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The Two-Hybrid System:
Finding Likely Partners for Lonely Proteins

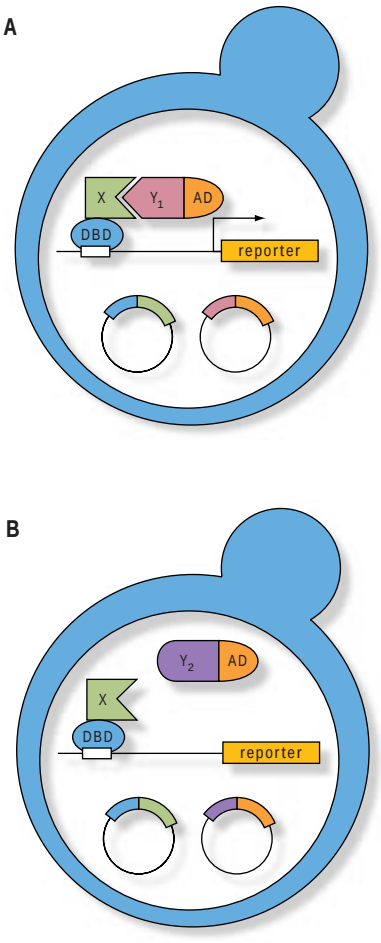

FIGURE 1. How the two-hybrid system works. *Panel A.* Protein X (the bait protein) is fused to a DNA-binding domain (DBD) and protein Y_1 is fused to a transcription activation domain (AD). Both hybrid proteins are expressed in a yeast cell from plasmids. Interaction of X and Y_1 leads to activation of a reporter gene that allows the yeast to grow on a defined medium. *Panel B.* Protein X and protein Y_2 do not interact; consequently, the reporter gene is not expressed.

A common response of the biologist first scoring a successful two-hybrid search is outright disbelief that an assay so simple can yield results so revealing of the innermost circuitry of a cell. That the method, described nearly a decade ago (1), often works precisely as depicted in its standard cartoon version *(figure 1)* is a testament to the tolerance of the eukaryotic transcriptional complex for proteins that have no business residing in the vicinity of a promoter.

Three features are key to why searching for partners with this assay means that your protein won't be home alone on a Saturday night. The first is that DNA-binding domains like those of the yeast Gal4 and *E. coli* LexA proteins can drag all sorts of unwitting protein X's to the regulatory region of a yeast reporter gene.

The second is that transcription activation domains derived from Gal4 or Herpes virus VP16, for example, can be recruited to this same reporter gene when fused to some protein Y, provided that Y can cozy up to X.

Finally, when the two hybrid proteins show up together at the reporter, the transcription machinery joins the party and the gene is expressed. While the readout of the assay is yeast that can grow on a defined medium or turn blue with the right substrate, the outcome of the experiment is a set of cDNA clones in hand encoding proteins that bind to your favorite protein X.

SOME HISTORY

As with any new technology, the two-hybrid system had its origins in a host of previous experiments and methodologies. One was the glorious history of genetic selections in microbial organisms: for bacteria and phage, these selections were essential to decipher the fundamental language underlying the flow of genetic information but are now used principally for the mundane manipulations of recombinant DNA. In yeast, complementation of cell cycle defects with human cDNA libraries (*e.g.*, reference 2) was a major contribution to an understanding of how animal cells control their division.

Two, the use of fusion proteins to analyze transcription, translation, protein localization, and other processes has a long and productive track record. In particular, β-galactosidase—the workhorse of fusion technology—played a central role both in the dissection of Gal4 protein function and as a simple reporter for Gal4-dependent transcription.

Three, by the late 1980s we were more than a decade into the revolution brought about by DNA cloning. Recombinant DNA-based methods were becoming ever more prevalent in the identification of genes through the screening of cDNA, genomic, or λ expression libraries.

Four, and of particular relevance, were the ground-breaking studies on the mechanism of transcriptional activation. That a transcription factor like Gal4 has small, discrete domains responsible for contacting DNA and for recruiting the general transcription machinery was itself a striking finding, but the demonstration that an artificial factor could be created, by fusing a DNA-binding domain to a transcription activation domain, was a remarkable result (3). While this "swap" experiment provided the concept of a covalent hybrid activator, other experiments (4) revealed that proteins with activation domains could work by associating noncovalently with DNA-bound proteins.

Finally, the pressure to generate fundable ideas in order to maintain a laboratory (as recounted in reference 5) provided a major stimulus to the development of this assay.

SOME VIRTUES

The two-hybrid system possesses several virtues that have led to its popularity. First, it came along at a timely moment, as a burgeoning supply of proteins to analyze was becoming available, initially from more traditional biochemical and genetic approaches and more recently from genome sequencing. Not only did the supply of proteins grow, but the demand for interacting partners accelerated as awareness increased of the role of protein complexes in growth control, signal transduction, and other cellular processes.

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Second, the yeast assay is easy to perform, allowing geneticists, cell biologists, and molecular biologists to succeed in a task formerly the sole province of competent biochemists. As a method that could be readily packaged into a set of strains, plasmids, libraries, and an accompanying guidebook, the yeast assay has unintentionally contributed to the current kit-based mentality of molecular biology. In this regard, hybrid proteins have many friends, including glutathione S-transferase, hexahistidine, and an assortment of epitope tags.

Third, the two-hybrid system is enormously adaptable, proving to be the basic platform upon which were built a series of related approaches (reviewed in 6) to analyze DNA-protein, RNA-protein, and small molecule-protein interactions. Other developments include reverse assays designed to identify mutations, peptides, or drugs that disrupt interactions, and three plasmid assays to express bridging proteins or modifying activities. In addition to transcription factor activity, the functions of other proteins like ubiquitin, β-galactosidase, and SOS have proven capable of reconstitution via a protein-protein interaction.

Finally, at a time when genomics is being superseded by proteomics, the method can be carried out on a grand scale. In the works are two-hybrid searches to identify interactions for the complete protein complements of model organisms such as yeast up to humans.

SOME OUTCOMES

What is the ideal outcome for a two-hybrid search with a "bait" protein dear to your heart? Twenty positives are found, defining six different proteins, each of these as a set of overlapping clones that defines a minimal domain of interaction. These six fall into three classes as follows. The first comprises three known proteins, one previously shown to bind to your bait by co-immunoprecipitation, and two others that make sense from the genetics of your system. These validate your search and provide confirmatory data for a long-held hypothesis. The second class consists of a totally surprising, but in retrospect plausible, well-known protein that links your bait to what was thought to be an unconnected cellular process. Further work confirms this unexpected cross-connection and suggests innovative possibilities for the treatment of human disease. The third class defines two novel proteins with no significant homology to any others in the database. These provide grist for John Miller, a graduate student in your lab, who turns them into a stellar degree.

While we know of no such single search, each of the individual outcomes has been described. For example, one early success was the identification of the Raf protein kinase as a partner for the oncoprotein Ras (7). Raf had been shown to be active downstream of Ras in *Drosophila*, *Xenopus*, *C. elegans*, and mammalian cells. Thus, the finding that an 81-residue region of Raf bound in the two-hybrid assay to wild-type, but not an effector domain mutant of Ras, was a satisfying result. Another early search, using the HIV gag protein as bait, unexpectedly turned up cyclophilins A and B (8), suggesting that these host proteins may act during the retroviral life cycle.

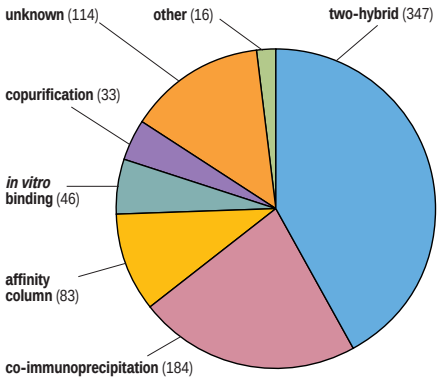

FIGURE 2. Frequency of methods used to detect protein-protein interactions of yeast proteins. Some of these interactions have been detected by multiple methods. Note that some methods such as affinity columns and *in vitro* binding are based on similar principles. The popularity of these methods to detect interactions may differ for proteins from other organisms.

Recently, a two-hybrid search with GATA4, a protein required for the upregulation of genes implicated in cardiac hypertrophy, found NF-AT3, a member of the family of nuclear factors of activated T cells (9). Just as T cell signaling requires dephosphorylation of NF-AT factors by the calcineurin protein phosphatase, the heart pathology involves calcineurin signaling through NF-AT3 and GATA4. Furthermore, the immunosuppressant cyclosporin A, which inhibits calcineurin in T cells, suppresses cardiac hypertrophy in an animal model of the disease.

The Two-Hybrid System continued

The two-hybrid system has become popular along with other simple methodologies that employ expression plasmids to attach convenient handles to proteins. In the yeast world, where use of the protocols required has no energy barrier, two-hybrid has been used to detect about half of the interactions, for which a method is provided, that are listed in the Yeast Proteome Handbook (10)*(figure 2)*. In the biological community as a whole, a clear sign of the system's commonality is the decreasing frequency with which it is cited in the literature. After a few years in the early '90s of exponential citation growth, references to the original description of the assay are now plateauing and should soon decrease, replaced by reviews, literature from supply companies, or common knowledge. Similarly, methods sections of papers no longer provide detailed descriptions of the search procedures, but merely indicate which version of the assay was used.

SOME FINAL THOUGHTS

As users well know, two-hybrid searches are not without pitfalls (*e.g.*, reference 11), and more than one promising hypothesis has evaporated following the performance of additional tests on a hopeful candidate from a library screen. But if you're persistent—if not with one bait then with another—it's likely that something worthwhile and perhaps even invaluable will turn up. Indeed, once you've mastered the technique, you can apply it rapidly to all manner of proteins in which you have a passing fancy. That the technique is nearly immune to the unique characteristics of proteins that make them so interesting is perhaps its greatest strength, as long as the two-hybrid practitioner realizes that more than the life or death of a yeast cell will be required for proteins to give up more of their secrets. FOCUS

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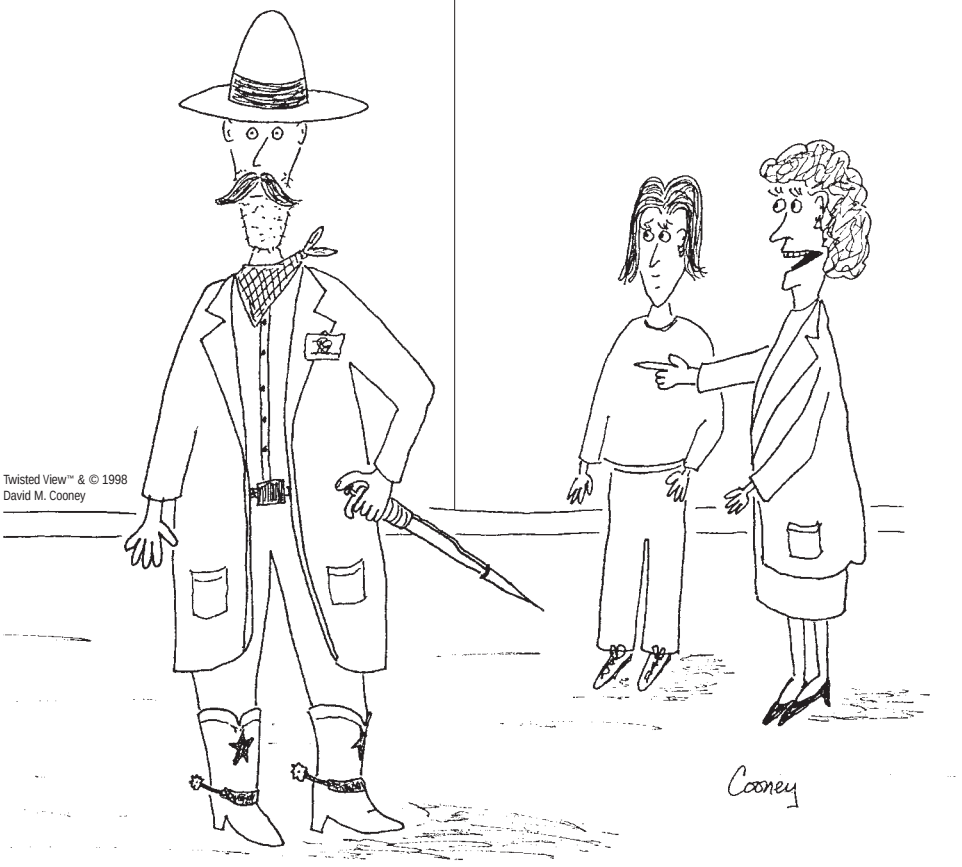

"That's Wyatt, he does all of our Western blots."