interaction between two proteins has been established, subsequent desirable pursuits include mapping of interaction domains and construction of mutant proteins to explore the physiological significance of the interaction. Domain mapping generally involves expression and two-hybrid analyses of truncated fusion proteins following construction of gene deletions with available restriction sites, exonuclease treatment, or PCR-directed mutagenesis. Fine-structure mapping and construction of mutant proteins that are not altered in properties other than the capacity for interaction require a site-directed approach based on available structural data or on an informative selection scheme following random mutagenesis.

A straightforward PCR procedure for random mutagenesis was described by Lehming et al. (32). They used PCR with Taq polymerase, which has an error frequency of one per thousand nucleotides (33), to generate a population of molecules with random errors in a gene of interest. The PCR products were cotransformed directly into yeast strain with a plasmid containing a gap within the cloned gene. Plasmid integrity is restored by homologous recombination in transformants selected with a plasmidborne prototrophic marker (Fig. 1). This method bypasses a subcloning step and passage through E. coli which might result in biased amplification of mutated sequences. The yeast transformants generated with this protocol po-
tentially express a variety of mutant forms of the protein of interest. Steffan et al. (34) recently reported adaptation of this technique with two-hybrid plasmids, followed by colony screens for loss of β-galactosidase activity, to identify mutant forms of a protein in the RNA polymerase I upstream activation factor that lose the ability to interact with TATA-binding protein.

In addition to colorimetric colony screens, several reverse two-hybrid systems have been developed to provide positive selection techniques (Table 1) to facilitate identification of mutant proteins that have lost the capacity for interaction. Leanna and Hannink (35) constructed a host yeast strain with both a genomic cyh2 locus that confers resistance to cycloheximide and a genomic CYH2 locus under control of a GAL1 promoter. Since the CYH2 allele is dominant, interacting Gal4 fusion proteins in this strain produce a Chx<sup>s</sup> phenotype. Thus, loss of interaction between the fusion proteins is scored as Chx<sup>r</sup>. Vidal et al. (36) also constructed a host strain to place expression of the URA3 gene under control of Gal4. In this system, interaction of two-hybrid chimeric proteins produces Ura<sup>+</sup> colonies, whereas loss of interaction in Ura<sup>-</sup> colonies can be positively selected as resistance to 5-fluoroorotic acid (FOA, Fig. 1). FOA is converted to a toxic intermediate by the URA3 gene product, orotidine-5'-phosphate decarboxylase. Huang and Schreiber (37) used the FOA selection scheme to identify small molecules that disrupt a protein/protein interaction in vivo. Their system is based on growth of transformants in multiple small volumes (nanodroplets) in the presence of organic chemicals released from beads in the medium by UV irradiation.

A positive selection scheme for loss of interaction has also been developed for the LexA system (38) (Table 1). In this case, an E. coli tet repressor gene under control of the lexA operator was introduced into the host yeast strain and a tetR operator sequence placed upstream of the yeast HIS3 gene. Loss of interaction can therefore be scored as His<sup>-</sup> prototrophy.

Among the mutant proteins identified with these counterselection schemes are likely to be a significant number of truncated polypeptides. While these may be useful in defining interaction domains, it is important to eliminate global effects of truncation on native structure. It may be possible to distinguish truncated and full-length mutant proteins using Western blot analysis of extracts from the yeast transformants with antisera specific for the polypeptide or an epitope tag. A subsequent elegant test of mutant proteins would be a

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**FIG. 1.** Mutagenesis and counterselection scheme. (1) Demonstration of protein A/protein B interactions in vivo: Leu<sup>+</sup>/Trp<sup>-</sup> colonies from a host yeast strain (genomic reporters are HIS3, lacZ, and URA3 genes with GAL1 promoters) cotransformed with both fusion plasmids display His<sup>-</sup>Ura<sup>-</sup> phenotypes and express β-galactosidase. Cotransformants with each fusion plasmid plus the other “empty” plasmid are His<sup>-</sup>Ura<sup>-</sup> and do not express β-galactosidase. (2) Random mutagenesis of the protein A gene in vitro: Using the AD fusion plasmid as template and primers based on plasmid sequences ~100 bp from the points of fusion, PCR is conducted with Taq polymerase to introduce random errors (+) during synthesis. (3) Recombination in the host yeast strain and selection for loss of interaction: Leu<sup>-</sup>/Trp<sup>-</sup> transformants are selected following transformation of the host yeast strain with PCR products, a gapped form of the AD plasmid, and the BD/protein B gene fusion plasmid. The Leu<sup>-</sup> phenotype indicates recombination in vivo between homologous sequences in the PCR products and the AD plasmid. The transformants are replica plated or replica streaked onto plates lacking uracil and onto plates containing 5-fluoroorotic acid. Ura<sup>-</sup>/FOA<sup>-</sup> colonies represent transformants that may express mutant forms of protein A that have lost the capacity for interaction with protein B.
functional assay for loss of interaction with the test protein of interest but for retention of interaction with another protein (34) or with another subunit.

These counterselection methods provide powerful approaches for identification of mutant proteins that have lost the capacity for interaction in two-hybrid assays. They will clearly facilitate both the mapping of interaction domains and the collection of mutant proteins for subsequent analyses of effects in vivo.

Sensitivity of Two-Hybrid Assays

A significant virtue of two-hybrid assays is the ability to detect weak interactions between proteins that may be difficult to detect or examine by in vitro methods. Estojak et al. (14) reported that assays with the LexA system can detect dimeric interactions with $K_d$ values ranging from $\sim1$ nM to $\sim1$ µM as measured by in vitro assays. However, they also reported that, while relative strengths of interaction in two-hybrid assays were generally indicative of the rank order of affinities measured in vitro, this relationship was not linear and appeared to depend on, among several variables, the reporter gene used for measurements. Nutritional reporters, in particular, have thresholds for colony growth that preclude measurement of interaction affinity as subtle changes in growth rate. Expression of the $\lambda$acZ reporter appears to be a more accurate indicator for strength of interaction, visually as time of development of blue colonies relative to controls or quantitatively as determined by enzyme assays (39).

There is some indication that higher-order oligomeric interactions are reflected in reporter gene expression (14). Thus, it is possible that two-hybrid assays can be optimized to assess strength of interactions in oligomeric complexes and to monitor changes in quaternary structure of mutant enzymes. Another possibility of interest is that these assays may be sufficiently sensitive to report subtle differences in physical contacts between subunits in mutant enzymes, for example, that retain catalytic activity but that have lost properties of cooperative interactions as measured with kinetic assays. As application of the two-hybrid and related systems becomes more widespread, issues regarding sensitivity and related concerns about the effects of intracellular milieu, e.g., organellar localization, are likely to receive increasing scrutiny.

ACKNOWLEDGMENTS

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