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# Two-Hybrid Systems

*Methods and Protocols*

*Edited by*

**Paul N. MacDonald**

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METHODS IN MOLECULAR BIOLOGY™

# Two-Hybrid Systems

## *Methods and Protocols*

Edited by

**Paul N. MacDonald**

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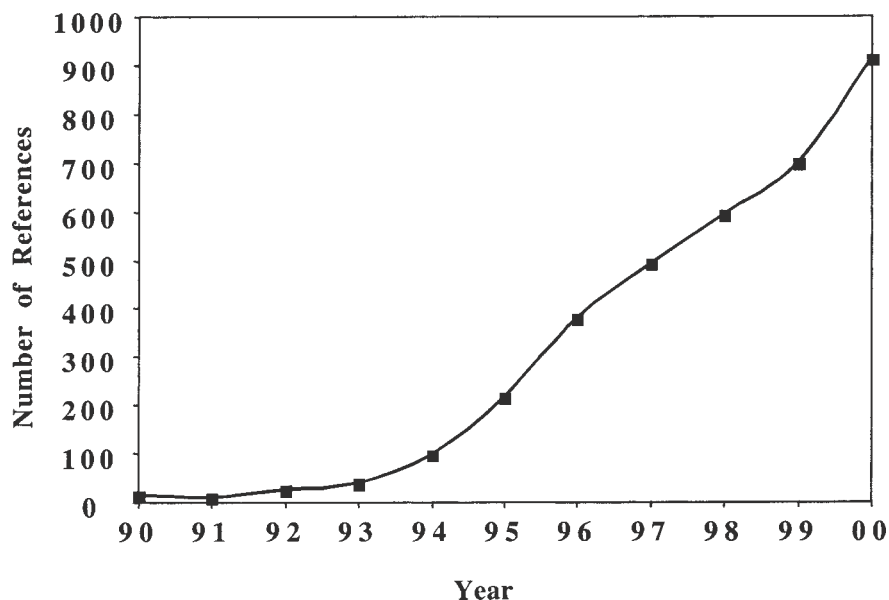
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## Preface

Many, if not all, essential biological processes require selective interactions between proteins. Complex signaling systems require sequential, ordered protein–protein interactions at essentially all levels of the signaling cascade. For example, peptide hormones interact with selective membrane receptor proteins, and autophosphorylation of the receptor then recruits other key regulatory proteins that initiate kinase cascades in which each phosphorylation event requires selective recognition of the protein substrate. The ultimate signaling effect, in many cases, is the regulation of RNA polymerase II-directed transcription in the nucleus, a process that involves numerous, multiprotein complexes important for transcription initiation, elongation, termination, and reinitiation. Defining, characterizing, and understanding the relevance of these protein–protein interactions is an arduous task, but substantial inroads have been made over the past 20 years. The development of more recent methodologies, such as mammalian expression systems, immunopurification schemes, expression cloning strategies, surface plasmon resonance (BiaCore), and nanosequencing technologies, has contributed a wealth of new insights into these complex multiprotein mechanisms and clearly accelerated the discovery process. Arguably, the yeast two-hybrid system has been one of the predominant and most powerful tools in this discovery process.

On a personal note, my specific interest in the yeast two-hybrid system developed in a manner probably not terribly different from that of many other investigators who were interested in the early 1990s in identifying and characterizing interactions between two proteins. While working in the laboratory of Mark R. Haussler, our interests centered on the vitamin D receptor (VDR), a member of the nuclear receptor family, and the mechanisms involved in VDR binding to DNA. Specifically, I was interested in identifying a nuclear factor that interacted with and conferred high-order binding of the VDR to DNA. We and other larger groups in the nuclear receptor field chose a traditional biochemical approach that focused on purifying and identifying the unknown nuclear accessory factor. Other laboratories used expression cloning strategies with purified radiolabeled proteins to screen cDNA expression libraries for clones encoding the interacting factor. Both approaches were comparatively large efforts at the time, requiring a tremendous number



**Fig. 1.** The number of publications over the past 10 years that were found in a search of PubMed using “two-hybrid” in the search window. The year 2000 value is projected based on the number of references found at the time of the search (September, 2000) and the number of remaining months in the year.

of person-hours. Both approaches eventually resulted in the successful identification of the factor as retinoid X receptor, a common heterodimeric partner for many of the class II nuclear receptors. Unfortunately, we were not one of the groups to first report the identification of RXR as the partner. Our smaller effort was, in no uncertain terms, “scooped.”

At about this same time, reports from the Fields laboratory on the successful use of the yeast two-hybrid system began to emerge and more beneficial yeast strains and vectors were being developed. The power of the system was inspiring to anyone working on trying to identify protein interaction partners. Here was a simple, direct screening assay that could uncover novel factors that interacted with your protein of interest. Millions of cDNAs could be screened in a single experiment, in a relatively short time, and with comparatively less effort. Following the initial screen, the cDNA clones encoding the putative interactors were already in hand and they could be directly sequenced and identified. The playing field seemed somehow leveled a bit by the two-hybrid system. More than twelve years have passed since the original description of the yeast two-hybrid system was reported, and few would disagree that this system has had a

tremendous impact on virtually every field of modern biology. Continuous refinements and novel innovations of the original systems over the past decade have only strengthened the utility of the approach. As illustrated in **Fig. 1**, it is obvious that many groups continue to adopt the two-hybrid system as a new approach in their laboratories and this trend will only continue to expand in the future as the era of functional genomics unravels over the next century.

Therefore, the overall goal for *Two-Hybrid Systems: Methods and Protocols* is to introduce the yeast two-hybrid system to students, research assistants, research associates, and other more senior investigators considering this as a new approach in their laboratories and research projects. Toward this end, I have assembled a collection of detailed descriptions of basic protocols and a compendium of experimental approaches in different biological systems that I hope reflects the utility of the system and its variations in modern biomedical research. My hope is that this will also serve as a useful reference for those laboratories that have extensive experience with the two-hybrid system. Thus, I invited several authors to discuss in more general terms some of the problems and strategies involved in the yeast two-hybrid assay as well as some of the alternative systems that have evolved from the original system that may prove useful to those more experienced two-hybrid laboratories.

*Two-Hybrid Systems: Methods and Protocols* is divided into four main sections. The first section is a compendium of general methodologies that are used in the two-hybrid system. Here, the reader will find in-depth discussion and detailed methodologies that serve as the foundation on which successful yeast two-hybrid experiments rest. Since many laboratories beginning two-hybrid approaches have not worked with yeast to a significant extent, this first section begins with a general introduction to handling yeast, a detailed compendium of media formulations, as well as an overview of the common strains of yeast and plasmid vectors that are used for two-hybrid work. This section ends with three chapters that describe the basic methodologies involved in introducing plasmids into yeast, interaction assays, and recovering the plasmids from yeast. This first section was intentionally designed to be somewhat repetitive in nature with components of the subsequent application chapters. The intent was to provide more in-depth methodological detail and variations of these fundamental techniques that serve as the backbone of any two-hybrid assay as well as to illustrate how these techniques are incorporated into individual applications. One well-known, recurring drawback of the two-hybrid system is the potential for artifacts and false positives. Thus, Section II provides a discussion of the various classes of false positives and the common mechanisms through which false-positives arise. This section also includes two chapters that focus on general strategies and detailed



protocols to confirm the authenticity of the interaction using in vitro protein–protein interaction assays. Part III includes four application chapters that describe how the yeast two-hybrid system was applied in various systems to identify interacting partners in important biological systems including the Smad and nuclear receptor pathways. Finally, Part IV describes various alternative strategies that have arisen out of the original yeast two-hybrid paradigm. These alternative strategies include the one-hybrid, split two-hybrid, three-hybrid, membrane recruitment systems, and mammalian systems. These alternative systems serve to illustrate the flexibility and refinements that are possible with the basic two-hybrid approach.

The authors and I hope that *Two-Hybrid Systems: Methods and Protocols* will prove a valuable addition to any laboratory that is interested in studying macromolecular interactions between proteins.

I would like to express my sincere gratitude to all the authors for their valuable, insightful contributions and for their patience in seeing this project to fruition. This book is a testament to their breadth of knowledge on the topic and the power of the two-hybrid approach. It is evident that both the basic system, as well as its many variants, will continue to play a predominant role in the characterization and identification of protein–protein interactions in the genomic and proteomic arenas of the 21st century.

***Paul N. MacDonald***



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I \_\_\_\_\_

## GENERAL METHODS

## The Two-Hybrid System

*A Personal View*

**Stanley Fields and Paul L. Bartel**

### 1. Origins of the Two-Hybrid Method

The two-hybrid system dates to early 1987, when Stanley Fields was a new assistant professor at the State University of New York at Stony Brook with a small National Science Foundation grant. The university had a seed grant program to fund ideas with commercial potential, and it struck us that we would be more likely to obtain such a grant than another federal grant. Unfortunately, the laboratory was working on pheromone response in the yeast *Saccharomyces cerevisiae*, in particular the role of a protein implicated in transcriptional induction, and the intricacies of yeast mating behavior seemed unlikely to excite the seed grant panel. However, our research interests kept us familiar with the current findings in transcriptional regulation. Specifically, we knew of two key results: one was the work of Brent and Ptashne (1) demonstrating that a hybrid transcriptional activator could be generated from the *E. coli* LexA repressor and the yeast Gal4 protein; the second was the work of groups such as Triezenberg et al. (2) suggesting that transcriptional activators could function by binding to DNA-bound proteins rather than directly to DNA. We toyed with various notions in the hope of linking yeast transcription to commercial potential. Late one afternoon, the idea came to use two different hybrid proteins, one containing a DNA-binding domain and one a transcriptional activation domain (AD), to detect protein-protein interactions. Thus was born the two-hybrid system, not

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as an incremental step in our continuing studies but in an instant. Along with the basic idea came the immediate realization of its most important consequence: it might be feasible to construct libraries of AD hybrids and search them to identify interacting proteins.

In March 1987, we submitted a grant on this idea and began gathering the necessary reagents. It did not take long to learn that the grant would not be funded; the review panel rated the research as having no possibilities for commercial development. Nevertheless, it seemed too persuasive an idea to abandon, and we continued both our experimental and fund-raising efforts, eventually getting the assay to work and Procter and Gamble to support us. This story can be viewed in either of two lights: the competition for grants works well because it drives the generation of good ideas, or the system does not work as well as it might because good ideas often are not funded. In any event, the history of the two-hybrid system is one of many examples in which small laboratories have made contributions, and, thus, it is critical to ensure that these kinds of laboratories can remain operational.

The original two-hybrid experiments were based on several suppositions for which there was then no experimental support. Specifically, we assumed the following:

1. Many proteins, in addition to transcriptional activators, were capable of maintaining their structural integrity as hybrid proteins.
2. The typical affinities of protein-protein interactions that would be studied in this system would be sufficient to reconstitute a transcriptional signal.
3. ADs would be accessible to the transcriptional machinery when present as hybrid proteins.
4. Nonnuclear proteins could be targeted to and function within the nucleus.

In addition, the view of transcriptional activation back then was simpler than it is now. If we had been aware of the need in this process for not only site-specific activators and the basal transcription factors, but all the additional complexity of TATA-binding protein-associated factors, mediator complexes, chromatin-affecting proteins, and the like, we might not have considered it as likely that an idea so simple as the two-hybrid system was workable. In fact, we encountered considerable skepticism, not only from protein chemists but also from molecular biologists.

In July 1988, the first two-hybrid test, the combination of the yeast proteins Snf1 and Snf4, was assayed. Although the results were somewhat encouraging, the transcriptional response resulting from the protein-protein interaction was barely above background. We considered this result likely to be owing to low expression of the hybrid proteins, but after spending several months swap-



ping promoters, we failed to obtain any increase in  $\beta$ -galactosidase expression. It was only when, in early 1989, we obtained a yeast strain from Grace Gill in Mark Ptashne's laboratory, GGY1 : 171, that the same Snf1 and Snf4 constructions yielded a significant signal.

By the time Paul Bartel came to the laboratory that August to interview for a postdoctoral position, the initial two-hybrid experiments had been published (3). It was an easy decision for him to join the laboratory to continue work on this system and, specifically, to use this approach to screen libraries for interacting proteins. In late 1989, we began to collaborate with Rolf Sternglanz, a colleague working in a laboratory upstairs, and Cheng-ting Chien, who was then a graduate student with Rolf. They had used the two-hybrid system to detect homodimerization of the yeast protein Sir4, which provided us with a test case for a library search. We developed a set of AD vectors that allowed us to produce fusions in all three reading frames, and we used these vectors to generate libraries from yeast genomic DNA. We screened the first library we had made for Sir4-binding proteins, and from just over 200,000 transformants we identified two positives that required the presence of the Sir4 fusion protein for a *lacZ* signal. Fortunately, one of these positives encoded Sir4, demonstrating that a library approach was feasible (4). We later determined that the other positive was, in fact, our first false positive (but not the last one). Thanks to the efforts of a number of researchers, two-hybrid searches are now much easier than they were in those days.

In the following years, the laboratory turned some of its attention to p53, using the two-hybrid system to screen for its protein partners and to identify p53 mutants that had lost the ability to associate with SV40 large T-antigen. As in many other laboratories, we began to explore other uses of the two-hybrid system, including the study of antibody-antigen and protein-peptide interactions. More recently, we have been engaged in developing global approaches to study protein-protein interactions and in generating a related system to study RNA-protein interactions.

## 2. Why Is the System Popular?

The two-hybrid system addresses one of life's fundamental questions: How does one find a meaningful partner? If a protein has a known function, new proteins that bind to it bring additional components into play, ultimately contributing to the understanding of the process under study. Alternatively, a protein's function may be obscure but the protein may be of obvious relevance; for example, its gene may be mutated in human disease. In this case, partners with known roles may turn up and provide essential clues. Thus, the method is a tractable and rapid form of genetics for organisms, such as mammals, that

cannot be readily manipulated, and it can accomplish some of the tasks that suppressor screens and similar genetic strategies can do in simple organisms.

Among its strongest features, the two-hybrid system has the virtue of being easy to perform. Once the essential steps of putting plasmids into yeast and getting them back out again, and distinguishing true positives from false ones, are learned, the method is no more difficult than other routine procedures in molecular biology. This simplicity means that once one protein has been successfully used in a search, it is possible to screen many more. In this sense, the method is largely insensitive to the individual properties of proteins that make them unique and interesting.

The popularity of the method is tremendously indebted to the contributions of laboratories such as those of Roger Brent, Steve Elledge, Dan Nathans, Richard Treisman, and Hal Weintraub, which early on began building vectors, libraries, and reporter strains. The willingness of these and other laboratories to freely provide these reagents meant that bugs in the procedure were worked out fairly quickly, new innovations came into play, numerous combinations of proteins were tested, and diverse proteins were used in searches. In addition to the yeast community, investigators in some fields, such as signal transduction and cell-cycle control, quickly adopted the technology and spread it to nearby laboratories. A few early successes, including searches with the retinoblastoma protein (5), the human immunodeficiency virus gag protein (6), Ras (7), and cyclin-dependent kinases (8–10), gave support to the idea that this could be a general method. The increasing availability of AD libraries also facilitated spread of the technology.

### 3. Unintended Consequences

While the possibility of carrying out library searches may have been implicit in the original idea, what we did not foresee was the potential for so many permutations and variations on the two-hybrid theme. The advent of one-hybrid systems (11,12) brought methodology to the analysis of DNA-protein interactions similar to what was becoming available for protein-protein interactions. Subsequently, three-hybrid systems were developed that may have comparable uses in the analysis of RNA-protein interactions (13) and small molecule-protein interactions (14,15). Specific protein-protein interactions could be analyzed to identify mutations that affected binding, particularly using a reverse two-hybrid assay (16,17). In other experiments, such mutations were correlated with structural information (18). The principle of using hybrid proteins to detect interaction was shown not to be limited to transcriptional activators. For example, the activities of ubiquitin (19), guanyl nucleotide exchange factor (20),  $\beta$ -galactosidase (21), dihydrofolate reductase (22), and adenylate cyclase (23) could also be split and reconstituted. Initial limitations to the two-hybrid

assay could be circumvented by mammalian-based systems, the presence of a small molecule, or the addition of a protein-modifying activity. The assay proved to be amenable, as well, to normally extracellular proteins (24) and to peptides (25,26). Finally, the ability to apply the two-hybrid method on a genomewide scale, initially for a small genome such as that of bacteriophage T7 (27) but later for much larger ones, means that many complexes and pathways may be amenable to this approach.

Phil Hieter has pointed out that yeast technologies, like yeast artificial chromosome construction and the two-hybrid system, have brought laboratories working on all kinds of biologic problems into contact with those working on yeast. While the initial contact is for technical advice, a side effect is the exchange of respective research ideas, sometimes resulting in collaborations. It is a delight to us to see that the two-hybrid system has brought together more than proteins.

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## Growth and Maintenance of Yeast

Lawrence W. Bergman

### 1. Introduction

On many occasions, baker's yeast (*Saccharomyces cerevisiae*) has been referred to as the *Escherichia coli* of the eukaryotic world. Yeast has been extensively characterized genetically and a complete physical map is now available. Much of the comparison to *E. coli* is based on the observations that culturing yeast is simple, economical, and rapid, with a doubling time in rich medium of approx 90 min. Cells divide mitotically by forming a bud, which is subsequently pinched off to form a daughter cell. Yeast can also be grown on a completely defined medium, which has allowed the isolation of numerous nutritional auxotrophs. This type of analysis has provided many mutations useful for genetic analysis and as selectable markers for plasmid manipulation.

Physiologically, yeast can exist stably in either haploid or diploid states, with the haploid cell being either of two mating types called **a** and  $\alpha$ . Diploid **a**/ $\alpha$  cells, formed by the fusion of an **a**-cell and an  $\alpha$ -cell, are stable mitotically. However, under conditions of carbon and nitrogen starvation, the diploid cell will undergo meiosis to produce four haploid spores. It is possible to recover all four haploid products of the meiosis individually, which may facilitate many types of studies.

The genome sequence of yeast is now known and the genome contains 16 linear chromosomes, ranging from approx 200 to 2200 kb. The functional units of the chromosomes have been identified, cloned, and characterized: origins of replication (ARS elements), centromeres (CEN elements), and telomeres. The combination of these elements with the auxotrophic selectable markers has led to the construction and utilization of numerous plasmids that vary in a number of properties (integrating vs extra chromosomal, high copy vs single copy, circular vs linear). Several of these plasmids are now commercially available, and

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procedures for the high-efficiency transformation of yeast with plasmid vectors and gene libraries have been available for more than 20 yr. Thus, the utility of the yeast as a vehicle for the two-hybrid system is evident.

The purpose of this chapter is to discuss the general laboratory principles used to grow and maintain yeast for use in the two-hybrid protein interaction assay. The goal is to provide a working knowledge of the general principles involved in working with yeast cells. Many of these general principles are highlighted throughout the more detailed protocols and formulations discussed in subsequent chapters.

## **2. Growth of Yeast Strains**

### **2.1. Growth in Liquid or Solid Medium**

Yeast can be grown in either liquid medium or on the surface of a solid agar plate. Yeast cells will grow on a minimal medium containing dextrose (glucose) as a carbon source and salts that supply nitrogen, phosphorus, and trace metals. Yeast cells grow much more rapidly in the presence of rich medium that contains reagents such as yeast extract and bactopectone. These provide many of the metabolites that the cells would synthesize when growing under minimal growth conditions. During log-phase growth in rich medium, yeast cells divide once approximately every 90 min. Early log phase is the period when cell densities are  $<10^7$  cells/mL. Mid-log phase is the period when densities are between  $1$  and  $5 \times 10^7$  cells/mL. Late log phase occurs when cell densities are between  $5 \times 10^7$  and  $2 \times 10^8$  cells/mL. The measurements of cell density are discussed later.

Detailed recipes for media that are commonly used for yeast are provided in Chapter 3. The rich medium yeast extract, peptone, dextrose (YPD) is most commonly used for growing yeast under nonselective conditions (e.g., when maintaining plasmid selection is not required). Note that some transformation procedures suggest a 4- 6-h period of growth in rich medium (despite the presence of plasmids in the cells) prior to the transformation process itself. In some cells, particularly those strains containing an *ade2* mutation, adenine may be added to YPD. Cultures of an *ade2* or *ade1* mutant will turn pink in 2 to 3 d. The formulas for synthetic complete media vary in the amount of adenine, such that some have amounts of adenine so high that colonies never turn pink or red. Autoclaving is usually carried out for 15 min at 15 lb/in.<sup>2</sup> but should be increased when larger volumes are prepared. It may be preferable to use a 20% solution of dextrose that has been autoclaved separately or filter sterilized, because this prevents caramelization or darkening of the medium and promotes optimal growth.

Minimal medium, also known as synthetic defined (SD) medium, supports the growth of yeast, which has no nutritional requirements. It contains yeast nitrogen base, ammonium sulfate, and dextrose. Minimal medium is commonly used when testing the mating type of yeast cells using specific mating tester strains (of both mating types). Minimal medium is most often used as a basal medium to which mixtures of amino acids and nucleoside precursors are added. SD dropout medium lacks a single (or several) nutrient that allows selection for maintenance of particular plasmids or selection for induction or repression of specific gene promoters. These two particular properties are the basis of the two-hybrid system.

In instances in which it is necessary to sporulate a diploid yeast cell, a nitrogen-deficient starvation medium containing acetate as a carbon source to promote respiration is used. A general formula for this medium is 1.0% potassium acetate, 0.1% yeast extract, and 0.05% dextrose, and sporulation of diploid cells can be carried out in liquid medium or on plates.

Wild-type yeast can use a variety of carbon sources other than glucose to support growth. In particular, raffinose and galactose are used under conditions to relieve glucose repression (in the case of raffinose) or to induce expression from a Gal4p-dependent promoter such as *GAL1* and *GAL10*. All are used at a concentration of 2.0% (wt/vol) (20 g/L) and are used to replace dextrose in either rich or defined medium.

As mentioned previously, yeast can grow on solid medium. For all plates, agar is added to a final concentration of 2.0% (20 g/L). To prevent agar breakdown during autoclaving, it is possible (although not necessary) to add a pellet of sodium hydroxide (per liter) to the medium-agar suspension. Care should be taken to avoid autoclaving the medium-agar suspensions longer than necessary, because this will cause agar breakdown leading to “soft” plates.

Specialty plates containing either 5-fluoro-orotic acid (5-FOA) or cycloheximide are used in negative selection experiments against the wild-type *URA3* or *CYH2* genes, respectively. Note that 5-FOA is quite expensive, it should be filter sterilized, and plates that contain 5-FOA should also contain uracil. Cycloheximide may be prepared as a filter-sterilized 10 mg/mL solution with the final working concentration in plates being 10 µg/mL.

Wild-type yeast grows well at 30°C with good aeration and glucose as a carbon source. Erlenmeyer flasks work well for growing liquid cultures, and baffled-bottom flasks are good but not necessary. Although small cultures may be grown in culture tubes, in many cases the cells will settle out from suspension. For optimal aeration and growth, the medium should constitute no more than 20% of the total volume of the flask, and growth should be carried out in a shaking incubator at 250–300 rpm. On solid YPD medium at 30°C, single colonies



may be seen after 24 h, but generally growth for at least 48 h is required prior to picking of colonies or replica plating. Growth on dropout medium is approx 50% slower than that observed in YPD.

## **2.2. Determination of Cell Density**

The approximate number of cells in a culture can be determined with a spectrophotometer by measuring the optical density (OD) at 600 nm. Cultures should be diluted such that the observed reading ( $OD_{600}$ ) is  $<1.0$ . In this range, an  $OD_{600} = 1.0$  is approximately equal to  $3 \times 10^7$  cells/mL. However, there is strain variability in this measurement, or it may be affected by overexpression of a particular gene product within a strain (such as a two-hybrid bait). It is best to determine this function by graphing the  $OD_{600}$  as a function of actual cell number that has been determined by counting in a hemocytometer or plating for viable colonies. Many transformation procedures utilize growth of the yeast culture to a certain cell density prior to harvesting.

## **3. Strain Preservation and Revival**

Yeast strains can be stored at  $-70^{\circ}\text{C}$  in 15% glycerol and are viable for more than 3 yr. Alternatively, they can be stored at  $4^{\circ}\text{C}$  on slants of rich medium for 6 mo to 1 yr. To prepare glycerol stocks, make a sterile solution of 30% glycerol (w/v). Pipet 1.0 mL of the solution into sterile 4-mL screw-cap vials. Add 1.0 mL of a late log or early stationary phase culture, mix, freeze on dry ice, and store at  $-70^{\circ}\text{C}$ . Revive the strain by scraping some cells off the frozen surface and streak onto plates. It is not necessary to thaw the entire vial. Cells can also be stored in a similar manner using 8% (v/v) dimethylsulfoxide (DMSO); however, the quality of the DMSO is critical. Yeast strains can be conveniently mailed as slants. Also, cells may be mailed after transfer to a piece of sterile Whatman 3MM paper. Dip the paper into a yeast culture or press onto a yeast colony using sterile forceps. Then wrap the filter paper in sterile aluminum foil and mail. The strain is revived by placing the paper onto the surface of an agar plate and incubating the plate at  $30^{\circ}\text{C}$  for several days.

## **4. Replica Plating**

Cells from yeast colonies grown on one medium can be tested for their ability to grow on another medium by replica plating. There are now several commercial sources for the purchase of both a replica-plating block and velveteen squares. A master plate containing the cells of interest is first printed onto sterile velvet. A copy of these cells on the velvet is then transferred to plates made with all the relevant selective media. In general, three or four copies may be made from a single master plate and up to five or six copies made from a single

square of velvet. This type of plating has application with the two-hybrid system for testing nutritional requirements (either different dropout media or different carbon sources), placing cells onto filter paper for  $\beta$ -galactosidase assays, or in mating studies.

## 5. Plasmid Segregation from Yeast

It is sometimes useful to generate a yeast strain that has only a single type of plasmid (as compared with multiple plasmids). As discussed, it is possible with certain plasmids to select against the presence of a plasmid (5-FOA or cycloheximide); however, this does not work for all plasmids. Alternatively, the yeast strain containing multiple plasmids is grown for several days in medium that maintains selection for the plasmid of interest but not on the plasmid you wish to lose. Under nonselective conditions, plasmids are estimated to be lost at a rate of 10–30% per generation. A diluted sample is then spread onto agar plates that will select only for the desired plasmid and after subsequent growth; individual colonies are picked and screened to verify loss of the unwanted plasmid and maintenance of the desired plasmid.

## 6. Mating Analysis and Two-Hybrid System

### 6.1. Diploid Construction

Diploid strains are constructed by mating strains of opposite mating types on the surface of agar plates. Mix cells from freshly grown colonies of each haploid parent in a small circle of approx 0.5 cm in diameter on an agar plate. The plates should allow growth of both haploid strains. Allow mating to occur for more than 4 h at 30°C. This time frame will allow mating of two strains containing plasmids without significant loss of the plasmid. Then, streak or replica the mating mixture onto a plate that will select for the genotype of the diploid. This type of procedure is particularly adaptable to testing an activation domain (AD) plasmid vs multiple baits.

### 6.2. Sporulation on Plates and in Liquid Medium

Starvation of diploid cells for nitrogen and carbon sources induces meiosis and the formation of spores. Sporulation can be induced in cells growing on solid or in liquid medium; however, there may be strain specifically as to whether the solid or liquid medium induces better formation of spores.

On plates, cells that have been grown on YPD or selective plates are patched onto a sporulation plate and incubated for 3–5 d at 25°C, because sporulation is usually less efficient at higher temperatures. Generally, formation of spores is monitored microscopically ( $\times 250$ –400) by looking for the presence of tetrads

(clusters of four small spheres within a small sac or ascus). The proportion of cells that undergoes sporulation as well as the fraction of four-spore asci varies from one strain to another.

As stated, some strains sporulate better in liquid culture. Often sporulation in liquid medium is completed within 48 hours and is not inhibited by higher incubation temperatures. Briefly, cells are grown in YPD or selective medium to late log phase or early stationary phase. One milliliter of culture is collected by centrifugation, washed with sterile water, and resuspended in 1.0 mL of sporulation medium. These cells are incubated for 2 to 3 d at 30°C with shaking (300–350 rpm) and examined microscopically for the formation of tetrads.

### **6.3. Random Spore Analysis**

Although it is possible to isolate individual haploid spores, this generally requires a light microscope with a movable stage, a micromanipulator, and a skilled yeast geneticist. As an alternative, haploid spores can be released from the ascus and plated directly onto agar plates. Cells are collected from either the sporulation plate or sporulation liquid culture and washed with sterile water. The preparation is treated with glusulase (an extract from snails) in sterile water for 20 min and then is plated directly on YPD plates or selective plates for the appropriate haploid. It is always necessary to test individual colonies obtained in this procedure to ensure that the cells are haploid. This can be accomplished by mating the selected cells with a pair of mating-type tester strains for the ability of the selected cells to form diploids (a property of haploid cells).

### **6.4. Use of Pretransformed AD Libraries**

It is now possible to obtain yeast cells that have been previously transformed with an AD library of interest. This circumvents the low-efficiency transformation of yeast (low as compared to *E. coli*) and substitutes a mating procedure to introduce the bait plasmid. In this procedure, an excess of yeast cells of one mating type containing the bait plasmid is allowed to mate with cells of the opposite mating type containing the AD plasmid. This occurs in solution for 20–24 h at 30°C in YPD supplemented with adenine (if necessary) with gentle aeration (30–50 rpm). This prevents the yeast from settling to the bottom of the flask, whereas rapid shaking reduces mating efficiency. The cells are then collected and plated to conditions that allow growth of only the diploid cells. Titration of the number of cells screened and the mating efficiency are necessary in this approach. Another valuable application of this protocol is screening of the same library with multiple baits, thus avoiding numerous large-scale transformations.

## Media Formulations for Various Two-Hybrid Systems

Michael Saghbini, Denise Hoekstra, and Jim Gautsch

### 1. Introduction

The two-hybrid system, which was originally developed by Fields (*1*), represents the only successfully tested tool to study protein-protein interaction in a living cell. *Saccharomyces cerevisiae*, the species of choice for this system, is a model eukaryotic organism with a stable, well-defined, and easy-to-manipulate genetic system. It is available in many different strains with stable nutritional marker mutations permitting rescue with vectors carrying the appropriate wild-type markers.

*S. cerevisiae* is essentially processed like bacteria. Culturing is simple, economical, rapid, and nonhazardous. This yeast grows nonselectively on rich medium (yeast extract, peptone, dextrose [YPD]) and selectively (i.e., with selected vectors) on synthetic defined (SD) minimal medium containing the appropriate dropout nutritional supplement. Supplements enhance the growth of yeast cells by providing amino acids and nucleotide precursors, thereby bypassing the need for *de novo* synthesis (*2,3*).

Media for *S. cerevisiae* are readily available from commercial sources as individual components, powder mixes, or pre-poured plates. The composition of rich medium is well defined, yet certain deletion mutant strains grow differently on YPD obtained from different sources. The components of minimal medium are also well defined with the exception of the nutrient supplements. With the advent of improved versions of the original two-hybrid system, different nutrient supplements have emerged, sometimes with minor variations (*4–7*). Making the various supplement mixes required for a successful screen, as most two-hybrid system suppliers recommend, is impractical and time-

**Table 1**  
**YPD Formulation**

Final concentration	Amount per liter (g)
1% Yeast extract	10
2% Peptone	20
2% Dextrose (D-glucose)	20
2% agar	20 (only solid medium)

consuming. As a matter of convenience, we have organized dropout nutrient supplements by closest match to readily available dropout mixtures (BIO101).

In an attempt to facilitate assignment of minimal medium required for a particular task in a two-hybrid screen, the genotypes of reporter strains are tabulated in an easy-to-read format. Media requirements are presented for the three major two-hybrid systems: system I (4), system II (5), and system III (6). Media requirements are also presented for the following specialized systems: the split and reverse hybrid systems (8,9), the trihybrid system with an inducible third party expression (10), the trihybrid system for detecting RNA-protein interactions (11), the trihybrid system for detecting protein-protein interactions dependent on posttranslational modifications (12), the SOS-based recruiting system (13), the one-hybrid system (14–16), and the mammalian two-hybrid system (17,18). In all cases, reference to commercially available two-hybrid systems is made when applicable.

## 2. Yeast Rich Medium (YPD)

### 2.1. Composition

YPD, also called YEPE, is the most commonly used rich medium for growing *S. cerevisiae* when special conditions are not required. It provides an excess of amino acids, nucleotide precursors, vitamins, and essential metabolites needed for optimal cell growth (Table 1). Yeast cells divide every ~90 min when grown in YPD during the exponential phase of the growth cycle (2,3). YPD can be prepared as a broth or a solid medium with the addition of agar to a final concentration of 2%. Sterile YPD broth can be stored at room temperature for several months. YPD plates can be stored in sealed plastic bags for more than 3 mo at room temperature or 4°C.

### 2.2. Source of YPD Makes a Difference in Growth Rate of Certain Mutant Yeast Strains

YPD has a defined composition yet there are many different sources for the individual components that define this medium. Figure 1 shows serial dilution

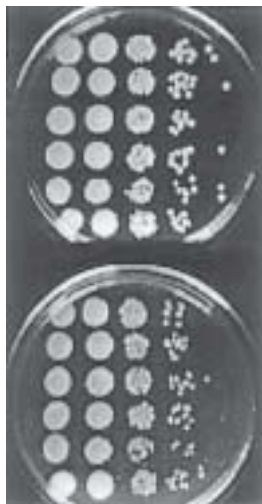


Fig. 1. Growth on YPD obtained from different sources. Several deletion mutant yeast strains are spotted at four increasing dilutions on YPD plates made from ingredients obtained from different sources (top plate vs bottom plate). Photograph is taken after 2 d of growth at 30°C.

of several mutant yeast strains grown on YPD obtained from different sources (top plate vs bottom plate). The slower growth rate observed at the highest dilution (bottom plate far right vs top plate) is an indication that not all YPD rich media are equivalent.

### **2.3. The Addition of Adenine to YPD Enhances Growth of Certain Yeast Two-Hybrid Strains**

Yeast reporter strains with a mutation in the *ADE2* gene (*ade2*) accumulate a red pigment and appear as red colonies (19). The accumulation of this pigment slows growth (20). The addition of adenine slows the accumulation of red pigment, leading to enhanced growth rate. **Table 2** lists the various yeast two-hybrid strains that are grown on YPD containing adenine, YPAD. Different amounts of adenine are sometimes used than the 0.003% recommended by Sherman (2).

### **2.4. Uses of YPD/YPAD in Two-Hybrid Screens**

The rich medium YPD/YPAD is used for propagation of reporter strains and for growth whenever selection is not required. It can be used with positive selection agents such as Zeocin™. This antibiotic is used to select recombinant yeast, bacteria, and insect and mammalian cells carrying the ble gene product

**Table 2**  
**Two-Hybrid Strains Grown on YPAD**

Strain	Reference	Recommended amount of adenine added to YPD
L40	<b>5</b>	0.01% Adenine
cdc25-2	<b>13</b>	0.004% Adenine sulfate
YRG-2	<b>21</b>	0.004% Adenine sulfate
S-260	<b>22</b>	0.003% Adenine sulfate
PJ69-2A	<b>7</b>	0.003% Adenine sulfate

([23]; Invitrogen). Rich medium is also used for recovery purposes prior to plating cells from a large-scale transformation or a mating assay on selective medium (4,5). Mating assays facilitate the processing of putative positive yeast clones obtained from two-hybrid screens.

### 3. Yeast SD Medium

#### 3.1 Definition

SD medium for yeast is also known as complete minimal and synthetic complete. It consists of a defined mixture of salts, vitamins, and a nitrogen source collectively known as yeast nitrogen base (YNB; ref. 24; Table 3) to which a carbon source, usually dextrose, is added along with nutrient supplements consisting of various amino acids and nucleotide precursors (Table 4). SD medium supports the vigorous growth of virtually all strains of *S. cerevisiae* with a doubling time of ~140 min during the exponential phase of growth (2,3). SD lacking nutrients is known as omission or dropout medium. It is used to select for yeast containing vectors with specific nutritional markers. For example, dropout medium lacking tryptophan and leucine (SD–trp–leu) selects for the presence of plasmids carrying the TRP and LEU markers. Dropout medium containing agar is referred to as SDA.

#### 3.2. Different Carbon Sources Used in SD Medium

Yeast cells can grow on a variety of carbon sources. The most commonly used carbon/energy source is D-glucose (dextrose). Other fermentable carbon sources also can be used such as galactose, maltose, fructose, and raffinose. They are added to a final concentration of 2% to either rich or minimal medium (2,3). Glucose-free galactose (<0.01% glucose) is particularly useful to induce transcription of genes fused to Gal1/Gal10 promoters. The incorporation of this inducible expression cassette into the library vector of yeast two-hybrid



**Table 3**  
**YNB Formulation**

Nitrogen Source	
Ammonium sulfate	5000 mg/L
Salts	
Potassium phosphate, monobasic	1000 mg/L
Magnesium sulfate	500 mg/L
Sodium chloride	100 mg/L
Calcium chloride	100 mg/L
Vitamins	
Biotin	0.002 mg/L
Pantothenate, calcium	0.4 mg/L
Folic acid	0.002 mg/L
Inositol	2 mg/L
Niacin	0.4 mg/L
PABA	0.2 mg/L
Pyridoxine, Hcl	0.4 mg/L
Riboflavin	0.2 mg/L
Thiamine, Hcl	0.4 mg/L
Trace Elements	
Boric acid	0.5 mg/L
Copper sulfate	0.04 mg/L
Potassium iodide	0.1 mg/L
Ferric chloride	0.2 mg/L
Magnesium sulfate	0.4 mg/L
Sodium molybdate	0.2 mg/L
Zinc sulfate	0.4 mg/L
Total	6.7 g/L

systems allows the detection of interacting proteins that are potentially toxic to yeast cells (6). Usually 1% raffinose is added to minimal medium containing 2% galactose to give the cells a better growth advantage without affecting the induction process. In addition, nonfermentable carbon sources can be used such as glycerol, ethanol, and acetate: 3% glycerol, 3% ethanol, 2% glycerol + 2% ethanol, and 2% potassium acetate (2,3).

### 3.3. Nutritional Supplements

The addition of a mixture of amino acids containing uracil and adenine to YNB containing a carbon source completes the SD growth medium. Dropout nutrient supplements can be made by mixing the various components, as per

**Table 4**  
**Nutritional Supplements for Various Yeast Two-Hybrid Systems**

Nutritional supplements <sup>c</sup>	CSM		HSM		BSM	
	(mg/L)	Ref.	(mg/L)	Ref.	(mg/L)	Ref.
Two-hybrid, trihybrid, and split-hybrid yeast reporter strains <sup>a</sup>	SFY526	(25)				
	CG1945	(26)				
	HF7c	(26)				
	Y190	(27)				
	Y187	(27)			S-260	(22)
	AH109 <sup>d</sup>				YRG-2	(21)
	CY770	(28)			cdc25-2	(13)
	Y153	(29)			EGY48	(32)
	AMR70	(5)			EGY191	(32)
	GGY : : 171	(30)			EGY194	
	YI 584,					
	YI596	(8)				
	Y1671	(8)	L40-coat	(11)		
	pJ69-4A	(7)	L40-ura	(11)	MaV95, 96, 97	
	YPB2	(31)	L40	(4)	103, and 203	(9)
One-hybrid yeast reporter strains <sup>b</sup>	W303	(33)				
	GGY1	(14)				
	YM4271	(16)				
	yWAM2	(15)				
	YM954,					
	YM955	(34)				
Aspartic acid	80		50		100	
Serine			50		375	
Glutamic acid					100	
Histidine	20		50		20	
Arginine	50		100		20	
Threonine	100		100		200	
Proline			50			
Cysteine			100			
Tyrosine	50		50		30	
Valine	140		50		150	
Methionine	20		50		20	
Lysine	50		100		30	
Isoleucine	50		50		30	
Leucine	100		100		60	
Phenylalanine	50		50		50	
Tryptophan	50		100		40	

**Table 4 (continued)**

Nutritional supplements <sup>c</sup>	CSM		HSM		BSM	
	(mg/L)	Ref.	(mg/L)	Ref.	(mg/L)	Ref.
Adenine		10		100		40
Uracil		20		100		20
Total medium (mg/L)		790		1250		1285

<sup>a</sup>The genotype for these strains is shown in **Table 5**.

<sup>b</sup>The genotype for these strains is shown in **Table 6**.

<sup>c</sup>All listed strains can use the CSM supplement mixture with no effect on growth rate. CSM, BSM, and HSM are available from Qbiogene in any dropout combination.

<sup>d</sup>Available from Clontech.

CSM, complete supplement mixture; BSM, Brent supplement mixture; HSM, Hollenberg supplement mixture.

formulation, and grinding to a fine powder with a ball mill or a mortar and pestle; they can also be obtained commercially.

Because accurate and homogeneous mixtures are essential for successful and reproducible two-hybrid screens, strict guidelines are needed for commercial manufacturing. The production process should follow good laboratory practice guidelines. Ideally, raw materials are carefully chosen for their ability to promote vigorous growth on yeast minimal medium. Computerized nutrient dropout recipes automatically calculate the amounts of raw materials needed for each dropout mixture while keeping track of inventory and lot number assignments. Bar-coded raw materials are carefully measured on computerized electronic scales to keep track of amounts and components used for each batch. After a careful check of composition by quality assurance personnel, the dropout mixes are individually milled to a fine powder. They are further verified by high-performance liquid chromatography (HPLC) analysis before packaging.

Different supplement mixtures, sometimes with minor variations, have been recommended with the advent of various versions of the original yeast two-hybrid system (4–7). Making these dropout supplements is a tedious and time-consuming process. **Table 4** matches the nutritional requirement for the various yeast reporter strains to dropout nutrient mixes produced by Qbiogene following the strict guidelines outlined in this subheading.

### 3.4. HPLC Analysis of Supplements

Nutritional supplements consist of a defined mixture of amino acids and nucleotide precursors (**Table 4**). Thus, it is possible to detect their components by HPLC analysis using commercially available kits. The kit used in our laboratory is the Waters AccQ-Fluor™ Reagent kit (cat. no. 052880). Amino acids are derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and

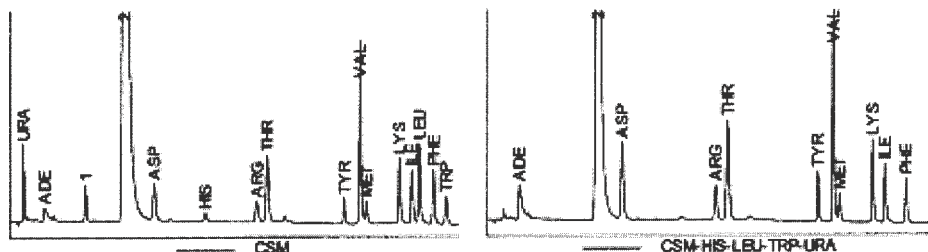


Fig. 2. HPLC analysis of nutrient supplement mixtures. Chromatograms for CSM and CSM-his-leu-trp-ura is shown. This is taken from the certificate of analysis for that quadruple dropout (Qbiogene). 1, underivatized TRP; 2, derivatizing agent.

separated by reverse-phase HPLC on a C18 column. They are then detected using a an ultraviolet detector at 554 nm.

The nucleotide precursors adenine and uracil are normally not detected once the nutrient supplement mixture is derivatized. However, the untreated adenine and uracil can be detected at 554 nm at concentrations used in minimal dropout medium. Injecting a derivatized mixture followed by an underivatized one allows the detection of adenine, uracil, and the rest of the nutrient supplement components on a single chromatogram (Fig. 2).

#### 4. Preparation and Storage of Specialized Yeast/Bacterial Minimal Medium Used in Two-Hybrid Screens

##### 4.1. Yeast Minimal Medium Containing 3-Amino-1,2,4-triazole

3-Amino-1,2,4-triazole (3-AT) is used to suppress leaky *HIS* reporter genes by competitively inhibiting the *HIS3* gene product (4). The amount of 3-AT employed is host strain and bait dependent, varying from a 0 to 100 mM final concentration. It must be empirically determined via titration experiments. The lowest possible concentration of 3-AT should be used to minimize the negative effect on transformation efficiency.

##### 4.1.1. Preparation of Medium Containing 3-AT

1. Prepare a 1 M 3-AT stock solution in H<sub>2</sub>O by heating at 50°C to dissolve all solids if necessary. Sterile filter and store at 4°C for ~1 mo.
2. Add the appropriate amount of 3-AT to autoclaved minimal medium ± agar that has been cooled to ~50°C, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing 3-AT should be poured thick (110–120 mL of medium/15-cm plate) to prevent drying during the screening process as the appearance of putative positive colonies is monitored for up to 10 d following a two-hybrid screen. Plates containing 3-AT are stable for ~1 mo when stored at 4°C in sealed plastic bags.

## 4.2. Yeast Minimal Medium Containing Cycloheximide

Cycloheximide blocks peptide elongation during translation. It is added to a final concentration of 1–10 µg/mL of medium depending on the yeast reporter strain used; for example, Y190 requires only 2.5 µg/mL (4). Cycloheximide acts to prevent the growth of cells that contain the wild-type *CYH2* gene. The loss of vectors containing the *CYH2* allele confers a growth advantage to cells that carry the resistant allele chromosomally (*cyh<sup>R</sup>*) when grown on medium containing cycloheximide (35). Incorporation of the *CYH2* gene into the bait vector permits the selective loss of that vector. This facilitates the introduction of nonspecific baits, by mating, to test the authenticity of interaction of putative positive yeast clones following a successful two-hybrid screen (4).

### 4.2.1. Preparation of Medium Containing Cycloheximide

1. Prepare a 10 mg/mL cycloheximide stock solution in H<sub>2</sub>O and sterile filter. Store at –20°C for later use.
2. Add the appropriate amount of cycloheximide to autoclaved minimal medium ± agar that has been cooled to ~50°C, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing cycloheximide are stable for 2 to 3 mo when stored at 4°C in sealed plastic bags.

## 4.3. Yeast Minimal Medium Containing 5-Fluoroorotic Acid

5-Fluoroorotic acid (5-FOA) is converted to a toxic product, 5-fluorouracil, by the *URA3* gene product. Yeast cells that contain the *URA3* marker grow on medium lacking uracil and are unable to grow on medium containing 5-FOA. This property is used to select for the loss of vectors carrying the wild-type marker in certain two-hybrid systems (11). It is also used to design a reverse two-hybrid system that positively selects for dissociation of interacting proteins (9).

### 4.3.1. Preparation of Medium Containing 5-FOA

1. Add up to 2 g of 5-FOA to 500 mL of H<sub>2</sub>O and dissolve by stirring at 50–60°C for ~1 h (see Note 1).
2. Add 5 mL of 2.4 mg/mL uracil and sterile filter (see Note 2).
3. Combine with 500 mL of 2X minimal dropout medium ± agar that has been autoclaved and cooled to ~50°C.
4. Swirl to mix and pour plates if preparing agar-containing medium. Plates containing 5-FOA are stable for 2 to 3 mo when stored at 4°C. The recipe can be scaled to any desired amount.

## 4.4. Yeast Minimal Medium Containing 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

*LacZ* is a common reporter gene in yeast two-hybrid strains. Checking for β-galactosidase activity can be accomplished in several ways with different

levels of sensitivity. The least sensitive detection method involves growing colonies on 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) plates. One yeast reporter strain (EGY48) can be used with this detection method (6). The filter lift assay is much more sensitive than growing cells on X-gal plates, and it is the most commonly used method (36). The liquid assay can be the most sensitive method if using a chemiluminescent substrate, but it requires a luminometer or scintillation counter (Galacton-Star®; Clontech)

#### 4.4.1. Preparation of X-Gal Medium Plates

1. Add dropout minimal medium powder, enough to make 1 L, to 900 mL of H<sub>2</sub>O. Add 20 g of agar, autoclave at 121°C for 15 min, and cool to ~50°C.
2. Add 2 mL of 20 mg/mL X-gal in *N,N*-dimethylformamide.
3. Add 100 mL of 1 *M* potassium phosphate, pH 7.0, that has been sterilized by autoclaving, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing X-gal are stable for 2 to 3 mo when stored protected from light at 4°C in sealed plastic bags.

#### 4.5. Yeast Minimal Medium Containing Tetracycline

Tetracycline is used in the split-hybrid system to modulate the tetracycline repressor (TetR) activity if the bait has intrinsic activation capabilities (8).

##### 4.5.1. Preparation of Medium Containing Tetracycline

1. Prepare a 25 mg/mL tetracycline stock solution in 50% ethanol. Store at –20°C for later use.
2. Add the recommended concentration of tetracycline to autoclaved minimal medium  $\pm$  agar that has been cooled to ~50°C and pour plates if preparing agar-containing medium. Plates containing tetracycline are stable for ~ 1 mo when stored protected from light at 4°C in sealed plastic bags.

#### 4.6. Bacterial Medium for Selection of Vectors Carrying Yeast Markers

An essential step in two-hybrid screens is determining the authenticity of the interaction between the newly uncovered library proteins and the bait. One strategy requires the isolation of library vectors from putative positive yeast clones to be later reintroduced with the original bait and nonspecific baits after amplification in bacteria. If both bait and library vectors carry the same bacterial antibiotic marker, as is the case with most two-hybrid systems, it is still possible to selectively recover the library vector. This selective recovery is based on the ability of the *TRP* and *LEU* yeast marker, carried on library vectors, to complement bacteria with a *trpC* and *leuB* mutation, respectively.

#### 4.6.1. Plates for Selective Recovery of Library Vectors Carrying TRP Marker: Bacterial Strain KC8 (6)

1. Add 11.3 g M9 minimal salts 1 part formulation (cat. no. 3037-012; Qbiogene), 0.74 g of CSM-trp (cat. no. 4510-012), and 20g of agar to 990 mL of H<sub>2</sub>O. Autoclave and cool to ~50°C.
2. Add 10 mL of 20% sterile glucose.
3. Add 1 mL of sterile filtered 10 mg/mL thiamine-HCl.
4. Add the appropriate amount of antibiotics. Swirl to mix and pour plates. Plates are stable for several months at 4°C in sealed plastic bags.

#### 4.6.2. Plates for Selective Recovery of Library Vectors Carrying LEU Marker: Bacterial Strains KC8, HB101, RR1, JA226, and C600

Prepare medium as in **Subheading 4.6.1** except use 0.69 g of CSM-leu (cat. no. 4510-512). If using HB101 or RR1, add 4 mL of 10 mg/mL sterile, filtered proline. If using C600, add 4 mL of 10 mg/mL sterile, filtered threonine and 4 mL of 10 mg/mL sterile, filtered proline.

### 5. Sterilization of Yeast Medium: Filtration vs Autoclaving

Autoclaving for 15 min at 121°C (15 psi) is the classic way to sterilize yeast minimal (SD and SD dropouts) and rich media (YPD) in either solid or liquid format. All standard components—yeast extract, peptone, dextrose, YNB, and nutritional supplements—are added together as per formulation to the appropriate amount of water before autoclaving. Temperature-sensitive compounds such as 3-AT, 5-FOA, cycloheximide, tetracycline, Zeocin, and X-gal are added to autoclaved medium after it has cooled to ~50°C from a sterile, filtered stock solution.

Autoclaving agar containing yeast minimal medium too long can lead to soft plates ([6]; personal observations). Yeast minimal and rich liquid media can be sterile filtered, resulting in faster preparation, less caramelization of carbohydrates, and faster growth of cells (2,3,37). To prepare sterile, filtered agar medium, a 2X agar solution (20 g/500 mL of H<sub>2</sub>O) is prepared and autoclaved for 15 min at 121°C. After cooling to ~50°C, it is mixed with 500 mL of a 2X sterile, filtered minimal dropout medium. The resulting plates exhibit a firm agar surface, more so than their autoclaved counterpart, and will support healthier growth. **Figure 3** shows a growth advantage for one of two randomly selected putative positive yeast clones grown at increasing dilutions on sterile, filtered (right plate, bottom half) vs autoclaved (left plate, bottom half) SD dropout plates. Prepoured sterile, filtered plates are commercially available for all yeast two-hybrid systems from Qbiogene.



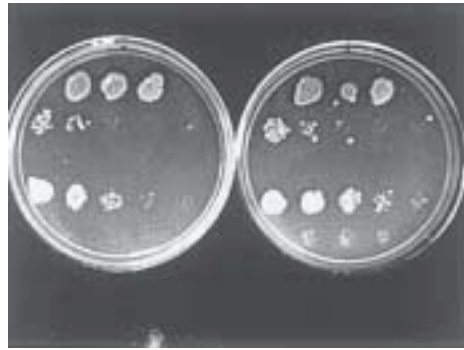


Fig. 3. Growth on sterile, filtered vs autoclaved SD dropout plates. Two randomly selected His<sup>+</sup> and LacZ<sup>+</sup> yeast clones from a two-hybrid screen (top half and bottom half of either plate) are plated at increasing dilutions on SD–trp–leu–his + 2 mM 3-AT prepared by autoclaving (left plate) and sterile filtration (right plate). The yeast reporter strain used is CG1945. Photograph is taken after 3 d of growth at 30°C.

## 6. Genotypes for Various Yeast Two-Hybrid Reporter Strains

Reporter strains with the *ade2* mutation exhibit a pink to red colony color in medium containing low adenine (19). The color may turn darker as the colony ages and the adenine becomes depleted. White colonies will normally form at a rate of 1 to 2% owing to spontaneous mutations that disrupt mitochondrial function. These petite colonies, which grow to a very small colony size (<1 mm in diameter), are to be avoided in two-hybrid screens.

It is generally good practice to verify the genotype of yeast reporter strains as to markers and reporter genes by growing on the appropriate dropout medium. For example, Y190 should display the phenotype Leu<sup>–</sup>, Trp<sup>–</sup>, Ura<sup>+</sup>, and Lys<sup>+</sup> when grown on SD–leu, SD–trp, SD–ura, and SD–lys, respectively. **Tables 5** and **6** show the genotypes for the various yeast two-hybrid and one-hybrid reporter strains, respectively.

## 7. Media for Various Yeast Two-Hybrid Systems

### 7.1. Introduction

Different two-hybrid systems have been devised in an attempt to improve on the original system introduced by Fields (1). Yeast strains with improved reporter genes and more user-friendly bait and library vectors were developed (systems I, II, and III). A two-hybrid system with a cytoplasmic interaction was introduced (13). Three-hybrid systems were developed with different applications (10–12). A one-hybrid system to identify DNA-binding proteins (14–16) and split-hybrid systems that select for dissociation of interacting pro-

teins (8,9) were also developed. These various systems use different yeast reporter strains and dropout minimal medium to carry out their desired function.

Yeast dropout minimal medium is designated as SD-nutrient. It consists of YNB containing ammonium sulfate, 2% D-glucose, and the appropriate nutrient dropout (**Table 4**)  $\pm$  agar. SD(gal)-nutrient represents a substitution of dextrose with 2% galactose. SD(gal/raff)-nutrient represents a substitution of dextrose with 2% galactose and 1% raffinose.

### **7.2. Media for the Three Major Two-Hybrid systems**

**Tables 7** and **8** give the media and vectors for systems I–III, respectively. **Table 9** lists the commercial two-hybrid systems based on systems I–III.

### **7.3. Media for SOS Recruiting System**

**Table 10** provides the media and their functions for the SOS recruiting system.

### **7.4. Media for Trihybrid Systems**

Three different trihybrid systems have been introduced: one with an inducible third-party expression under the control of a methionine regulated promoter (**10**), one to identify RNA-binding proteins (**11**), and one for the identification of protein-protein interaction dependent on posttranslational modifications (**12**).

#### **7.4.1. Media and Their Functions for Trihybrid System with an Inducible Third-Party Expression**

**Table 11** provides the media and their functions for the trihybrid system with an inducible third-party expression.

#### **7.4.2. Media for Trihybrid System to Identify RNA-Protein Interactions**

**Table 12** provides the media for identifying RNA-protein interactions in the trihybrid systems. An alternative version of the trihybrid system for the analysis of RNA-protein interactions has been reported (**39**). It uses the yeast reporter strain CG-1945. The equivalent of LexA-MS2 fusion protein and MS2-RNAx hybrid RNA are expressed from a single TRP vector. The library is expressed from a LEU vector. Libraries for these two systems have the same selectable markers yet they cannot be interchangeably used because one is LexA based (**11**) and the other is GAL 4 based.

#### **7.4.3. Media for Trihybrid System for Identifying Protein–Protein Interaction Dependent on Posttranslational Modifications**

**Table 13** provides the media for identifying protein–protein interactions dependent on posttranslational modifications.

**Table 5**  
**Genotypes for Yeast Two-Hybrid, Trihybrid, and Split-Hybrid Reporter Strains**

Strain <sup>a</sup>	Type	Markers	Other	Reporter
<i>Y190<sup>b</sup> (27)</i>	<i>a</i>	<i>trp, leu, cyh<sup>R</sup></i>	<i>ade2-101, ura3-52</i> <i>gal4, gal80, his3Δ200</i>	<i>URA3::GAL1-lacZ</i> <i>LYS2::GAL1-HIS3</i>
<i>Y187 (27)</i>	$\alpha$	<i>trp, leu, his</i>	<i>ade2-101, met<sup>-</sup></i> <i>gal4, gal80, ura3-52</i>	<i>URA3::GAL1-lacZ</i>
<i>L40<sup>b</sup> (38)</i>	<i>a</i>	<i>trp, leu</i>	<i>ade2, Lys2-801am</i> <i>GAL4, his3Δ200</i>	<i>URA3:: (Lex A op)<sub>8</sub>-lacZ</i> <i>LYS2:: (Lex A op)<sub>4</sub>-HIS3</i>
<i>L40-ura3<sup>b</sup> (11)</i>	<i>a</i>	<i>trp, leu, ura</i>	<i>ade2, his3Δ200</i> <i>ura3-52</i>	<i>ura3:: (Lex A op)<sub>8</sub>-lacZ</i> <i>LYS2:: (Lex A op)<sub>4</sub>-HIS3</i>
<i>AMR70 (38)</i>	$\alpha$	<i>trp, leu, his</i>	<i>Lys2-801am</i>	<i>URA3:: (lexAop)<sub>8</sub>-LacZ</i>
<i>EGY48 (32)</i>	$\alpha$	<i>trp, his, ura</i>		<i>(LexA op)<sub>6</sub>-LEU2</i>
<i>EGY191 (32)</i>	$\alpha$	<i>trp, his, ura</i>		<i>(LexA op)<sub>2</sub>-LEU2</i>
<i>cdc25-2 (13)</i>	$\alpha$	<i>trp, leu, ura, his</i>	<i>ade2-101, lys2-801</i> <i>GAL+</i>	<i>Cdc25-2</i>
<i>HF7C (26)</i>	<i>a</i>	<i>trp, leu</i>	<i>ade2-101, lys2-801</i> <i>Gal4-542, gal80-538</i> <i>ura3-52, his3Δ200</i>	<i>URA3:: (GAL4-17mer)<sub>3</sub>-CYC1-LacZ</i> <i>LYS2:: GAL1-HIS3</i>
<i>YI584<sup>b</sup> (8)</i>	<i>a/α</i>	<i>trp, leu</i>	<i>ade2/ade2</i> <i>hisΔ200/hisΔ200</i>	<i>URA3:: (LexA op)<sub>8</sub>-TetR</i> <i>LYS2:: (Tet op)<sub>2</sub>-HIS3</i>
<i>SFY 526 (25)</i>	<i>a</i>	<i>trp, leu, his</i>	<i>ade2-101, lys2-801</i> <i>gal4-542, gal80-538</i> <i>can<sup>I</sup>, ura3-52</i>	<i>URA3:: GAL1-LacZ</i>
<i>AH109<sup>b</sup></i>	<i>a</i>	<i>trp, leu</i>	<i>gal4Δ gal80Δ</i> <i>ura3-52, his3Δ200</i>	<i>URA3:: MEL1-LacZ</i> <i>LYS2:: GAL1-HIS3</i> <i>GAL2-ADE2</i>

<i>PJ69-4A (7)</i>	<i>a</i>	<i>trp, leu, ura,</i>	<i>gal4Δ gal80Δ</i> <i>his3-200</i>	<i>met2 : : GAL7-LacZ</i> <i>LYS2 : : GAL1-HIS3</i> <i>GAL2-ADE2</i>
<i>GGY : : 171 (30)</i>	<i>α</i>	<i>leu, his</i>	<i>ade2, met<sup>-</sup>, tyr1,</i> <i>gal4Δ gal80Δ, ura3-52</i>	<i>URA3 : : GAL1-lacZ</i>
<i>YPB2<sup>b</sup> (31)</i>	<i>a</i>	<i>trp, leu,</i>	<i>ade2-101, lys2-801, can<sup>R</sup></i> <i>gal4-542, gal80-538</i> <i>ura3-52, his3Δ200</i>	<i>URA3 : : GAL17mer-CYC1-lacZ</i> <i>LYS2 : : GAL1-HIS3</i>
<i>S-260 (22)</i>	<i>α</i>	<i>trp, leu, ura</i>	<i>ade2-101, can1-100, ho</i>	<i>ura3 : : (Col E1 op)<sub>6</sub>-LacZ</i>
<i>MaV103b (9)</i>	<i>a</i>	<i>leu, trp, cyh2<sup>R</sup></i>	<i>can1<sup>R</sup>, ade2-101</i> <i>ura3-52, his3Δ200</i> <i>gal4Δ gal80Δ</i>	<i>SPAL10 : : URA3</i> <i>GAL1-LacZ</i> <i>LYS2 : : GAL1-HIS3</i>

<sup>a</sup>YI596b is similar to YI584b except that the Tet repressor gene is driven by the ADH promoter: URA3 : : (LexA op)<sub>8</sub>-ADH-TetR (DeMaggio, personal communication).

YI671<sup>b</sup> is similar to YI596 except that it is haploid: MAT *α* (DeMaggio, personal communication).

Y153<sup>b</sup> (29) is identical to Y190 except that it is cycloheximide sensitive: *CYH2*.

CY770<sup>b</sup> (28) is derived from Y190 by selection on 5-FOA medium to select for loss of URA : : GAL1-LacZ.

YRG-2 (21) is a derivative of HF7C selected for high-efficiency transformation. It has the same genotype as HF7C.

CG1945 (26) is a derivative of HF7C selected for cycloheximide resistance: *cyh<sup>R</sup>*.

MaV203<sup>b</sup> (9) is similar to MaV103 except that it is MAT $\alpha$ .

MaV95, 96, 97<sup>b</sup> (9) are similar to MaV103 with the exception of fewer SPAL sites 5, 7, and 8, respectively.

EGY194 is similar to EGY48 except that it has four *lexA* operators in the *LEU2* reporter gene: (*LexA op*)<sub>4</sub>-*LEU2*.

<sup>b</sup>The *HIS* reporter gene has different levels of basal expression requiring different amounts of 3-AT to suppress background growth in two-hybrid screens.

**Table 6**  
**Genotypes for Yeast One-Hybrid Reporter Strains**

Strain	Type	Markers	Other
YM954 (34)	<i>a</i>	<i>trp, leu, ura, his, lys</i>	<i>ade2-101, can<sup>R</sup>, gal4Δ542, gal80Δ538</i>
YM955 (34)	<i>a</i>	<i>trp, leu, ura, his, lys</i>	<i>ade2-101, can<sup>R</sup>, gal4Δ542, gal80Δ538, tyr1-501</i>
GGY1 (14)	$\alpha$	<i>leu, ura, his</i>	<i>ade2, tyr, Δgal4, Δgal80</i>
W303-1a (33) <sup>a</sup>	<i>a</i>	<i>trp, leu, ura, his</i>	<i>ade2-1, can1-100</i>
yWAM2 (15)	$\alpha$	<i>trp, leu, his</i>	<i>ade2-101, lys2-801, CYH2, Δgal4, Δgal80</i>
YM4271 (16)	<i>a</i>	<i>trp, leu, his, ura, lys</i>	<i>URA3::GAL1-LacZ, ade2-101, ade5, tyr1-501, Δgal4, Δgal80</i>

<sup>a</sup>W303-1b (33) is similar to W303-1a except that it is MAT  $\alpha$ .

### 7.5. Media for Split/Reverse Two-Hybrid Systems

Two systems that positively select for dissociation of interacting proteins have been defined. The split-hybrid system (*see Table 14*) uses the *HIS3* reporter gene under the control of the Tet R repressor. Interaction between the bait and prey leads to repressor production, which inhibits transcription of the *HIS3* reporter gene, preventing growth on medium lacking histidine. Dissociation restores histidine prototrophy (8). The reverse two-hybrid system (*see Table 15*) uses the URA3 counterselectable marker to positively select for dissociation of interacting protein in the presence of 5-FOA (9).

### 7.6. Media for One-Hybrid Systems

Several different versions of the one-hybrid system have been developed that use the following reporter genes: *LacZ* (14,33), *HIS3* (15), *HIS3* and *LacZ* (16,34,40). These reporter genes are under the control of the target DNA fragment to which binding proteins are to be identified. Recombinant reporter genes can either be incorporated into the yeast genome (14,16,40) or exist on autonomously replicating vectors (14,33,34). The same libraries used for two-hybrid screens are used for the one-hybrid system if the markers are compatible. Commercially available one-hybrid systems include Matchmaker one-hybrid system (Clontech) (*see Table 16*).

**Table 7**  
**Media for Systems I–III and Their Functions**

System I	System II	System II	Medium function
SD–trp	SD–trp	SD–his	Bait selection
SD–leu	SD–leu	SD–trp	Library selection
SD–trp–leu	SD–trp–leu	SD–his–trp–ura	Bait + library selection
SD–leu+	SD–leu or SD–leu–ura+	SD–trp	Bait loss
Cycloheximide	10 mg/L Adenine <sup>a</sup>		
SD–trp–his	SD–trp–leu–his–ura–lys	SD–ura + X–gal	Testing bait for
±3–AT	±3–AT	SDgal/raff–ura	autoactivation
		+ X–gal	of reporter genes <sup>c</sup>
		SDgal/raff–ura–	
		his–trp+/–leu	
SD–trp–leu–his	SD–trp–leu–his–ura–lys	SDgal/raff–his–	Screening bait + library
(±3–AT)	(±3–AT)	trp–leu–ura	for activation of
		SD–his–trp–	reporter genes
		leu–ura	
		SD–ura	<i>lacZ</i> reporter
			vector selection <sup>d</sup>
Y190/Y187	L40 <sup>b</sup> /AMR70	EGY48/EGY191	Reporter strains
CSM	CSM/HSM	CSM/BSM	Recommended
			nutritional supplement
			(Table 4)

<sup>a</sup>Select for segregants that have lost the bait vector carrying the *ADE2* gene by a change in colony color from white to red.

<sup>b</sup>Minimal medium contains added succinate: 10 g of succinic acid + 6 g of NaOH/L of dropout medium.

<sup>c</sup>Autoactivation is ideally carried out in the presence of bait and activation domain vector. Selection for these vectors may not need to be maintained during the testing process, as is the case for the *lacZ* assay of system III.

<sup>d</sup>The *LacZ* reporter gene is integrated into the yeast genome in systems I and II.

**Table 8**  
**Vectors for Systems I–III**

	System I	System II	System III
Bait vectors	pAS1/2	pBTM116	pEG202
Library vectors	pACT1/2	pVP16	pJG4–5

**Table 9**  
**Commercial Two-Hybrid Systems Based on Systems I–III (4–6)**

System I	System III
Matchmaker I, II (Clontech) Strains Y153, Y187, CG1945, HF7c, SFY526	DupLEXA (Origene) Matchmaker LexA (Clontech) Strain EGY48
HybriZap (Stratagene) Strain YRG2 <sup>a</sup>	Hybrid hunter (Invitrogen) <sup>c</sup> Strains EGY48 and L40 Bait selected with Zeocin
Matchmaker system3 <sup>b</sup> (Clontech) Strain AH109	

<sup>a</sup>Minimal medium contains 1 M sorbitol: 182.2 g of sorbitol/L of medium (Stratagene).

<sup>b</sup>Omission of adenine selects for expression of third reporter gene, *ADE2*.

<sup>c</sup>Bait is selected with Zeocin. Medium for L40 strain contains added succinate: 10 g of succinic acid + 6 g of NaOH/L of dropout medium.

**Table 10**  
**Media and Their Functions for SOS Recruiting System**

Medium	Function
SD–leu	Bait selection/expression
SD–ura	Repressed library selection
SDgal/raff–ura	Expressed library selection
SD–leu–ura	Bait + repressed library selection
Sdgal/raff–leu–ura	Screening bait + library for growth at 36–37°C
Yeast reporter strain: <i>cdc25–2</i>	
Bait vector: expresses bait–hSOS fusion protein	
Library vector: expresses myristoylated fusion protein	
Nutritional supplement: CSM/BSM	

**Table 11**  
**Media and Their Functions for Trihybrid System with Inducible Third-Party Expression**

Medium	Function
SD-trp + 1 mM methionine (met)	Protein 1 and 2 selection <sup>a</sup>
SD-trp-met	Protein 1 and 2 selection/expression
SD-leu	Protein 3 selection/expression
SD-trp-leu + 1 mM met	Protein 1 and 3 expression
SD-trp-leu-his + 1 mM met	Screening protein 1 and 3 for interaction
SD-trp-leu-met	Protein 1, 2, and 3 expression
SD-trp-leu-his ± 1 mM met	Screening all three expressed proteins for interaction
Yeast reporter strains: L40/HF7C	
Bait vectors: pLex9-3H (L40)/ pGBT9-3H/B (HF7C)	
Library vectors: pVP16 (L40)/ pGAD-GH (HF7C)	
Nutritional supplement: CSM/HSM	

<sup>a</sup>1, Suppressed; 2, repressed.

**Table 12**  
**Media for Identifying RNA-Protein Interactions in Trihybrid System**

Medium	Function
SD-trp	LexA-MS2 fusion protein vector selection
SD-ura	MS2-RNAx-hybrid RNA vector selection
SD-leu	Library selection
SD-trp-leu-ura	Selection of all three vectors
SD-trp-leu-ura-his ± 3-AT	Screening for library protein interaction with RNAx
SD-trp-leu + 5-FOA	MS2-RNAx-hybrid RNA vector loss
Yeast strains: L40-ura <sup>a</sup>	
Nutritional supplement: CSM/HSM	

<sup>a</sup>Strain L40 coat can also be used; it has the LexA-MS2 fusion protein vector integrated into its genome. If using this strain, do not omit tryptophan from the medium.



**Table 13**  
**Media for Identifying Protein–Protein Interactions Dependent on Posttranslational Modifications**

Medium	Function
SD–trp	Repressing bait selection
SD–ura	Repressing library selection
SD–leu	Modifying enzyme vector selection
SDgal–trp–leu	Testing modified bait for activation of reporter gene
SD–leu–trp–ura	Screening library for interaction to modified bait
SDgal–trp–leu–ura	
Yeast reporter strain: S-260	
Nutritional supplement: CSM/BSM	

**Table 14**  
**Media for Splithybrid System**

Medium	Function
SD–trp	Bait selection
SD–leu	Prey selection
SD–trp–leu	Bait + prey selection
SD–trp–leu–lys–ura	
SD–trp–leu–lys–ura±his ±tet ±3–AT	Modulation with 3–AT and tetracycline
SD–trp–leu–lys–ura–his ±3–AT ±tet	Positive selection for dissociation
Yeast strains: Y1584, Y1596, Y1671	
Bait vector: pBTM116	
Prey vector: pVP16, PVP16–LacZ	
Nutritional supplement: CSM	

**Table 15**  
**Media for Reverse Two-Hybrid System<sup>a</sup>**

Medium	Function
SD–leu	Bait selection
SD–trp	Prey selection
SD–leu–trp	Bait + prey selection
SD–leu–trp + 5–FOA	Selecting for disruption of protein interaction

**Table 15 (continued)**

Medium	Function
SD–trp–leu–ura	Selecting for positive interaction (LacZ)
SD–trp–leu–his $\pm$ 3–AT	Selecting for positive interaction (HIS3)
Yeast reporter strain: MaV103/203	
bait vector: pPC97–CYH2	
Prey vector: pMV257	
Nutritional supplement: CSM/BSM	

<sup>a</sup>This system is available from Gibco-BRL (ProQuest™ Two-Hybrid System).

**Table 16**  
**Media for One-Hybrid Systems**

Medium	Function
SD–leu, SD–trp, or SD–ura	DNA–reporter gene selection (A)
SD–trp or SD–leu	Library selection (B)
SD–A–B $\pm$ his <sup>a</sup>	A and B selection to screen for DNA-binding proteins
SD(gal/raff)–A–B $\pm$ his <sup>a</sup>	A selection and B selection/expression to screen for DNA-binding proteins
Yeast strains: YM954/YM955 (33), W303–1a (32), GGY1 (12), YM4271 (14), yWAM2/PCY2 derivative (13),	
Nutritional supplement: CSM	

<sup>a</sup>A and B represent the nutrient to be dropped out for selection; for example, if the DNA reporter gene construct has the URA marker and the library carries the LEU marker, the following medium is used for a library screen: SD–ura–leu  $\pm$  his or SD(gal/raff)–leu–ura  $\pm$  his (histidine is dropped only if using the *HIS3* reporter gene).

### 7.7. Media for Mammalian Two-Hybrid System.

The mammalian two-hybrid system, like its yeast counterpart, uses the reconstitution of a transcription factor to drive expression of a detectable reporter gene. Reporters used in this system include the chloramphenicol acetyltransferase (17) and the luciferase (41) genes. Bait, library, and reporter vectors are introduced into mammalian cells by transient transfection (42). Different mammalian cell lines have been successfully used in this system (17,18,41,43,44) (see Table 17).

**Table 17**  
**Cell Lines and Media for Mammalian Two-Hybrid System**

Cell line	Medium <sup>a</sup>
293 cells (43)	Low-glucose DMEM with 2 mM glutamine + 10% FCS
CHO (17)	MEM alpha + 10% FCS
Hela (45)	MEM + 10% FCS
CV1 (18)	DMEM + 10% FCS
SAOS2 (44)	DMEM + 10% FCS
C3H10T1/2 (41)	DMEM + 10% FCS
Jurkat (41)	RPMI + 2 mM glutamine + 1 mM Na pyruvate + 10% FBS

<sup>a</sup>The various media listed are commercially available as sterile liquid solutions or powder mixes that must be reconstituted in H<sub>2</sub>O and sterilized by filtration (e.g., Gibco-BRL, Fisher, VWR, Thomas, Beckton Dickinson, HyClone). FCS is added shortly before use. The following antibiotics can be added to prevent bacterial contamination: 20–50 µg/mL of gentamicin sulfate or 100 µg/mL of penicillin + 100 µg/mL of streptomycin (42). A mammalian two-hybrid system is available from Clontech and Stratagene. DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FCS, fetal calf serum.

Mammalian cells are much more finicky than bacteria or yeast and are susceptible to contamination by these organisms. They have a long generation time (12–48 h). They require much stricter sterile techniques for maintenance and propagation. Culturing is usually done in sterile hoods following good sterile techniques. Only high-quality purified water is used to prepare medium. Sterile disposable items are used as much as possible. A special set of glassware is often dedicated for tissue culture purposes, because even a trace amount of common laboratory chemicals can be toxic to mammalian cells. Following these guidelines ensures a healthy population of cells resulting in efficient transfections, which are essential for successful library screens (42).

## 8. Notes

1. One gram of 5-FOA (0.1% final concentration) is traditionally used to select for the loss of URA marker (3). A range of 0.05–0.2% 5-FOA is used in the reverse two-hybrid system (9).
2. Uracil is omitted from this medium if one is screening for dissociation of interacting proteins using the reverse two-hybrid system.

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## Yeast Two-Hybrid Vectors and Strains

Philip James

### 1. Introduction

Since its introduction by Fields and Song (*1*), the two-hybrid system has been widely used to identify and explore interactions between proteins. The extreme popularity of this method has led to numerous modifications. These include many new plasmids and strains for use in the traditional yeast transcription-based assay and the adaptation of the two-hybrid system to other organisms (*2–4*) and to protein-nucleic acid interactions (one- and three-hybrid systems) (*5–7*), as well as the development of assays that do not depend on transcription (*8,9*). These latter modifications are described in detail in Chapters 15–20. This chapter focuses on the many options available for performing a traditional yeast transcription-based two-hybrid experiment.

Transcription-based two-hybrid systems require three tools: one plasmid encoding a protein domain with promoter-specific DNA-binding activity, a second plasmid encoding a protein domain that serves as a transcription activator, and a yeast strain containing transcription-activated reporter genes. The gene encoding a protein of interest (the “bait”) is cloned as an in-frame fusion to the DNA-binding domain (DBD), and genes encoding additional proteins of interest, or a library of proteins (the “target”), are cloned as in-frame fusions to the transcription activation domain (AD). Both plasmids are then transformed into an appropriate yeast strain. The bait protein is expressed as a fusion to the DBD and is thus localized to the promoter of a reporter gene in the yeast nucleus. The target protein (or library of proteins) is expressed as a fusion to the transcription AD. Interaction between the bait and target results in the colocalization of the transcription AD to the DNA of the host strain, activating transcription of the adjacent reporter gene and generating a phenotypic signal.

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Traditional yeast two-hybrid assays are most often performed using one of two major systems. One system is based on the separable DBD and transcription AD of the yeast Gal4 transcription factor (**10**), and the other is based on the bacterial LexA operator binding protein (**11**). In either system there are a variety of plasmids and yeast strains available, and the ability to mix the two systems under certain circumstances further expands the choices available to the researcher. In any experiment the DNA-binding protein and the yeast strain must be matched such that DNA binding will occur upstream of the yeast strain reporter genes. However, a particular Gal4 (or LexA) strain is generally compatible with any Gal4 (or LexA) bait vector (**12**). In addition, any transcription AD can be used with any bait plasmid or yeast strain, although other elements of the AD vector may limit compatibility. This is an important feature because it allows users to choose among a wide variety of available two-hybrid libraries, regardless of their choice of strain and bait plasmid.

The purpose of this chapter is to describe the available two-hybrid vectors and yeast strains, their advantages and disadvantages, and the considerations involved in choosing among them. This chapter focuses primarily on published reagents; however, many of the reagents discussed here, as well as novel modifications, are also available commercially (*see Note 1*). These sources should also be examined when choosing the appropriate system for your application. Many of the reagents discussed in this chapter were originally developed as matched sets (yeast strain, binding domain, and AD vectors). Because of the ability to mix and match different reagents, each of these three tools is discussed separately to allow users to more easily compare what is available and decide which combination is best suited to his or her needs. Cases in which using a matched set of reagents can provide extra functionality are described in **Subheading 1.1**. Finally, a protocol is presented for the construction and testing of fusion plasmids and performing screens using one particular yeast strain, PJ69-4A.

## **1.1. Two-Hybrid Vectors**

### **1.1.1. Functional Considerations**

Performing a two-hybrid experiment is a complex process involving many different steps, and there are a number of potential problems ranging from merely aggravating to potentially insurmountable. There are the technical problems of cloning genes of interest into properly designed plasmids and then introducing those plasmids into yeast. Once inside the cell, a variety of *in vivo* criteria must be met, which can sometimes be addressed through plasmid design. The first criterion is that protein fusions must be expressed from the plasmids at appropriate levels—high enough to generate an interaction signal but not so



high as to cause toxicity. Vectors that vary in promoter strength and copy number are useful for overcoming such expression problems. Expressed protein fusions also must be stable, localized to the nucleus, and capable of dimerization (both Gal4 and LexA bind DNA as dimers). They must be folded properly to produce the interaction site, and that site cannot be occluded by the DBD or transcription AD. If a problem is suspected in one of these areas, it may be possible to overcome it by switching from a Gal4 to a LexA system (or vice versa) or by using a vector that allows N-terminal rather than C-terminal fusions. Because it is not necessary that the protein be fully functional, problems such as stability, localization, and folding might also be overcome by using smaller fragments of the protein of interest. This is especially true when the domain structure of the protein is known; if little information is available, there is a risk of eliminating the binding site when the protein is fragmented. Once an interaction has been detected, there are more technical hurdles. Validation will require the isolation of yeast that have lost one or both plasmids, rescue of plasmids from yeast into *Escherichia coli*, and sequencing. In many cases it will be necessary to carry out additional cloning for either controls or subsequent screening.

In recent years, the number and variety of available two-hybrid vectors have rapidly increased. Although this makes choosing more difficult, these recent modifications help address many of the impediments to a successful two-hybrid experiment. The following sections discuss the different components of two-hybrid vectors and their roles in the assay as well as the specific DBD and transcription AD vectors that are available at this time.

### 1.1.2. Vector Components

Each two-hybrid vector is made up of a variety of components that affect the overall functionality of the system. The success of a particular screen can depend on the choice of vectors, and the particular traits that are most desirable will vary among different screens. To aid in this decision, each of the following sections discusses one component of the vector and its contribution to the success of the experiment.

#### 1.1.2.1. THE VECTOR BACKBONE

The vector backbone includes features such as the bacterial and yeast origins of replication and markers for phenotypic selection and is often an afterthought in vector selection. However, in addition to some functional considerations, backbone features can make life considerably easier. The most important factor in choosing a vector backbone is to ensure that the yeast marker is suitable for selection in the yeast strain of choice. Thus, a vector

carrying the *LEU2* yeast marker must be paired with a yeast strain that carries a *leu2* mutation. It is also imperative that *LEU2* not be used as a reporter gene in this case, because the same gene cannot be used to select for both the presence of the plasmid and the interaction.

Proper choice of yeast markers can also simplify parts of the screening process. An important step in eliminating false positives is to isolate cells that have lost either the bait or target plasmid and demonstrate that the reporter signal requires the presence of both plasmids. Vectors that carry the yeast markers *URA3* or *CYH2* can be selected against (*see Note 2*), turning the tedious plasmid loss screen into a selection. The *URA3* gene may have an advantage in this case because it allows both positive and negative selection (hence, a second marker gene is not used up) and the *CYH2* gene is somewhat toxic to the cells at high copy (*13,14*).

It will also be necessary to recover the target plasmid out of yeast for analysis once positive interactions have been identified. This is most often done by preparation of total yeast DNA using the glass bead method (*15,16*) followed by transformation into *E. coli* for amplification. Both the bait and target plasmids are recovered by this method; identification of the correct plasmid is simplified by the ability to differentiate the bait and target plasmids through selection in *E. coli*. This can be accomplished by using target plasmids that are marked by the yeast *LEU2* gene, which can be selected in *E. coli* by complementation of the *leuB6* mutation (*see Note 3*). In addition, a number of vectors have now been introduced that carry bacterial drug-resistance genes against kanamycin, chloramphenicol, or zeomycin as an alternative to ampicillin.

The majority of two-hybrid vectors utilize the yeast 2 $\mu$  origin of replication to maintain plasmids at high copy number (15–30 copies per cell). The elevated copy number has the advantage of increased protein expression levels, but the disadvantage that plasmid copy number is variable and may not be the same for bait and target. A consideration when using these vectors is that after initial transformation, cells begin with low plasmid copy numbers and take several generations to build to high copy. Thus, for some interactions in which the copy number is critical, cells may require time to amplify the plasmids before they are able to activate more stringent reporters such as *ade2* or *LexAop(1x)* (*see Subheading 1.2.*).

A few centromere (CEN)-based vectors are available. These are maintained at low copy number (one to five copies per cell) and are useful for reducing expression levels when protein toxicity is a concern (*17*). In addition, the copy number of CEN vectors is more stable and the bait and target are more likely to be expressed at equivalent levels. Although the vast majority of two-hybrid screens have utilized 2 $\mu$  vectors, Durfee et. al. (*18*) have recently shown that

for at least some interactions, CEN-based vectors provide stronger interaction signals in spite of much lower protein expression.

#### 1.1.2.2. MULTIPLE CLONING SITES

In most cases, the polylinker region of a particular vector will not have a major effect on the success or failure of a two-hybrid experiment. However, choosing vectors with the optimal cloning sites can greatly increase the efficiency of your experiment. Although it is always possible to add restriction sites to your gene using polymerase chain reaction (PCR), doing so requires that the gene be sequenced to ensure that mutations have not been introduced (a control that is ignored in too many cases). With the expanded polylinkers now available, almost any restriction site in your gene can be used for cloning, particularly if sites can be made blunt with Klenow polymerase. In addition, many bait and target vectors with identical polylinkers are now available. These matched sets simplify the swapping of inserts between bait and vector, which can be useful to verify interactions, to begin additional screens with newly identified proteins, or to produce truncated versions of the bait or target when mapping interaction domains.

One case in which the choice of polylinker may determine the success of the experiment is in choosing the position of the fusion site. Most two-hybrid vectors produce fusions in which the protein of interest is fused to the carboxyl terminus of the transcription factor domain. However, when proteins require their amino-terminal domain for interaction, this orientation may block the interaction site. In at least some cases, the use of amino-terminal fusion vectors has allowed the detection of such interactions (19,20). Switching from carboxyl-terminal to amino-terminal fusions may also be a useful strategy in cases in which problems with the stability or folding of the fusion protein are suspected.

#### 1.1.2.3. PROMOTERS AND TERMINATORS

The majority of two-hybrid vectors available today utilize the yeast *ADHI* promoter to drive expression of the fusion protein, although a few use the *PGK1*, *GAL1*, or *GAL10* promoters. The *ADHI* promoter is available in full-length and truncated (referred to as *ADHI\**) forms. The *ADHI\** form of the promoter results in lower expression levels than the full-length form (21), allowing the adjustment of fusion protein levels through vector choice. The higher expression provided by the full-length *ADHI* promoter allows easier detection of fusion proteins on Western blots and may enhance detection of some weak interactions; however, it may also increase problems with protein toxicity and background activation of reporters by the bait. The *ADHI\** pro-

moter reduces the problems of bait toxicity and may eliminate background problems associated with autoactivation by the bait in some cases (13,22). Regarding detection of interactions, there is a common belief that higher expression of fusions will increase the number of interactions identified, and this has been demonstrated in a few cases (21). However, in most cases the same interactions are detected with either high or low expression (23), and in some cases, low expression actually results in better detection of interactions (18). Thus, the decision on promoter strength is not a straightforward one, and in some cases it may be worth testing both.

Some vectors also use the *GALI* or *GALI0* promoter to drive expression of the fusion protein. These promoters have the advantage of being tightly regulated by the addition or removal of galactose from the growth medium. As a result, they can be used to assay toxic fusion proteins that could not otherwise be screened, by rapid induction and assay of reporter activation (24). In addition, the *GAL* promoters provide a simple test for false positives, since activation should occur only in the presence of the inducer galactose. The *GAL* promoters are only compatible with the LexA-based system, since Gal4-based yeast strains require that the galactose induction system be knocked out by mutation.

#### 1.1.2.4. EPITOPE TAGS

Prior to the beginning of a two-hybrid experiment, it is often desirable to check for the expression of a stable bait fusion protein by Western blotting. This can be done using antibodies directed against the DBD and transcription AD (these are commercially available; see Note 4); however, many of these are not particularly good antibodies. Gal4 antibodies, in particular, are unable to detect expression from the truncated *ADHI\** promoter. This is clearly owing to the antibodies rather than low expression levels since in many cases the fusion protein is detectable using antibodies directed against the bait domain. To aid with this problem, a number of vectors incorporate epitope tags into the fusion protein, most often using the hemagglutinin (HA) epitope. Epitope tags can be used for detection of the fusion protein on Western blots and also for coimmunoprecipitation experiments intended to verify identified interactions. Nevertheless, it is important not to place too much emphasis on these tests. In some cases, detection has failed (the epitope may not be available because of the folding of the fusion protein, or low expression may prevent detection), but two-hybrid screening has been successful anyway (25). Also, successful detection of the expressed protein does not imply that the interaction domain is accessible and properly folded; thus, the screen may fail in spite of the presence of the fusion. An additional problem some laboratories have observed is

that false positives can arise owing to interaction with the HA epitope (13,22). If antibodies against DNA-binding or transcription activation domains are inadequate, the best option may be an antibody against your protein of interest; however, epitope tags are also helpful in many cases.

#### 1.1.2.5. FUSION DOMAINS

In choosing the actual fusion domains that will be used in a two-hybrid screen, it is absolutely essential that the DBD selected is able to bind the reporter gene promoters in the yeast strain selected. In addition, some functional differences among the different available domains need to be considered. In many cases, the choice of which DBD or transcription AD to use in a particular screen will be of little consequence. The different fusion domains work similarly, and most interactions tend to either work or fail to work in all systems. Protein expression levels and the particular yeast strain used often have a larger effect on sensitivity (26). There are, however, several potential problems that can sometimes be addressed by switching to an alternate fusion domain. These include poor stability or incorrect folding of the fusion protein, or obstruction of a binding site. Unfortunately, these are difficult to diagnose and deciding which domain might help is largely a matter of trial and error. Some interactions work better using LexA, others with Gal4. In addition, some interactions are directional, that is, they do not work if the DBD and transcription AD inserts are reversed (27). In difficult cases such as these, it is up to the investigator to judge how much effort to invest in testing different combinations.

Several DBDs are available for two-hybrid screening. The ones most commonly used are derived from the Gal4 and LexA proteins. Both bind to DNA as dimers and show increased efficiency when multiple binding sites are present in the reporter gene promoters. The major difference between the two is that Gal4 contains a nuclear localization signal (NLS). LexA fusions apparently enter the nucleus in spite of this, although with lowered efficiency, making low copy number CEN-based vectors less practical for the LexA system (28). Also, it is possible that LexA baits enter the nucleus because they are below the size exclusion limit of the nuclear pore; thus, it may be prudent when using larger LexA-based baits to include an NLS in the construct. Recently, a third DBD system has been introduced based on the bacteriophage  $\lambda$  repressor protein cI (29). Although it can be used as a stand-alone system, the cI-based reagents were developed for use in combination with a LexA system, allowing the use of a second bait for simultaneous screening or as a built-in control (*see Note 5*). Finally, a bait vector that makes use of the DBD of the estrogen receptor (ER) protein is also available (30).

Unlike the DBD vectors, there is no requirement for the transcription AD to be compatible with other reagents, and all the transcription activation vectors incorporate an NLS from the SV40 large T antigen. Three transcription ADs are commonly used, and all can be used with any of the different DNA-binding systems, as long as other features are compatible. This is fortunate because it allows access to the numerous two-hybrid libraries that have been made in a variety of different vectors. The major difference among the vectors is the strength of activation provided. B42 is a relatively weak transcription activator, whereas Gal4 is moderate and VP16 is strong (28). Although strong activation might be expected to provide increased sensitivity, it has been shown that strong ADs are somewhat toxic in yeast (31). Thus, the B42 and Gal4 transcription ADs are the ones most commonly used.

### 1.1.3. DBD Vectors

Many of the currently available DBD vectors and their features are listed in **Table 1**. In the Gal4 system, a variety of DBD vectors have been developed based on the original pGBT9 vector of Bartel et al. (32), a high copy number 2 $\mu$ -based vector containing the truncated and less active form of the *ADHI* promoter (*ADHI\**) and the *TRP1* marker gene. Improvements include providing the polylinker region in all three reading frames to aid in cloning, in both the forward and reverse orientations (pGBT9[x] and pGBT9rev[x]) (33) or with the addition of a unique *ClaI* site (pGBD-C[1,2,3] and pGBDU-C[1,2,3]) (23). In the pGBDU-C(1,2,3) vectors, the *TRP1* marker has also been replaced by the *URA3* gene to allow counterselection, which greatly simplifies plasmid loss assays. The pGBDK-C(1,2,3) (van Hemert, M. and van Hensden, P., personal communication) vectors carry the bacterial *Kan<sup>R</sup>* gene to allow easy identification of bait and target plasmids after rescue from yeast. The pGBREP plasmid is unique in that it has the SSB24 repressor sequence fused to the DBD (34), which serves to dampen the activity of bait proteins that cause high background. This may be a promising approach to screening difficult baits, but the effectiveness is likely to vary with different baits and the repressor may also suppress activation caused by real interactions. Also unique is the pGB-MEL1 plasmid (35), which contains the *MEL1* gene encoding yeast  $\alpha$ -galactosidase in the pGBT9 vector. The *MEL1* gene is driven by a native Gal4-inducible promoter and the enzyme can be assayed using a chromogenic substrate. Thus, pGB-MEL1 serves as both a bait vector and a reporter gene. Finally, pOBD-2 is a low copy number CEN-based vector that has been modified to allow recombinational cloning of bait gene inserts (36,37).

A second family of Gal4-based DBD vectors utilizes the full-length *ADHI* promoter and thus expresses fusion proteins at higher levels. Most popular among these are the pAS1 and pAS2 vectors (38,39). Each is a *TRP1*-marked,



2 $\mu$ -based plasmid and contains a hemagglutinin epitope tag; the latter also carries a *CYH2* marker gene to allow counterselection of the plasmid. pODB8 (14) is derived from pAS2 and lacks the *CYH2* gene but shows improved stability and protein expression. Several CEN-based vectors also use the full-length *ADH1* promoter. pCD.1 and pCD.2 (18) have features similar to those of pAS1 while pPC62 and pPC97 (17) have unique polylinkers and use the *LEU2* marker gene instead of *TRP1*. Finally, the pNGBD-U(1,2,3) vector series was recently developed in this laboratory and allows the fusion of a bait protein to the amino-terminus of the Gal4 DBD. The polylinker is provided in all three reading frames, and the 2 $\mu$  origin, *ADH1* promoter, and *URA3* marker gene are used. The base vector is one of a series developed by Mumberg et al. (40) that allows easy swapping of the expression cassette to utilize any combination of 2 $\mu$  or CEN-based vectors; the *CYC1*, *ADH1*, *TEF2*, or *GPD* promoters; and the *TRP1*, *LEU2*, *URA3*, and *HIS3* marker genes. The start codon for the fusion gene can be provided by the bait protein or by a *SpeI* polylinker site made blunt by mung bean nuclease.

Although it is also very popular, the LexA-based two-hybrid system has not generated the same variety of DBD vectors. Most experiments use either pBTM116 (41) or pEG202 (24), which differ mainly in the yeast marker used (*TRP1* and *HIS3*, respectively) and the promoter strength (pEG202 provides higher expression). pFBL23 was developed from pBTM116 and allows fusion of a bait protein to the amino-terminus of the LexA DBD (19). pMW101 and pMW103 (42) are versions of pEG202 carrying alternate antibiotic resistance markers for selection in *E. coli*, while pJK202 is identical to pEG202 except that a nuclear localization sequence is included in the fusion protein (11), which may be especially useful for large baits. pGILDA contains the fusion cassette of pEG202 moved into a CEN-based vector and driven by the galactose-inducible GAL1 promoter and, thus, may allow the expression of baits that are toxic in yeast (43).

Recently, a new system has been introduced based on the DNA-binding activity of the bacteriophage  $\lambda$  repressor protein cI (29). Although functional as a stand-alone system, the cI components were designed to serve primarily as a complement to the existing LexA-based system. The combination of these two systems enhances both the screening capabilities and the ability to eliminate false positives. The cI system DBD vectors include pGKS3, a close relative of pEG202, and pGKS6 and pGKS8. The latter two use the *zeo<sup>R</sup>* gene as a marker for both yeast and *E. coli*, and pGKS8 also carries the cIop<sub>(3x)</sub>-gusA reporter gene.

Finally, the pBL1 vector (30) is used in a system based on the DBD of the human ER protein. It uses the *PGK1* promoter and carries an epitope tag from the F fragment of the ER.

**Table 1**  
**DBD Vectors**

Vector	DBD	Promoter	Yeast markers	<i>E. coli</i> marker	MCS	Other features	Reference
pGBT9	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	—	<b>32</b>
pGBT9(x)	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	Multiple reading frames	<b>33</b>
pGBT9rev(x)	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>PstI</i> , <i>SalI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>EcoRI</i>	Multiple reading frames	<b>33</b>
pGBD-C(1,2,3)	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>ClaI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	Multiple reading frames	<b>23</b>
pGBDU-C(1,2,3)	Gal4	<i>ADHI</i> *	<i>URA3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>ClaI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	Multiple reading frames, counterselectable	<b>23</b>
pGBDK-C(1,2,3)	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Kan<sup>R</sup></i>	<i>EcoRI</i> , ( <i>SmaI</i> ), <i>BamHI</i> , ( <i>ClaI</i> ), <i>SalI</i> , ( <i>PstI</i> ), <i>BglII</i>	Multiple reading frames	<sup>a</sup>
pGBREP	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	Includes SSB24 repressor	<b>34</b>
pOBD-2	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>PvuII</i> , <i>NcoI</i> , <i>SmaI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	CEN-based, recombinational cloning	<b>36, 37</b>
pGB-MEL1	Gal4	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	Includes <i>MEL1</i> reporter gene	<b>35</b>
pAS1	Gal4	<i>ADHI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>NdeI</i> , <i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i>	HA tag	<b>38</b>
pAS2	Gal4	<i>ADHI</i>	<i>TRP1</i> , <i>CYH2</i>	<i>Amp<sup>R</sup></i>	<i>NdeI</i> , <i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	HA tag, counterselectable	<b>39</b>
pODB8	Gal4	<i>ADHI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>NdeI</i> , <i>NcoI</i> , <i>SfiI</i> , <i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	HA tag	<b>14</b>



pPC62	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp<sup>R</sup></i>	<i>SalI</i> , ( <i>ClaI</i> ), ( <i>HindIII</i> ), ( <i>EcoRV</i> ), ( <i>EcoRI</i> ), <i>PstI</i> , <i>SmaI</i> , ( <i>BamHI</i> ), <i>SpeI</i> , <i>XbaI</i> , <i>NotI</i> , <i>SacII</i>	CEN based	<b>17</b>
pPC97	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp<sup>R</sup></i>	<i>SalI</i> , <i>SmaI</i> , <i>EcoRI</i> , <i>BglII</i> , <i>SpeI</i> , <i>NotI</i> , <i>AatII</i> , <i>SacI</i> , <i>SacII</i>	CEN based	<b>17</b>
pCD.1	Gal4	<i>ADHI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>NdeI</i> , <i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	HA tag, CEN based	<b>18</b>
pCD.2	Gal4	<i>ADHI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>NdeI</i> , <i>NcoI</i> , <i>AvrII</i> , <i>BglII</i> , <i>BclI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>SalI</i> , <i>PstI</i>	HA tag, CEN based	<b>18</b>
pNGBD-U(1,2,3)	Gal4	<i>ADHI</i>	<i>URA3</i>	<i>Amp<sup>R</sup></i>	<i>XbaI</i> , <i>SpeI</i> , <i>BamHI</i> , <i>SmaI</i> , ( <i>PstI</i> ), <i>EcoRI</i> , ( <i>EcoRV</i> ), <i>HindIII</i> , <i>ClaI</i> , <i>SalI</i>	Fusion to the N-terminus of Gal4, multiple reading frames	<sup>b</sup>
pBTM116	LexA	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	—	<b>41</b>
pFBL23	LexA	<i>ADHI</i> *	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>BamHI</i> , <i>SalI</i>	Fusion to N-terminus of LexA	<b>19</b>
pEG202	LexA	<i>ADHI</i>	<i>HIS3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , ( <i>SmaI</i> ), <i>BamHI</i> , ( <i>SalI</i> ), <i>NcoI</i> , <i>NotI</i> , <i>XhoI</i> , ( <i>SalI</i> ), ( <i>PstI</i> )	—	<b>24</b>
pJK202	LexA	<i>ADHI</i>	<i>HIS3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , ( <i>SmaI</i> ), <i>BamHI</i> , ( <i>SalI</i> ), <i>NcoI</i> , <i>NotI</i> , <i>XhoI</i> , ( <i>SalI</i> ), ( <i>PstI</i> )	Includes NLS	<b>11</b>

<sup>a</sup>M. vanHemert and P. vanHensden, unpublished data.

<sup>b</sup>P. James, unpublished data.

(continued)

**Table 1 (continued)**

Vector	DBD	Promoter	Yeast markers	<i>E. coli</i> marker	MCS	Other features	Reference
pMW101	LexA	<i>ADHI</i>	<i>HIS3</i>	<i>Cam<sup>R</sup></i>	<i>EcoRI</i> , ( <i>SmaI</i> ), <i>BamHI</i> , ( <i>SalI</i> ), <i>NcoI</i> , <i>NotI</i> , <i>XhoI</i> , ( <i>SalI</i> ), ( <i>PstI</i> )	—	<b>42</b>
pMW103	LexA	<i>ADHI</i>	<i>HIS3</i>	<i>Kan<sup>R</sup></i>	<i>EcoRI</i> , ( <i>SmaI</i> ), <i>BamHI</i> , ( <i>SalI</i> ), <i>NcoI</i> , <i>NotI</i> , <i>XhoI</i> , ( <i>SalI</i> ), ( <i>PstI</i> )	—	<b>42</b>
pGILDA	LexA	<i>GALI</i>	<i>HIS3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>BamHI</i> , <i>NcoI</i> , <i>NotI</i> , <i>SalI</i> , <i>XhoI</i>	CEN-based, galactose-inducible promoter	<b>43</b>
pGKS3	cI	<i>ADHI</i>	<i>HIS3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI</i> , <i>BamHI</i> , <i>NcoI</i> , <i>NotI</i>	—	<b>29</b>
pGKS6	cI	<i>ADHI</i>	<i>zeo<sup>R</sup></i>	<i>zeo<sup>R</sup></i>	<i>EcoRI</i> , <i>SacI</i> , <i>BglII</i> , <i>PvuII</i> , <i>KpnI</i> , <i>SacII</i> , <i>NotI</i>	—	<b>29</b>
pGKS8	cI	<i>ADHI</i>	<i>zeo<sup>R</sup></i>	<i>zeo<sup>R</sup></i>	<i>EcoRI</i> , <i>SacI</i> , <i>BglII</i> , <i>PvuII</i> , <i>KpnI</i> , <i>SacII</i> , <i>NotI</i>	Includes cIop(3x)-gusA reporter gene	<b>29</b>
pBL1	ER	<i>PGK1</i>	<i>HIS3</i>	<i>Amp<sup>R</sup></i>	<i>XhoI</i> , <i>SacII</i> , <i>BamHI</i> , <i>EcoRI</i>	ER(F) tag	<b>30</b>

Parentheses indicate that the restriction site is not unique; MCS, multiple cloning site.

#### 1.1.4. Transcription AD Vectors

The transcription AD vectors are listed in **Table 2**. Like the DBD vectors, the transcription AD vectors fall into several families. Gal4-based vectors that use the truncated *ADHI*\* promoter are derived from the original pGAD424 vector of Bartel et al. (32) and use the same polylinker as the pGBT9-derived DBD vectors, allowing simple cloning between the two. Again, several vector sets offer improved cloning efficiency by providing the polylinker in all reading frames and in both orientations (33). The pGAD-C(1,2,3) series also introduces a unique *ClaI* site that is especially useful for library construction, accepting the partial digestion products of five different restriction enzymes (23). pOAD is a partner of the pOBD-2 DBD vector; it is CEN based and has been designed to facilitate recombinational cloning of target protein inserts (36,37).

A second family of vectors contains the Gal4 transcription AD and the full-length *ADHI* promoter. pGADGH, pGAD10 (32), and pACT1 (38) are the earliest versions and provide a variety of polylinker choices. pACT2 is an improved version of pACT1 with an expanded polylinker and an HA epitope tag (39). The pACT vectors are also available in the form of a  $\lambda$  phage. Libraries can be constructed in the phage and then excised out in the plasmid form. pC-ACT.1 and pC-ACT.2 are CEN-based versions of pACT2 (18); these three vectors share common polylinkers with the DBD vectors pCD.1 and pCD.2. pNGAD-L(1,2,3) is a new set of *LEU2*-marked vectors from this laboratory that allows fusion of target proteins to the amino-terminus of the Gal4 AD. They share a common polylinker with pNGBD-U(1,2,3), and like those vectors, the expression cassette can be easily swapped into CEN-based; *CYC1*, *TEF2*, or *GPD* promoter-driven, or *URA3*-, *TRP1*-, or *HIS3*-marked vector backbones. Finally, pPC86 is a CEN-based Gal4 AD vector that shares a common polylinker with the DBD vector pPC97 (17). pPC86 is marked by the *TRP1* gene; all the other Gal4 AD vectors listed here are marked by *LEU2*, which can be used to complement the *leuB6* mutation in *E. coli*, thereby distinguishing bait and target plasmids during plasmid rescue.

AD plasmids designed for the LexA system use either the B42 or VP16 transcription AD. A number of vectors are based on the plasmid pJG4-5 (44) and contain the B42 transcription AD driven by the *GAL1* promoter, a *TRP1* marker gene, and an HA epitope tag. pMW102 and pMW104 are variants that include the *Kan<sup>R</sup>* and *Cam<sup>R</sup>* genes, respectively, as an alternative to *Amp<sup>R</sup>* for plasmid rescue (42). The pNB42 vector series incorporates the pEG202 polylinker into pJG4-5 in forward and reverse orientations as well as in all three reading frames and allows fusion of a target protein to the amino-terminus of the B42 activation domain (20). VP16 is a strong AD that may cause toxicity when overexpressed.

**Table 2**  
**Transcription AD Vectors**

Vector	AD	Promoter	Yeast marker	<i>E. coli</i> marker	MCS	Other features	Reference
pGAD424	Gal4	<i>ADHI</i> *	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	—	<b>32</b>
pGAD424(x)	Gal4	<i>ADHI</i> *	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	Multiple reading frames	<b>33</b>
pGAD424rev(x)	Gal4	<i>ADHI</i> *	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>BglII</i> , <i>PstI</i> , <i>SalI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>EcoRI</i>	Multiple reading frames	<b>33</b>
pGAD-C(1,2,3)	Gal4	<i>ADHI</i> *	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>ClaI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	Multiple reading frames	<b>23</b>
pOAD	Gal4	<i>ADHI</i> *	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>EcoRI</i> , <i>PvuII</i> , <i>NcoI</i> , <i>SmaI</i> , <i>SalI</i> , <i>PstI</i> , <i>BglII</i>	CEN-based, recombinational cloning	<b>36, 37</b>
pC-ACT.1	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>NdeI</i> , <i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	HA tag, CEN based	<b>18</b>
pC-ACT.2	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>NdeI</i> , <i>NcoI</i> , <i>AvrII</i> , <i>BglII</i> , <i>BclI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>SalI</i> , <i>PstI</i>	HA tag, CEN based	<b>18</b>
pGADGH	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	<i>SpeI</i> , <i>BamHI</i> , <i>SmaI</i> , ( <i>PstI</i> ), <i>EcoRI</i> , ( <i>EcoRV</i> ), ( <i>HindIII</i> ), <i>EcoRI</i> , ( <i>EcoRV</i> ), ( <i>HindIII</i> ), ( <i>ClaI</i> ), <i>SalI</i> , <i>XhoI</i> , <i>ApaI</i> , ( <i>KpnI</i> )	—	<b>32</b>
pGAD10	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	( <i>BglII</i> ), <i>XhoI</i> , <i>BamHI</i> , <i>EcoRI</i> , ( <i>BglII</i> )	—	<b>32</b>
pACT1 <sup>a</sup>	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	( <i>BglII</i> ), ( <i>EcoRI</i> ), <i>BamHI</i> , <i>XhoI</i> , ( <i>BglII</i> )	—	<b>38</b>
pACT2	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp</i> <sup>R</sup>	( <i>NdeI</i> ), <i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>EcoRI</i> , <i>SacI</i> , <i>XhoI</i> , ( <i>BglII</i> )	HA tag	<b>39</b>
pPC86	Gal4	<i>ADHI</i>	<i>TRP1</i>	<i>Amp</i> <sup>R</sup>	<i>SalI</i> , <i>SmaI</i> , <i>EcoRI</i> , <i>BglII</i> , <i>SpeI</i> , <i>NotI</i> , <i>AatII</i> , <i>SacI</i> , <i>SacII</i>	CEN based	<b>17</b>

pNGAD-L(1,2,3)	Gal4	<i>ADHI</i>	<i>LEU2</i>	<i>Amp<sup>R</sup></i>	<i>XbaI, SpeI, BamHI, SmaI, PstI, (EcoRI), (EcoRV), HindIII, (ClaI), SalI</i>	Fusion to N-terminus of Gal4, multiple reading frames	P. James, unpublished data
pJG4-5	B42	<i>GALI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI, XhoI</i>	HA tag, galactose-inducible promoter	<b>44</b>
pMW102	B42	<i>GALI</i>	<i>TRP1</i>	<i>Kan<sup>R</sup></i>	<i>EcoRI, XhoI</i>	HA tag, galactose-inducible promoter	<b>42</b>
pMW104	B42	<i>GALI</i>	<i>TRP1</i>	<i>Cam<sup>R</sup></i>	<i>EcoRI, XhoI</i>	HA tag, galactose-inducible promoter	<b>42</b>
pNB42(1,2,3)	B42	<i>GALI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI, (SmaI), BamHI, (SalI), NcoI, NotI, XhoI, (SalI), (PstI)</i>	HA tag, multiple reading frames, fusions to the N-terminus of B42	<b>20</b>
pNB42 (1R,2R,3R)	B42	<i>GALI</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>(PstI), (SalI), XhoI, NotI, NcoI, (SalI), BamHI, (SmaI), EcoRI</i>	HA tag, multiple reading frames, fusions to the N-terminus of B42	<b>20</b>
pVP16	VP16	<i>ADHI</i>	<i>LEU2</i>	<i>Amp<sup>R</sup></i>	<i>BamHI, (SmaI), (KpnI), (SacI), NotI</i>	—	<b>45</b>
pSD.06(a,b,c)	VP16	<i>GALI</i>	<i>URA3</i>	<i>Amp<sup>R</sup></i>	<i>EcoRI, (EcoRV), (HindIII), (ClaI), SalI, XhoI, (ClaI)</i>	Multiple reading frames, CEN based	<b>46</b>
pWITCH(T)	VP16	<i>GALI10</i>	<i>TRP1</i>	<i>Amp<sup>R</sup></i>	<i>(SacI), BamHI, SpeI, (BstXI), EcoRI, PstI, (EcoRV), (BstXI), NotI, XhoI, SphI, (XbaI)</i>	V5 tag, CEN based, galactose-inducible promoter	<b>47</b>
pWITCH(U)	VP16	<i>GALI10</i>	<i>URA3</i>	<i>Amp<sup>R</sup></i>	<i>(SacI), BamHI, SpeI, (BstXI), EcoRI, (PstI), (EcoRV), (BstXI), NotI, XhoI, SphI, (XbaI)</i>	V5 tag, CEN based, galactose-inducible promoter	<b>47</b>
pASV(x)	VP16	<i>PGKI</i>	<i>LEU2</i>	<i>Amp<sup>R</sup></i>	<i>SfiIA, SalI, XhoI, SfiIB</i>	Multiple reading frames, lox sites for lambda	<b>30</b>

Parentheses indicate that the restriction site is not unique; MCS, multiple cloning site.

<sup>a</sup>pACT1 is also known by the name pSE1107.

This may be a particular problem with pVP16 and pASV(x), both of which use the 2 $\mu$  origin and a constitutive promoter (30,45). pASV(x) can also be used for library construction in phage  $\lambda$ . pSD.06(a,b,c) (46) and the pWITCH vectors (47) are CEN based and use inducible GAL promoters that may help avoid toxicity. The pSD.06(a,b,c) polylinker is available in all reading frames, and the pWITCH vectors include a V5 epitope tag, a choice of *TRP1* or *URA3* marker, and promoters for both in vivo and in vitro expression of the fusion protein.

Note that all the Gal4 AD vectors are compatible with any of the available DBD systems, but this is not true for the B42 and VP16 AD vectors. This is not owing to the ADs themselves, which should be fully functional in a Gal4-based system. However, except for pVP16 and pASV(x), the B42 and VP16 fusions are driven by Gal4-inducible promoters, and these will not be expressed in a Gal4 system yeast strain.

## 1.2. Two-Hybrid Strains

### 1.2.1 Functional Considerations

The yeast strain used in a two-hybrid experiment often has a greater impact on the outcome than any other factor. It will largely determine how efficiently interactions can be detected and how much work is necessary to separate real interactions from artifacts. First, though, the strain must meet certain basic requirements. The reporter genes in a two-hybrid strain must utilize the same promoter system as the DBD vector, so that the bait protein will be bound to the reporter gene promoters. If the Gal4 system is used, the strain should contain mutations in the native genes encoding the *GAL4* transcription factor and the *GAL80* transcription repressor. The strain must also contain the auxotrophic mutations that are to be complemented by the bait and target plasmids and should be capable of high transformation efficiency. Other markers may be needed to introduce additional plasmids or for plasmid loss assays. Also, many two-hybrid experiments are now using mating protocols to bring together bait and target (13,48,49) in a diploid strain. These methods are especially useful for high-throughput or iterative screens and can also be used to combine reporter genes from different strains. When mating protocols are used, the two haploid strains must be of opposite mating type and contain markers that will allow selection of the mated diploids (often the bait and target plasmid markers can be used for this purpose). In addition, the markers in the two haploids should be compatible; that is, a reporter gene in one haploid should not be complemented by the other haploid.

Beyond these technical considerations, an ideal yeast two-hybrid strain has two properties. First, the reporter genes used in the strain should be highly

sensitive so that even weak protein-protein interactions can be detected. Second, those same reporter genes should be highly selective, such that real interactions are identified and false positives are eliminated. These two goals tend to work against one another; how well a strain can accomplish both will play a major role in the success of an experiment.

Sensitivity in two-hybrid experiments can be affected by altering the expression levels of bait and target proteins as discussed previously, but in most cases the reporter genes present in the yeast strain will have a greater effect. Some reporter genes are inherently more sensitive than others, but the reasons for this are not always clear. Sensitivity can also be directly controlled by altering the number of bait protein binding sites in the promoter of a reporter gene, a method that has been utilized extensively in the LexA system (27). There are, of course, limits to the sensitivity of two-hybrid experiments. It is likely that interactions with  $K_d$ s weaker than 10–50  $\mu M$  are not detectable (27), and weak interactions generally have lower plating efficiencies that effectively reduce their representation in two-hybrid libraries.

Unfortunately, the most sensitive reporters also tend to be the least selective, generating large numbers of false positives. There are many types of false positives and methods for eliminating them, as discussed in Chapters 8–10. However, because these methods involve a large amount of work, it is best to minimize the number of false positives initially identified. The most effective way to accomplish this is to utilize multiple reporter genes, preferably containing different promoter regions. The Gal4 system has an advantage in this regard because several different Gal4-inducible promoters are available (23,35); however, this goal can also be accomplished by combining reporters from different systems (29).

#### 1.2.1.1. USEFUL GENETIC MARKERS

A wide variety of genetic markers are available in yeast. Among the most common in two-hybrid strains are *trp1*, *leu2*, *ura3*, *his3*, *lys2*, *ade2*, *cyh2*, and *can1*. Although this list may seem extensive, it is possible to run short of markers. Two markers will be required for selection of the bait and target plasmids, and others will be used to introduce reporter constructs into the strain or to serve as reporters themselves. In addition, some two-hybrid interactions are dependent on a third protein to form a complex (e.g., the Ste5/Ste7/Ste11 complex [50–52]) or on a protein modification such as tyrosine phosphorylation (53). In such cases, an additional marker may be needed to introduce a third plasmid. Also, some markers such as *cyh2* and *can1* are useful only for negative selections, such as for plasmid loss. Thus, it is wise when choosing a two-hybrid strain to plan ahead for all the genetic markers that may be needed in an

experiment. The *ura3* and *ade2* markers are especially useful because each provides both positive and negative assays. The *URA3* gene can be used for both positive and negative selections, and *ADE2* can be used for positive selection and in a colony color-based negative screen (see **Note 2**). In addition, a recently introduced gene conferring resistance to the antibiotic zeomycin will be particularly helpful since it will function in any yeast strain regardless of existing markers.

#### 1.2.1.2. REPORTER CONSTRUCTS

When the first two-hybrid strains appeared roughly a decade ago, they contained only a single *lacZ* reporter gene. Improvements since then are considerable, in both number and quality, and provide the opportunity to eliminate many false positives by using more than one reporter construct. This strategy is most effective when the reporter constructs also use different promoters, because many false positives result from a specific interaction with sequences within the promoter region. Unfortunately, many reporters use common promoters and thus fail to take full advantage of this opportunity.

In the Gal4 system, almost all the reporters are expressed from a *GAL1* promoter. The *HIS3* reporters are the most sensitive, but also the least selective, generating large numbers of false positives. *HIS3* reporters are also leaky and the competitive inhibitor 3-amino-1,2,4-triazole (3-AT) is usually required to eliminate background growth. Those that require the least 3-AT provide an advantage since high levels of 3-AT (25–50 mM) will reduce the sensitivity of the reporter and can be toxic to the newly transformed cells. Other reporters in the Gal4 system include *ADE2*, *LEU2*, *URA3*, *CYH2*, *lacZ*, and *MEL1*. The *ADE2* reporter is especially useful because it is the most selective, eliminating almost all false positives in a simple plate assay (23). It has the added advantage of being the only reporter to use the *GAL2* promoter. Although it is too stringent to identify some interactions in freshly transformed cells, even weak interactions are able to activate the *ADE2* reporter once the plasmids have become established. Thus, it provides a powerful secondary screen in combination with more sensitive reporters.

The *URA3* and *CYH2* reporters are especially noteworthy because they have been developed for negative selections (i.e., select for the loss of an interaction) when performing reverse two-hybrid experiments (54–56). *URA3* is the more versatile of the two because it can also be used for positive selections and has been placed behind a unique set of Gal4-SPO13 promoters of varying sensitivity. The *lacZ* and *MEL1* reporters provide color assays that are best used for verification or quantitation of identified interactions. *lacZ* is an almost universally used reporter and is present in many strains with a *GAL1* or modified



*GAL1* promoter. One *lacZ* reporter uses the *GAL7* promoter (23), which may more effectively screen out false positives. This reporter suffers from high background in qualitative filter assays but performs very well in quantitative liquid assays of  $\beta$ -gal activity. The *MEL1* reporter has seen little use to date and is less sensitive than *lacZ* reporters but appears very promising. *MEL1* encodes the yeast  $\alpha$ -galactosidase, a secreted protein whose activity can be assayed without lysing the cells using the chromogenic substrate X- $\alpha$ -gal (35). *MEL1* is present as an endogenous gene in many two-hybrid yeast strains (see Note 6) and its native promoter is induced by Gal4. For strains that do not contain the endogenous gene, *MEL1* can be introduced on a plasmid such as pGB-MEL1.

In the LexA system, most selections use *LEU2* and *lacZ* reporters, but a *HIS3* reporter is available as well (45). The *LEU2* reporters all use the endogenous *LEU2* promoter in which the native UAS region has been replaced by LexA operator-binding sites. Similarly, most *lacZ* reporters use a *GAL1* promoter in which the Gal4-binding sites have been replaced by LexA operator-binding sites. Rather than using different reporter genes, the LexA system generates a range of reporter sensitivity by introducing different numbers of LexA-binding sites into the reporter gene promoters. *LEU2* reporters are driven by two, four, or six LexA operator-binding sites and *lacZ* reporters by two, four, or eight. The most sensitive of these allows detection of interactions with  $K_d$ s of  $>1 \mu M$ , but, at the same time, they generate increased background compared with more stringent reporters (27). The most stringent reporters may be useful for screening baits that present high-background problems.

While the *LEU2* reporters are integrated in a single copy in the genome of the yeast strain, the *lacZ* reporters may be integrated or introduced as plasmid-based reporters. Table 3 lists the plasmid-borne reporter constructs available for the LexA and other systems. The LexA-LacZ reporter plasmids can be used with any of three bacterial antibiotic resistance markers, *Amp<sup>R</sup>*, *Kan<sup>R</sup>*, or *Cam<sup>R</sup>*, which can help differentiate them from the target plasmid during plasmid rescue. In addition, Cormack et al. (57) have recently introduced a plasmid-borne GFP reporter gene driven by eight LexA operator-binding sites. This reporter is easy to assay but less sensitive than *LEU2* or *lacZ*.

The cI-based two-hybrid system was designed as a complement to the LexA system and uses *LYS2*, *lacZ*, and *gusA* reporters, each driven by a *GAL1* promoter containing three cI operator-binding sites (29). The *gusA* gene encodes  $\beta$ -glucuronidase and is used in a colorimetric assay with the substrate X-gluc. The *lacZ* and *gusA* reporters are plasmid borne, and the system is designed to allow LexA-*LEU2*, LexA-*lacZ*, cI-*LYS2*, and cI-*gusA* to be assayed in a single yeast strain. The resulting strain should provide a powerful screen against false

**Table 3**  
**Plasmid-Borne Reporters**

Vector	Reporter	Yeast origin	Yeast marker	<i>E. coli</i> marker	Reference
pSH18-34	LexAop(8x)-lacZ	2μ	URA3	Amp <sup>R</sup>	27
pJK103	LexAop(2x)-lacZ	2μ	URA3	Amp <sup>R</sup>	27
pRB1840	LexAop(1x)-lacZ	2μ	URA3	Amp <sup>R</sup>	27
pMW107	LexAop(8x)-lacZ	2μ	URA3	Cam <sup>R</sup>	42
pMW112	LexAop(8x)-lacZ	2μ	URA3	Kan <sup>R</sup>	42
pMW108	LexAop(2x)-lacZ	2μ	URA3	Cam <sup>R</sup>	42
pMW109	LexAop(2x)-lacZ	2μ	URA3	Kan <sup>R</sup>	42
pMW110	LexAop(1x)-lacZ	2μ	URA3	Cam <sup>R</sup>	42
pMW111	LexAop(1x)-lacZ	2μ	URA3	Kan <sup>R</sup>	42
pcIop-lacZ	cIop(3x)-GAL1 <sub>TATA</sub> -lacZ	2μ	URA3	Amp <sup>R</sup>	29
pRG2	cIop(3x)-GAL1 <sub>TATA</sub> -gusA	2μ	URA3	kan <sup>R</sup>	29
pGKS8	cIop(3x)-gusA	2μ	zeo <sup>R</sup>	zeo <sup>R</sup>	29
pGB-MEL1	MEL1	2μ	TRP1	Amp <sup>R</sup>	35
pGNG1	LexAop(8x)-GFP	2μ	URA3	Amp <sup>R</sup>	57

positives if a bait is cloned into both LexA and cI vectors, and it will allow the simultaneous screening of two different baits, with each serving as a specificity control for the other.

### 1.2.2. Two-Hybrid Yeast Strains

The currently available two-hybrid yeast strains and their features are listed in **Table 4**. Because mating protocols for two-hybrid screening are gaining popularity, many of the strains are listed with isogenic mating partners, although it is possible and sometimes advantageous to perform matings between unrelated strains as well. Within the Gal4 system, SFY526 (58) and PCY2 (17) contain only a *lacZ* reporter and are best suited to quantitative analysis of known interactions. SFY26 does not contain an endogenous *MEL1* gene, and PCY2 has not been tested.

For two-hybrid screening, the most selective Gal4-based strains are PJ69-4A and its isogenic partner PJ69-4α (23) (see **Note 7**). These strains contain three different reporter genes, and each is expressed from a different Gal4-inducible promoter. As a result, nearly all false positives are quickly eliminated with simple phenotypic assays. The *GAL1-HIS3* reporter is extremely sensitive yet requires very little of the competitive inhibitor 3-AT (1–3 mM). *GAL1-HIS3* is generally used for the initial selection of positive interactions and may generate large numbers of false positives. However, secondary screening with the

**Table 4**  
**Yeast Two-Hybrid Strains**

Strain	Promoter system	Reporter genes	Genetic markers	Genotype	Reference
PJ69-4A	Gal4	<i>ADE2, HIS3, lacZ, MEL1</i>	<i>trp1, leu2, ura3, met2</i>	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>	<b>23</b>
PJ69-4α	Gal4	<i>ADE2, HIS3, lacZ, MEL1</i>	<i>trp1, leu2, ura3, met2</i>	<i>MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>	<i>See Note 7</i>
SFY526	Gal4	<i>lacZ</i>	<i>trp1, leu2, his3, lys2, ade2, can1</i>	<i>MATa trp1-901 leu2-3,112 his3-200 ade2-101 lys2-801 can1<sup>R</sup> gal4-542 gal80-538 URA3 GAL1-lacZ</i>	<b>58</b>
YPB2	Gal4	<i>HIS3, lacZ</i>	<i>trp1, leu2, ade2, can1</i>	<i>MATa trp1 leu2 his3 ade2 can1<sup>R</sup> gal4 gal80 LYS2::GAL1-HIS3 URA3::GAL4 17mers)-lacZ</i>	<b>32</b>
HF7c	Gal4	<i>HIS3, lacZ</i>	<i>trp1, leu2, ade2</i>	<i>MATa trp1-901 leu2-3,112 his3-200 ade2-101 gal4-542 gal80-538 URA3::GAL4<sub>(3x17mers)</sub>-CYC1<sub>TATA</sub>-lacZ LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3</i>	<b>59</b>
Y153	Gal4	<i>HIS3, lacZ, MEL1</i>	<i>trp1, leu2, ade2</i>	<i>MATa trp1-901 leu2-3,112 his3-200 ade2-101 gal4Δ gal80Δ LYS2::GAL1-HIS3 URA3::GAL1-lacZ</i>	<b>38</b>
Y187	Gal4	<i>lacZ, MEL1</i>	<i>trp1, leu2, his3, ade2</i>	<i>MATα trp1-901 leu2-3,112 his3-200 ade2-101 gal4 gal80 URA3::GAL1-lacZ</i>	<b>39</b>
Y190	Gal4	<i>HIS3, lacZ, MEL1</i>	<i>trp1, leu2, ade2, cyh2</i>	<i>MATa trp1-901 leu2-3,112 his3-200 ade2-101 cyh2<sup>R</sup> gal4Δ gal80Δ LYS2::GAL1-HIS3 URA3::GAL1-lacZ</i>	<b>39</b>
YD116	Gal4	<i>URA3, lacZ</i>	<i>his3, leu2, trp1, lys2, ade2, can1</i>	<i>MATa ura3-52 his3-200 leu2 trp1-901 can1<sup>R</sup> gal4Δ512 gal80Δ338 lys2-801::UAS<sub>G</sub>-His3-lacZ ad e2-101::GAL1-URA3</i>	<b>18</b>
YD119	Gal4	<i>URA3, lacZ</i>	<i>his3, leu2, trp1, lys2, ade2, can1</i>	<i>MATα ura3-52 his3-200 leu2 trp1-901 can1<sup>R</sup> gal4Δ512 gal80Δ338 lys2-801::UAS<sub>G</sub>-His3-lacZ ade2-101::GAL1-URA3</i>	<b>18</b>

(continued)

**Table 4 (continued)**

Strain	Promoter system	Reporter genes	Genetic markers	Genotype	Reference
PCY2	Gal4	<i>lacZ</i>	<i>trp1, leu2, his3, lys2, ade2</i>	<i>MATα gal4Δ gal80Δ trp1-Δ63 his3-200 leu2 ade2-101 lys2-801 URA3 : :GAL1-lacZ</i>	<b>17</b>
MaV52	Gal4	<i>HIS3, lacZ, MEL1</i>	<i>trp1, leu2, ura3, can1, ade2, cyh2</i>	<i>MATa ura3-52 trp1-901 leu2-3,112 his3-200 ade2-101 gal4Δ gal80Δ can1<sup>R</sup> cyh2<sup>R</sup> LYS2 : :GAL1-HIS3 GAL1-lacZ</i>	<b>55</b>
MaV95 <sup>a</sup>	Gal4	<i>HIS3, URA3, lacZ, MEL1</i>	<i>trp1, leu2, ade2, can1, cyh2</i>	<i>MATa ura3-52 trp1-901 leu2-3,112 his3-200 ade2-101 gal4Δ gal80Δ can1<sup>R</sup> cyh2<sup>R</sup> LYS2 : :GAL1-HIS3 GAL1-lacZ SPAL5 : :URA3</i>	<b>55</b>
62 MaV96 <sup>a</sup>	Gal4	<i>HIS3, URA3, lacZ, MEL1</i>	<i>trp1, leu2, ade2, can1, cyh2</i>	<i>MATa ura3-52 trp1-901 leu2-3,112 his3-200 ade2-101 gal4Δ gal80Δ can1<sup>R</sup> cyh2<sup>R</sup> LYS2 : :GAL1-HIS3 GAL1-lacZ SPAL7 : :URA3</i>	<b>55</b>
MaV97 <sup>a</sup>	Gal4	<i>HIS3, URA3, lacZ, MEL1</i>	<i>trp1, leu2, ade2, can1, cyh2</i>	<i>MATa ura3-52 trp1-901 leu2-3,112, his3-200 ade2-101 gal4Δ gal80Δ can1<sup>R</sup> cyh2<sup>R</sup> LYS2 : :GAL1-HIS3 GAL1-lacZ SPAL8 : :URA3</i>	<b>55</b>
MaV99 <sup>a</sup>	Gal4	<i>HIS3, URA3, lacZ, MEL1</i>	<i>trp1, leu2, ade2, can1, cyh2</i>	<i>MATa ura3-52 trp1-901 leu2-3,112 his3-200 ade2-101 gal4Δ gal80Δ can1<sup>R</sup> cyh2<sup>R</sup> LYS2 : :GAL1-HIS3 GAL1-lacZ SPAL10 : :URA3</i>	<b>55</b>
CL9	Gal4	<i>CYH2, lacZ</i>	<i>his3, leu2</i>	<i>MATα gal4 gal80 his3 trp1-901 ura3-52 leu2-3,112 cyh2<sup>R</sup> TRP1 : :GAL1-CYH2 URA3 : :GAL1-lacZ</i>	<b>54</b>
CBY12a	Gal4	<i>LEU2, lacZ</i>	<i>trp1, his3, ade2</i>	<i>MATa gal4Δ gal80Δ trp1 his3 ade2 leu2 : :UAS<sub>GALI</sub>-LEU2 URA3 : :UAS<sub>GALI</sub>-lacZ</i>	<b>49</b>
CBY12α	Gal4	<i>LEU2, lacZ</i>	<i>trp1, his3, ade2</i>	<i>MATα gal4Δ gal80Δ trp1 his3 ade2 leu2 : :UAS<sub>GALI</sub>-LEU2</i>	<b>49</b>

	CBY14a	Gal4	<i>HIS3, lacZ</i>	<i>trp1, leu2, ade2</i>	<i>URA3::UAS<sub>GALI</sub>-lacZ</i> <i>MATa gal4Δ gal80Δ trp1 leu2 ade2 LYS2::UAS<sub>GALI</sub>-HIS3</i>	<b>49</b>
	CBY14α	Gal4	<i>HIS3, lacZ</i>	<i>trp1, leu2, ade2</i>	<i>URA3::UAS<sub>GALI</sub>-lacZ</i> <i>MATα gal4Δ gal80Δ trp1 leu2 ade2 LYS2::UAS<sub>GALI</sub>-HIS3</i>	<b>49</b>
	CTY10-5d	LexA	<i>lacZ, MEL1</i>	<i>trp1, leu2, his3, ade2</i>	<i>URA3::UAS<sub>GALI</sub>-lacZ</i> <i>MATa trp1-901 leu2-3,112 his3-200 ade2 gal4Δ gal80Δ URA3::lexA-lacZ</i>	<b>41</b>
	L40	LexA	<i>HIS3, lacZ</i>	<i>trp1, leu2, ade2</i>	<i>MATa trp1 his3 leu2 ade2 LYS2::lexAop(4x)-HIS3</i> <i>URA3::lexAop(8x)-lacZ</i>	<b>45</b>
	EGY48	LexA	<i>LEU2</i>	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(6x)-LEU2</i>	<b>24</b>
	EGY191	LexA	<i>LEU2</i>	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(2x)-LEU2</i>	<b>27</b>
	EGY195	LexA	<i>LEU2</i>	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(4x)-LEU2</i>	<b>27</b>
	EGY40	LexA	None (negative control)	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(0x)-LEU2</i>	<b>24</b>
63	SKY48	LexA, cI	<i>LEU2, LYS2</i>	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(6x)-LEU2 cIop(3X)-LYS2</i>	<b>29</b>
	SKY191	LexA, cI	<i>LEU2, LYS2</i>	<i>trp1, his3, ura3</i>	<i>MATα trp1 ura3 his3 lexAop(2x)-LEU2 cIop(3X)-LYS2</i>	<b>29</b>
	SKY473	LexA, cI	<i>LEU2, LYS2</i>	<i>trp1, his3, ura3</i>	<i>MATa trp1 ura3 his3 lexAop(4x)-LEU2 cIop(3X)-LYS2</i>	See <b>Note 5</b>
	PL1	ER	<i>URA3</i>	<i>trp1, leu2, his3</i>	<i>MATα ura3-ΔI his3-Δ200 leu2-ΔI trp1::ERE<sub>1x</sub>-URA3</i>	<b>30</b>
	PL3	ER	<i>URA3</i>	<i>trp1, leu2, his3</i>	<i>MATα ura3-ΔI his3-Δ200 leu2-ΔI trp1::ERE<sub>3x</sub>-URA3</i>	<b>30</b>

<sup>a</sup>SPAL(x) indicates an SPO13 promoter containing (x) GAL4-binding sites.

highly selective *GAL2-ADE2* reporter eliminates most or all of the false positives. The remaining positive colonies are assayed for activation of the *GAL7-lacZ* reporter. This reporter is best analyzed using quantitative liquid assays; the number of positives remaining at this step is generally quite manageable. In addition, PJ69-4A contains an endogenous *MEL1* gene, which can serve as a fourth reporter or be used as an alternative to *GAL7-lacZ*. Finally, unlike most two-hybrid strains, PJ69-4A is *ura3*<sup>-</sup>; therefore, if a bait plasmid marked by *URA3* is used, plasmid loss assays can be carried out as a selection on 5-FOA medium.

Another frequently used set of Gal4-based strains are Y153, Y190, and Y187 (38,39). Y153 and Y190 contain *GAL1-HIS3* and *GAL1-lacZ* reporters while the mating partner Y187 contains only *GAL1-lacZ*. All three strains also contain the endogenous *MEL1* gene, which can serve as an additional reporter with a different promoter. The *GAL-HIS3* reporter in these strains requires high levels of 3-AT (25–50 mM). Additional pairs of mating strains in the Gal4 system are YD116/YD119 (18), CBY12a/CBY12 $\alpha$ , and CBY14a/CBY14 $\alpha$  (49), which contain *GAL1-URA3*, *GAL1-LEU2*, and *GAL1-HIS3* reporters, respectively. All contain *lacZ* reporters as well, but the *MEL1* status of these strains has not been tested. YPB2 (32) and HF7c (59) are additional choices containing *HIS3* and *lacZ* reporters. The *HIS3* reporters in these strains require only low levels of 3-AT, but neither contains the endogenous *MEL1* gene.

Several Gal4-based strains can be used for negative selection to identify the disruption of an interaction in the reverse two-hybrid system. CL9 uses a *GAL1-CYH2* reporter for this purpose (54), whereas the *URA3* reporters in YD116/YD119 and the MaV strain series (55) can be used for both positive and negative selections. The MaV strains should prove especially useful because they contain three reporter genes and the *URA3* reporter is driven by unique *GAL-SPO13* promoters that vary in sensitivity based on the number of Gal4-binding sites present. Because they are derived from Y153, the MaV strains will require high levels of 3-AT for the *HIS3* reporter but should also contain the endogenous *MEL1* gene.

In the LexA system, the EGY strain series is the most frequently used. Strains in the EGY series contain integrated *LEU2* reporters driven by promoters with two, four, or six LexA operator-binding sites. Plasmid-borne *lacZ* reporters with one, two, or eight LexA operator-binding sites are introduced by transformation. This combination allows screening to be conducted over a wide range of sensitivity levels. An additional advantage of the LexA system is the ability to use target plasmids in which the activation domain fusion is conditionally expressed from the inducible *GAL1* (or *GAL10*) promoter. This ability aids in the identification of false positives and may allow the screening of toxic

proteins. Additional LexA strains are L40 (**45**), which is also commonly used and contains *HIS3* and *lacZ* reporters, and CTY10-5d (**41**), which contains only a *lacZ* reporter and is suitable for quantitative analysis of known interactions.

The SKY48 and SKY191 strains recently introduced by Serebriiskii et al. (**29**) are of special note. These strains combine LexA-based and cI-based reporters to generate a new “dual bait” system. Each strain contains integrated LexA-LEU2 and cI-LYS2 reporters and can be transformed with four plasmids: a LexA-*lacZ* reporter plasmid, pGKS8 containing a cI-fused bait and a cI-*gusA* reporter, a LexA bait plasmid, and an AD target plasmid. The resulting system can be used to screen two baits simultaneously, both negatively controlled against the other. In addition, if the same bait protein is fused to both LexA and cI, the dual bait system should provide a powerful screen for the elimination of false positives.

Finally, the PL1 and PL3 yeast strains are used in the ER-based system (**30**). Each contains a *URA3* reporter gene with a promoter containing one or three ER-binding sites. The *URA3* promoter allows positive or negative selections and can also be used for quantitative assay of orotidine-5'-monophosphate decarboxylase activity.

## 2. Materials

### 2.1. Growth and Maintenance of PJ69-4A

1. Plate incubator and shaking incubator set to 30°C.
2. 15% Glycerol in water, sterilized by filtration or autoclaved.
3. Yeast extract, peptone, dextrose (YPD) medium: 10 g/L of Bacto yeast extract, 20 g/L of Bacto peptone, and 20 g/L of D-glucose in 1 L of distilled water. For plates add 18 g of Bacto agar. Do not add adenine to the YPD media as some recipes suggest, because it will interfere with the red phenotype of PJ69-4A. Autoclave for 25 min. Note that D-glucose caramelizes when autoclaved too long. The medium has a distinct smell and color when this happens and should not be used. Always take the medium out of the autoclave as soon as it is done. Allow to cool to 65°C before pouring plates.
4. Synthetic complete (SC) medium and dropout medium: 6.7 g/L of Difco yeast nitrogen base without amino acids and with ammonium sulfate, 20 g/L of D-glucose, 20 mL of 50X tyrosine stock, and 20 mL of 50X appropriate dropout stock (for SC use Trp dropout stock) in 1 L of distilled water. For plates add 18 g of Bacto agar. Autoclave for 25 min and let cool to 65°C. Add 10 mL of 100X Trp stock and pour plates (do not add Trp stock if Trp is one of the amino acids to be left out).
5. 50X Dropout stocks: Delete from the recipe the ingredients that are to be dropped out. For example, to make 50X Leu, Ura dropout stock (for SC–Leu–Ura medium), do not add uracil or leucine. For 50X Trp dropout stock, include all of

the following: 400 mL of ddH<sub>2</sub>O, 0.4 g of adenine (Ade), 0.4 g of uracil (Ura), 0.4 g of histidine (His), 0.4 g of arginine (Arg), 0.4 g of methionine (Met), 0.4 g of inositol (Ino), 1.2 g of lysine (Lys), 1.6 g of leucine (Leu), 1.6 g of isoleucine (Ile), 1.0 g of phenylalanine (Phe), 2.0 g of glutamic acid (Glu), 2.0 g of aspartic acid (Asp), 3.0 g of valine (Val), 4.0 g of threonine (Thr), and 7.5 g of serine (Ser). Mix in a 500-mL bottle with a stir bar until dissolved (about 45 min), autoclave for 25 min, and store at 4°C.

6. 100X Trp stock: Add 3.2 g of tryptophan (Trp) to 400 mL of distilled water. Filter sterilize into a sterile 500-mL bottle and store at 4°C.
7. 50X Tyrosine stock: Add 1.5 g of tyrosine (Tyr) and 16.67 mL of 6 *N* NaOH to 1 L of distilled water. The NaOH is included to help the tyrosine dissolve. Autoclave for 25 min and store at room temperature.

## **2.2. Construction of Two-Hybrid Plasmids**

1. DBD or transcription AD vector (*see Subheading 1.1.*).
2. Gene fragment encoding protein of interest.
3. Standard molecular biology reagents and supplies (60).

## **2.3. In Vitro Verification of Two-Hybrid Plasmids**

1. Standard molecular biology reagents and supplies (60).

## **2.4. Introduction of Bait Plasmid into PJ69-4A**

1. Liquid YPD medium.
2. Plasmid DNA.
3. SC-Ura dropout plates.
4. 1X TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (autoclaved).
5. 1X TE/LiAc: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM LiAc (autoclaved).
6. 10X TE/LiAc: 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 M LiAc (autoclaved).
7. Single-stranded DNA (ssDNA) (2 mg/mL): Dissolve salmon sperm or calf thymus DNA in sterile 1X TE at a concentration of 2 mg/mL and mix vigorously until dissolved (several hours). Aliquot and place in a boiling water bath for 10 min. Cool quickly on ice and store at -20°C.
8. 50% Polyethylene glycol (PEG): Mix 50 g of PEG (mol wt = 3,350) with 35 mL of ddH<sub>2</sub>O in a beaker and stir until dissolved (30 min). Transfer to a 100-mL graduated cylinder. Rinse the beaker with a small amount of distilled water, and add to the PEG solution. Bring the volume to exactly 100 mL with ddH<sub>2</sub>O. Filter sterilize and store in a tightly capped bottle.
9. LiAc/ssDNA/PEG mix: Make fresh each time. For each transformation (two plasmids into two yeast strains, with “no DNA” controls for each strain, equals six transformations), mix 240 µL of 50% PEG, 36 µL of 10X TE/LiAc, 36 µL of 2 mg/mL ssDNA, and 48 µL of ddH<sub>2</sub>O.



### 2.5. *In Vivo* Verification of Bait Plasmids

1. 2X Sample buffer: 20% glycerol, 2% sodium dodecyl sulfate (SDS), 100 mM Tris, pH 6.8. Add 40  $\mu$ L of  $\beta$ -mercaptoethanol ( $\beta$ -ME)/mL at the time of use.
2. Glass beads: 0.45- to 0.55-mm glass beads should be soaked several hours to overnight in an excess of 2 *N* HCl, then washed extensively with ddH<sub>2</sub>O. When water washing restores the pH above 5.0, the beads can be brought to neutral pH using Tris solutions. Wash several more times with water to reduce the Tris to <10 mM. Bake in an oven until dry.
3. Antibody to recognize the bait fusion protein.
4. Standard Western blotting reagents.
5. SC–Ura–Leu–His plates containing 3 mM 3-AT. 3-AT should be made as a 1 *M* stock in sterile ddH<sub>2</sub>O and added to the cooled medium just prior to plate pouring. Store at –20°C.
6. Z buffer: 16.1 g/L of Na<sub>2</sub>HPO<sub>4</sub>, 5.5 g/L of NaH<sub>2</sub>PO<sub>4</sub>, 0.75 g/L of KCl, 0.246 g/L of MgSO<sub>4</sub>. Adjust the pH to 7.0. At the time of use, add  $\beta$ -ME at 2.7 mL/L.
7. *o*-Nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) (4 mg/mL) in Z buffer; store at –20°C.

### 2.6. Introduction of a Library into PJ69-4A

1. The same materials are required as in **Subheading 2.4.**, with the addition of SC–Ura–Leu–His plates containing 3 mM 3-AT (*see Subheading 2.5.*). To scale up the LiAc/ssDNA/PEG mix, use: 6.7 mL/10 mL of 50% PEG, 1 mL/10 mL of 10X TE/LiAc, 1 mL/10 mL of 2 mg/mL ssDNA, and 1.3 mL/10 mL of ddH<sub>2</sub>O.

### 2.7. Selection of Interacting Proteins

1. SC–Ura–Leu–Ade plates.
2. Replica-plating apparatus.
3. Sterile velvets: Velvet fabric should be cut into 6-in. squares that are sewn around the edges to prevent fraying. Wash the velvets after each use in a standard washing machine without soap. After drying, wrap the velvets in aluminum foil in packs of about 10 and sterilize in an autoclave on a dry-load setting.
4. Whatman 50 (or Whatman 1) and Whatman 3 filter paper circles.
5.  $\beta$ -galactosidase assay buffer: 60 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 60 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 10 mM KCl, 1 mM MgSO<sub>4</sub>. Immediately prior to use, add 5.8  $\mu$ L of  $\beta$ -ME and 20  $\mu$ L of 2% X-gal in dimethylformamide for every 2 mL of assay buffer.

### 2.8. Elimination of False Positives Using Plasmid Loss Assays

1. YPD, SC–Ura, SC–Leu, SC–Leu–His + 3 mM 3-AT, and SC–Leu–Ade plates.
2. Sterile velvets and replica-plating apparatus (*see Subheading 2.7.*).
3. 5-Fluoro-orotic acid (5-FOA) plates (*see Subheading 2.1.* for solutions): Mix together 450 mL/L of ddH<sub>2</sub>O, 20 mL/L of 50X tyrosine stock, 20 mL/L of 50X

be cut efficiently. PCR-induced mutations, unfortunately, are common, so all PCR-generated gene fragments should be verified by sequencing before proceeding with a screen.

### **3.3. *In Vitro* Verification of Two-Hybrid Plasmids**

Prior to using a new plasmid, one must verify that the construction proceeded correctly. At a minimum, the investigator should confirm that all restriction sites expected to be restored by the ligation are present. This may include novel sites that were not previously present; for instance, the ligation of Klenow-filled *Bam*HI and *Bgl*III sites creates a new *Cla*I site. If no sites are restored by the construction, the fusion site between the DBD or transcription AD and the protein of interest should be sequenced to confirm that it is in-frame (*see Note 8*).

### **3.4. Introduction of Bait Plasmid into PJ69-4A**

A simplified transformation protocol (**61**) is sufficient for transforming single plasmids into PJ69-4A. This method will yield several thousand transformants. Transform the empty bait vector and a known activator into PJ69-4A at the same time to use in future control experiments (*see Note 9*).

1. Inoculate a 5-mL liquid culture of PJ69-4A into YPD and grow overnight with shaking at 30°C.
2. Pellet 1 mL of cells for each plasmid to be transformed, using Eppendorf tubes and spinning 5 s at top speed in a microfuge.
3. Resuspend the cells in 1 mL of 1X TE/LiAc and repellet.
4. Resuspend each pellet in 100 µL of 1X TE/LiAc. Incubate for 15 min on a benchtop with no agitation.
5. Pellet the cells again and remove all the supernatant with a pipetman.
6. Add 100 ng of plasmid DNA to the pellet, and then add 360 µL of the LiAc/ssDNA/PEG mix.
7. Mix thoroughly by pipetting up and down, followed by brief vortexing.
8. Incubate for 30 min on the benchtop with no agitation.
9. Incubate for 20 min in a 42°C water bath.
10. Pellet the cells in a microfuge.
11. Draw off all the supernatant with a pipetman.
12. Resuspend the pellet completely in 500 µL of sterile 1X TE.
13. Spread 100 µL of cell suspension onto an SC-Ura dropout plate (*see Note 10*). Let dry and then incubate inverted 2 to 3 d at 30°C.

### **3.5. *In Vivo* Verification of Bait Plasmids**

Once the fusion protein is transformed into yeast, two potential problems might disrupt a screen. One is that the fusion protein is not properly expressed

and thus the screen will not work. The other is that the fusion protein is expressed but that it is capable of activating the reporters by itself, causing a high background. The best way to verify proper expression is to demonstrate function, either by showing that a known interaction works or by complementing a mutant phenotype in yeast. Most often though these options do not exist, and Western blotting is used to check expression of the fusion protein.

1. Grow a 5-mL overnight culture of PJ69-4A containing the bait plasmid in liquid SC-Ura.
2. Pellet the cells and resuspend in 100  $\mu$ L of 2X sample buffer.
3. Transfer to an Eppendorf tube and add 50  $\mu$ L of glass beads.
4. Vortex hard for 1 min.
5. Place in a boiling water bath for 5 min; cool quickly on ice.
6. Spin down cell debris for 10 s in a microfuge and load 10  $\mu$ L of the supernatant on an SDS-polyacrylamide gel.
7. Electrophorese and transfer to nitrocellulose.
8. Immunoblot with antibodies against the fusion protein (*see Note 11*).

Note that Western blotting only determines whether a protein is present, not whether it is properly folded or functional. Also, it is not uncommon for a fusion protein to be undetectable by Western blotting yet be successful in a two-hybrid screen.

To check a bait for background activation, the strain should contain both the bait and an empty transcription AD vector. Introduce this second plasmid by transformation (*see Subheading 3.4.*) or mating (*see Subheading 3.10.*), plating on double dropout medium such as SC-Ura-Leu to select for both plasmid markers. At the same time, add this second plasmid to PJ69-4A containing the empty bait vector. Test the ability of the cells to grow on SC-Ura-Leu-His with 3 mM 3-AT and SC-Ura-Leu-Ade, and for activity in a  $\beta$ -galactosidase liquid assay (**62**):

1. Grow a 5-ml culture in SC-Ura-Leu. Spin down 1.0 OD<sub>600</sub> U of cells in an Eppendorf tube (less may be required for very strong activators).
2. Resuspend the pellet in 1 mL of Z buffer.
3. Add 10  $\mu$ L of 0.1% SDS and 50  $\mu$ L of chloroform. Vortex hard for 10 s.
4. Place the samples in a 28°C water bath.
5. Add 200  $\mu$ L of ONPG (4 mg/mL in Z buffer; store at -20°C). Note the time of addition.
6. When the solution is pale yellow, stop the reaction by adding 500  $\mu$ L of 1 M Na<sub>2</sub>CO<sub>3</sub>. Note the time of addition.
7. Spin in a microfuge for 5 min and measure the OD<sub>420</sub> of the supernatant.  $\beta$ -gal units = (OD<sub>420</sub>  $\times$  1000)/(total OD<sub>600</sub>s of cells  $\times$  incubation time in minutes).

An ideal bait will show no growth on plates, either in patches or as single colonies, and will produce the same level of  $\beta$ -gal activity as the empty vectors. Some baits will be strong activators, however, and others will activate weakly, producing tiny or rare single colonies. If a bait plasmid does cause background activation, it may not be possible to continue with the experiment, although possible solutions do exist (*see Note 12*).

### **3.6. Introduction of a Library into PJ69-4A**

Transformation of a library requires a large-scale, high-efficiency transformation procedure. The protocol described here follows closely the methods described by Agatep et al. (61). Because of the large scale of the experiment and because the cells will be incubated for long periods (14 d or longer), a proper sterile technique is extremely important. Even a few contaminants will be able to overgrow the plates before the experiment is completed. If contaminants do occur (and are not too numerous), they can be surgically removed from the plates early in the incubation. In addition, because of the length of the incubation, plates should be poured a little thicker than usual to prevent them from drying out. The number of desired transformants will vary with the particular library being screened, but it is best to err on the high side. Although in theory it is easy to calculate the number required for 99% coverage of the library, in practice fivefold coverage of a genome almost never results in five isolates of each target.

#### **3.6.1. Library-Scale Transformation of PJ69-4A**

This protocol generally produces a total of 3–15 million transformants in PJ69-4A.

1. For each library to be transformed, inoculate PJ69-4A containing the bait plasmid into a 25-mL SC–Ura liquid culture and grow overnight with shaking at 30°C.
2. In the morning, use the overnight culture to inoculate 100 mL of prewarmed liquid YPD to an OD<sub>600</sub> of 0.2. If more than 10 mL of the overnight culture is required, concentrate first by centrifugation to avoid excessive dilution of the YPD.
3. Grow the cells for 4 to 5 h with shaking at 30°C until the OD<sub>600</sub> is 1.0.
4. Pellet the cells in two sterile 50-mL Falcon tubes in a tabletop centrifuge (5 min at 600g (2000 rpm) is usually sufficient). Resuspend the pellet in 50 mL of 1X TE/LiAc and repellet.
5. Resuspend the cells in 1 mL of 1X TE/LiAc and transfer to a sterile 15-mL Falcon tube.
6. Incubate for 15 min on the benchtop with no agitation.
7. Pellet the cells again and remove all the supernatant with a sterile pipet.

8. Add 100  $\mu\text{g}$  of library DNA to the pellet, and then add 7 mL of the LiAc/ssDNA/PEG mix.
9. Mix thoroughly by pipetting up and down, followed by brief vortexing.
10. Incubate for 30 min on the benchtop with no agitation.
11. Incubate for 20 min with the portion of the tube containing the cell suspension fully submerged in a 42°C water bath. Mix by inversion every 5 min.
12. Pellet the cells in a tabletop centrifuge.
13. Draw off all the supernatant with a sterile pipet.
14. Resuspend the pellet completely in 8 mL of sterile 1X TE.
15. Dilute 10  $\mu\text{L}$  of the cell suspension into 1 mL of 1X TE, and plate 80  $\mu\text{L}$  of the dilution on an SC–Ura–Leu plate. Each 100 colonies that grow on this plate corresponds to one million total transformants screened.
16. Spread 200  $\mu\text{L}$  of cell suspension onto each of 40 standard size SC–Ura–Leu–His dropout plates containing 3 mM 3-AT (*see Subheading 2.5.*). Let dry and then incubate inverted at 30°C.

### 3.7. Selection of Interacting Proteins

As already stated, the primary transformants containing the bait and library plasmids should be spread onto SC–Ura–Leu–His dropout plates containing 3 mM 3-AT. It is important to select for the presence of both plasmids as well as reporter activation because cells can become His<sup>+</sup> via plasmid-independent mechanisms. Although the amount of 3-AT required will vary for different baits, with some requiring none, experience has shown that in PJ69-4A 3 mM 3-AT is sufficient for all baits except those with true background problems.

The SC–Ura–Leu–His plates should be incubated for 14 d in a 30°C incubator (*see Note 13*). The plates can be protected from drying out by placing a plastic bag over the top of the stack, leaving the bag open at the bottom. By the end of this incubation, there are frequently hundreds of colonies per plate of many different sizes and morphologies. Each SC–Ura–Leu–His plate should be replica plated onto an SC–Ura–Leu–Ade plate using sterile velvets and a replica-plating apparatus. Ade<sup>+</sup> colonies should appear after 1–3 d of incubation at 30°C.

The *GAL2-ADE2* reporter is very selective, and generally only a few of the many His<sup>+</sup> colonies will prove to be Ade<sup>+</sup> as well (*see Note 14*). One class of false positives that has appeared in several screens and does often grow on SC–Ura–Leu–Ade are colonies that have a dry, crusty morphology on plates and are very flocculent in liquid culture. To date, none of these colonies has been shown to represent a real interaction, and they should be discarded. Colonies that are both His<sup>+</sup> and Ade<sup>+</sup> should next be tested for activation of the *GAL7-lacZ* reporter using the liquid  $\beta$ -gal assay described in **Subheading 3.5**. The number of His<sup>+</sup> Ade<sup>+</sup> colonies that remains is usually low and can easily

be handled in liquid assays. However, if more than 50 positives are to be tested a filter assay may be preferable. The *GAL7-lacZ* reporter does produce high background in standard filter assays; however, the following modified procedure has been successful in overcoming this problem.

### 3.7.1. $\beta$ -Galactosidase Filter Assay for PJ69-4A

1. Overlay an SC–Ura–Leu–Ade plate with a circular piece of Whatman 50 or Whatman 1 filter paper. Use sterile toothpicks to spread a small patch of each colony to be tested onto the surface of the filter paper. Incubate at 30°C.
2. Once the patches are grown, lift the filters from the plates.
3. Using a long forceps, submerge the filter in liquid nitrogen for about 10 s.
4. Lay the filter on a paper towel, yeast side up, and allow to thaw completely (3–5 min).
5. Repeat **steps 3 and 4** two more times.
6. Place a circle of Whatman 3 paper in a Petri dish and saturate completely with 2 mL of  $\beta$ -gal assay buffer. Pour off any excess.
7. Overlay the thawed filter yeast side up onto the Whatman 3 filter. Seal the Petri dish with parafilm and incubate at 30°C.

The filter should be observed periodically for up to 8 h (*see Note 15*). Reporter activation will result in blue color development, which often is best observed from the bottom of the plate. Finally, positive colonies can also be assayed for activation of the endogenous *MEL1* gene as described in Aho et al. (35), either in addition to or in place of the  $\beta$ -gal assays.

At this point, it is a good idea to make a permanent stock of each positive colony to be stored at –70°C. Active cultures should be maintained under selection for interaction (i.e., on SC–Ura–Leu–Ade plates) with frequent restreaking (*see Subheading 3.1.*).

### 3.8. Elimination of False Positives Using Plasmid Loss Assays

In some cases, all the colonies that remain as positives at this stage represent real interactions. However, some false positives can remain, and it is important to check for these using plasmid loss assays. The basic principle of these assays is that a real interaction should require both interacting proteins; an ability to activate the reporters in the absence of either plasmid is indicative of a false positive. Two features of PJ69-4A make these assays straightforward. One is retention of the *ura3* allele, which allows the use of a *URA3*-marked bait, and the other is the color phenotype associated with the *GAL2-ADE2* reporter. First, if a bait plasmid marked by *URA3* has been used, then loss of the bait can be selected using 5-FOA medium.

1. Grow a patch of each positive overnight on an SC–Leu plate.
2. Taking cells from the SC–Leu plate, spread a thin patch of each positive onto a 5-FOA plate. Incubate at 30°C for 3 d.
3. Single colonies should arise on the 5-FOA plate within each patch. Test several colonies from each patch for the ability to grow on SC–Leu–His + 3 mM 3-AT and SC–Leu–Ade (note that because the bait plasmid is gone, double dropout medium replaces the triple dropout). An ability to grow indicates a false positive that should be discarded.

This test determines whether the library plasmid can activate the reporters by itself. An equally important determination is whether the bait plasmid can activate the reporters by itself. Although the bait plasmid was tested for this activity in **Subheading 3.5.**, it is possible for some baits to become activators via mutation (unpublished observations; and [63]). This typically occurs in baits that contain acidic regions that may become exposed as a result of the mutation and can sometimes account for a majority of the positive colonies.

A second plasmic loss assay takes advantage of the color phenotype associated with the *GAL2-ADE2* reporter gene. In yeast, *ade2* mutants form red colonies on YPD plates because of the accumulation of a pigmented pathway intermediate. Because PJ69-4A uses the *GAL2-ADE2* reporter, colonies are normally red on YPD but are restored to the normal white color in the presence of an interaction. This color phenotype can be used in a simple screen for plasmid loss.

Do the following for each positive to be tested:

1. Grow as a patch overnight on a YPD plate.
2. Resuspend the cells from the YPD plate in sterile ddH<sub>2</sub>O in an Eppendorf tube.
3. Spread on a new YPD plate an appropriate dilution of cells to produce 200–300 single colonies per plate. Incubate for 3 d at 30°C. The plates should contain a mix of all red, all white, and red and white sector colonies.
4. Using a single sterile velvet for each YPD master plate, replica plate the colonies to SC–Ura, SC–Leu, and YPD plates. Incubate at 30°C overnight.

To analyze the results of this assay, use the SC–Ura and SC–Leu plates to identify colonies that have lost one or the other plasmid (failure to grow on these plates). For a real interaction, each Ura+Leu– or Ura–Leu+ colony should produce a red colony on YPD. If a candidate produces Ura– or Leu– colonies that are white on YPD, it should be discarded as a false positive. In addition, check whether all the Ura+Leu+ colonies are white. The presence of red colonies that contain both plasmid markers may indicate that more than one library plasmid is present (*see Subheading 3.9.*).



### 3.9. Rescue of a Library Plasmid from Yeast

The library plasmid can be recovered from remaining positive candidates using the protocol of Ward (16). The library plasmid is differentiated from the bait plasmid by selecting for complementation of the *E. coli leuB6* mutation by the yeast *LEU2* gene. If a *LEU2* marked bait or a *leuB6* strain is not used, it may be possible to use a plasmid with an alternate antibiotic marker. Otherwise, it will be necessary to screen the plasmids recovered by restriction digest. This can be difficult since the bait and library plasmids often are not recovered at the same rate.

1. Grow a 5-mL liquid culture of each positive in SC–Ura–Leu–Ade (see **Note 16**).
2. Spin down 3 mL in two successive spins in an Eppendorf tube. Remove the supernatant.
3. Resuspend the pellet in 300  $\mu$ L of 4% Triton X-100, 2.5 M LiCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA.
4. Add 300  $\mu$ L of phenol:chloroform:isoamyl (25:24:1).
5. Add 100  $\mu$ L of glass beads.
6. Vortex for 2 min at top speed.
7. Spin for 5 min.
8. Recover the aqueous phase and reextract with 500  $\mu$ L of phenol:chloroform:isoamyl (25:24:1) two to three times.
9. Treat the aqueous phase with Wizard Cleanup resin (Promega) according to the instructions, recovering the DNA in 50  $\mu$ L.
10. Transform HB101 *E. coli* cells (or other *leuB6* mutant strain) by electroporation using 1  $\mu$ L of DNA (see **Note 17**). If desired, the DNA can also be concentrated by EtOH precipitation prior to transformation.
11. Plate the cells on M9 plates supplemented with 0.02% proline to select for complementation of the *leuB6* mutation by the yeast *LEU2* gene.
12. Because it is common for positives to contain more than one library plasmid, DNA should be prepared from several transformants and compared by restriction digest. If multiple plasmids are found, each should be tested as described in **Subheading 3.10**.

### 3.10. Verification of Interaction by Reconstruction

One remaining class of false positives are those that are dependent on the presence of a bait plasmid but are not specific to a particular bait (26). To eliminate these, and to demonstrate that the correct library plasmid has been rescued, each library plasmid is reintroduced into PJ69-4A in combination with the correct bait and several controls (see **Note 18**). Each bait/library combination is then tested for reporter gene activation as described in section **Subheading 3.7**. A true positive should activate transcription in the presence of the



original bait but not with control baits. If multiple baits result in activation of reporters, the candidate is a false positive. If there is no activation, even in the presence of the original bait, the correct library plasmid may not have been rescued, and the protocol in **Subheading 3.9.** should be repeated.

Library and bait plasmids can be reintroduced by transformation as described in **Subheading 3.4.** Another option is to use a mating strategy with PJ69-4 $\alpha$ . A number of different permanent stocks of PJ69-4 $\alpha$  can be kept, each containing a different bait plasmid. PJ69-4A isolates containing various library plasmids can then be mated to the strains containing different baits and the resulting diploids tested for reporter activation. Note that this procedure can also be carried out prior to plasmid rescue, by using the Ura-Leu+ colonies isolated in **Subheading 3.8.**

1. Using sterile toothpicks, draw parallel lines of PJ69-4A cells across a YPD plate, with each line of cells containing a different library plasmid. Repeat this on a second YPD plate using PJ69-4 $\alpha$  containing different bait plasmids. Incubate overnight at 30°C.
2. Replica plate the PJ69-4A cells onto a fresh YPD plate. Using a fresh velvet, replica plate the PJ69-4 $\alpha$  cells onto the same YPD plate, but turn the plate 90° so that the lines cross at right angles. Incubate at 30°C overnight.
3. Replica plate onto an SC-Ura-Leu plate and incubate at 30°C for 1 to 2 d. Growth on the SC-Ura-Leu plate should occur only at the points where lines cross. Each diploid patch should be streaked out on SC-Ura-Leu plates to obtain single colonies and then tested for reporter activation as in **Subheading 3.7.**

### 3.11. Further Analysis of Positives

Once true positives have been identified and rescued, the library inserts should be sequenced (*see Note 8*) and analyzed. While a two-hybrid interaction is observed *in vivo*, it does not represent a physiologic situation. Thus, further experiments will be necessary to demonstrate the biologic significance of the interaction. This may involve attempts to cosediment or coimmunoprecipitate the native bait and target proteins, or to demonstrate a genetic interaction between the two. Further two-hybrid experiments may also be useful, e.g., using bait or target truncations to map the interaction domain, or examining whether existing mutations or phenotypes correlate with disruption of the interaction. Sequencing results may also suggest the need for additional experiments, such as biochemical assays, to determine enzyme/substrate relationships. Occasionally, sequencing provides disappointing results by identifying a target that does not coexist in biologic time and space with the bait. In these cases, the result may suggest the need for tests of similar proteins with more biologic significance.

#### 4. Notes

1. Clontech, Invitrogen, Stratagene, Origene, Display Systems Biotech, Gibco/Life Technologies, Bio101, and Mo Bi Tec are among the companies from which two-hybrid reagents are available.
2. Yeast strains containing the wild-type *URA3* gene convert the compound 5-FOA into a toxic product, killing the cells. The *CYH2* gene encodes the L29 ribosomal protein and confers sensitivity to the translation-inhibiting drug cycloheximide. In a yeast strain that contains a *ura3* or *cyh2*<sup>R</sup> mutation, inclusion of the appropriate inhibitor in the medium can be used to create a negative selection against plasmids carrying the wild-type gene. An additional system for assaying plasmid loss is based on the color phenotype associated with the *ADE2* gene. *ade2* mutants accumulate a pigmented intermediate of the adenine biosynthetic pathway and thus form red colonies on YPD medium. Restoration of the wild-type *ADE2* function returns the colonies to their normal white color. Thus, the presence or absence of plasmids that affect *ADE2* function can be determined based on colony color.
3. Some common laboratory strains of *E. coli* that carry the *leuB6* mutation include HB101, RR1, C600, KC8, and MC1066. Leucine prototrophy is selected by plating cells on M9 medium supplemented for any additional auxotrophies. HB101 and RR1 require only proline, at a final concentration of 0.02%.
4. In addition to many of the companies that sell two-hybrid reagents (*see Note 1*), Santa Cruz Biotechnology, Zymed, Babco, and Upstate Biotechnology carry antibodies of interest for two-hybrid screening.
5. The Web site from the laboratory of Erica Golemis ([www.fccc.edu/research/labs/golemis/](http://www.fccc.edu/research/labs/golemis/)) has much useful information on both the LexA and cI two-hybrid systems, including a listing of promiscuous proteins that occur as false positives in many screens.
6. Only a few strains have been tested for the endogenous *MEL1* gene. PJ69-4A and Y190 contain a functional *MEL1* whereas HF7c and SFY526 do not (from [www.clontech.com/techinfo](http://www.clontech.com/techinfo)).
7. PJ69-4 $\alpha$  was generated from PJ69-4A by HO-mediated mating type switching. This was done independently by Shane Cutler in the laboratory of Joseph Heitman and by Daniel Lockshon in the laboratory of Stan Fields.
8. Inserts in the pGBDU-C(x) or pGBD-C(x) bait vectors can be sequenced with the primer 5'-AAGAGAGTAGTAACAAAG-3'. Inserts in the pGAD-C(x) target vectors can be sequenced with the primer 5'-TTCGATGATGAAGATACC-3'. These primers can also be used to sequence other plasmids derived from pGBT9 and pGAD424.
9. Negative control strains should contain the empty bait and target vectors, because these sometimes behave differently than a strain with no plasmids. A common positive control vector is pCL1, which contains the wild-type *GAL4* gene (**I**).
10. In this protocol, I am assuming the use of a *URA3*-marked DBD plasmid and a *LEU2*-marked AD plasmid. Other markers can be used and will require an adjustment to the appropriate dropout medium.

11. Antibodies directed against Gal4 are available from a number of companies (*see Note 4*) but tend to be rather poor. In particular, they are unlikely to detect proteins expressed from the truncated *ADHI\** promoter. The best antibodies for immunoblotting are those directed against the bait fragment itself. These often detect bait proteins when Gal4 antibodies cannot. In some cases, the detection ability of Gal4 antibodies can be enhanced by first using them to immunoprecipitate the bait protein, and then loading the precipitate on a gel for Western blotting.
12. Although it is tempting to proceed with a screen when a bait activates weakly, many false positives will arise as a result of changes in bait copy number or protein stability (**26**). One of the strengths of the PJ69-4A strain is that some baits that activate *GAL1-HIS3* do not activate *GAL2-ADE2*, allowing screening with that reporter. It also may be possible to switch to the LexA system and use a high-stringency reporter such as *LexAop(2x)-LEU2* or *LexAop(1x)-lacZ*. Another popular approach is to use smaller fragments of the protein of interest in the bait in hopes of eliminating the activating region; however, there is a risk of eliminating an interaction domain as well. It may also be possible to dampen the *HIS3* reporter with increasing amounts of 3-AT, or to use the pGBREP vector to dampen the activity of the bait itself (**34**).
13. Occasionally a screen produces very few colonies that grow quickly on SC-Ura-Leu-His, and the incubation is terminated when it becomes clear that no new colonies are arising. Often these are all false positives, and these screens may involve baits that are not being functionally expressed. More often new colonies continue to appear, even after as long as 28 d. Colony growth is not predictive of real interactions, because some colonies that are large and healthy prove to be false positives, and some that are small and late to appear turn out to be real.
14. The *GAL2-ADE2* reporter is the greatest strength of PJ69-4A. It is highly selective (90% of colonies selected as Ade+ turn out to be His+ and  $\beta$ -gal+ as well) and yet remains sensitive, since even weak interactions when tested directly display an Ade+ phenotype. Although *GAL2-ADE2* can be used as the primary selection in a library screen, it is not recommended as a general method because some interactions are unable to activate this reporter in freshly transformed cells. These same interactions are Ade+ when grown first on SC-Ura-Leu and then transferred to SC-Ura-Leu-Ade. The likely explanation is that for some interactions the 2 $\mu$  plasmids must reach high copy number to provide sufficient activation of the *GAL2-ADE2* reporter. Because the plasmids are transformed into the cells as single copies, establishment of normal copy numbers may take several generations. Once the plasmids are established, however, the Ade phenotype is very reliable.
15. The additional freeze/thaw cycles used here increase the signal generated by positive colonies but do not affect the background, which will still be a problem beyond about 8 h of incubation. However, most positive interactions will turn blue within 2–4 h under these conditions. Some very weak interactions may still be difficult to detect. The assay should be terminated by removing and drying the top filter paper circle, which can then be stored indefinitely.

16. It is not uncommon for a positive candidate to contain more than one library plasmid species. For this reason positives should always be maintained on media that select for activation of a reporter gene. If positives are maintained on SC-Ura-Leu, they will quickly eliminate all but one library plasmid, and it will often not be the plasmid of interest.
17. Recovery of plasmids out of yeast generally works at low efficiency. It is essential to use highly competent (at least  $10^7$  transformants/ $\mu$ g of DNA) *E. coli* in order to succeed. In addition, the DNA preparation contains inhibitors that affect chemically competent *E. coli* more severely than electrocompetent cells. Given cells of equal competency, electroporation typically produces 10-fold more transformants than calcium- or rubidium-treated cells.
18. Control baits can contain any protein that is not expected to interact in your particular system. They can be collected from colleagues or obtained from most companies that sell two-hybrid reagents.

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## High-Efficiency Transformation of Plasmid DNA into Yeast

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### 1. Introduction

The yeast two-hybrid system was first introduced by Fields and Song (1) in 1989. They showed that plasmids expressing the *GAL4<sub>BD</sub>-SNF1* and *GAL4<sub>AD</sub>-SNF4* fusion proteins activated a *GAL1-lacZ* reporter gene. The first application of the system involved cotransformation of two plasmids into the strain GGY1::171—one plasmid containing the *GAL4<sub>BD</sub>* fused to sequences from the 3' end of *SIR4* and the other containing *GAL4<sub>AD</sub>* fused to fragments of yeast genomic DNA (2). The cotransformation was accomplished by the newly reported high-efficiency lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ssDNA/PEG) protocol (3) to generate 220,000 transformants and identified two positive clones. The efficiency of this transformation protocol was  $2 \times 10^4$  transformants/(g of plasmid· $10^8$  cells) (3), and the frequency of cotransformation was shown to range from 30 to 40% (4). Screening a mammalian cDNA library by the yeast two-hybrid system requires a large number of transformants. For example, the detection of proteins that interacted with huntingtin, the Huntington disease protein, involved a screen of  $4 \times 10^7$  transformants from an adult human brain cDNA library (5). We have reported several modifications and improvements to the LiAc/ssDNA/PEG protocol so that it can easily generate the numbers of transformants required for such screens.

Transformation of intact yeast cells following exposure to LiAc was first reported in 1983 (6). The yield of transformants was 400/μg of plasmid DNA/ $10^8$  cells, lower than that obtained with contemporary spheroplasting techniques (7,8). The addition of single-stranded carrier DNA to the transfor-

mation mix increased yields 50-fold (3). Reduced exposure to LiAc prior to transformation increased efficiency to  $1 \times 10^6$  transformants/ $\mu\text{g}$  of plasmid/ $10^8$  cells (9). Optimization of the levels of plasmid, carrier DNA, and cell concentration (10) as well as elimination of TE buffer from the protocol (11) increased yield of transformants to between 2 and  $5 \times 10^6/\mu\text{g}$  plasmid/ $10^8$  cells for most strains (12). We have also shown that solutions of carrier DNA can be used without sonication and phenol/chloroform purification and that the solutions of LiAc and PEG can be sterilized by autoclaving rather than filtration (13). The protocol can also be applied to cultures grown or replica plated into the wells of microtiter plates (14).

In our laboratory, we employ three versions of the LiAc/ssDNA/PEG protocol in the course of a two-hybrid screen. We use the rapid transformation protocol to introduce the bait plasmid into a yeast strain. This plasmid ( $GAL4_{BD}:YFG$ ) contains either the coding sequence for  $GAL4_{BD}$  or the *lexA* gene fused to a gene of interest (your favorite gene or *YFG*). For this procedure high-efficiency is not necessary, although we have improved this protocol significantly. The high-efficiency transformation protocol is used to determine the appropriate concentration of the prey or library plasmid for transformation of the yeast strain carrying the bait plasmid. We will assume that the prey plasmid ( $GAL4_{AD}:cDNA$ ) carries the sequence coding for the Gal4p DNA-activating domain fused to sequences from a cDNA or genomic DNA library. Finally, the two-hybrid screen transformation protocol, a scaled-up version of the previous protocol, is used to generate the required number of transformants for effective screening of a complex cDNA or genomic library. In our experience, serial transformation of the bait and library plasmids is more efficient than cotransformation, and we prefer to transform them into the yeast strain sequentially. However, cotransformation of a yeast strain with both plasmids may be necessary if the bait plasmid affects the growth of your yeast strain. Although most of the yeast strains used for two-hybrid screens transform well by all three protocols, we have found that some (e.g., GGY1 : : 171) are refractory compared to others (e.g., Y190) (12) (see Note 1).

## 2. Materials

### 2.1. Equipment

1. Incubator at 30°C for growth of yeast on agar plates.
2. Shaking incubator at 30°C for growth of yeast in culture tubes and flasks.
3. Centrifuge to harvest yeast cultures at 3000g in 50- or 250-mL bottles.
4. Spectrophotometer or hemocytometer to determine cell titer.
5. Boiling water bath to denature carrier DNA.
6. Ice/water bath to chill denatured carrier DNA.

**Table 1**  
**YPAD Medium**

Component	YPAD agar	2X YPAD broth
Difco Bacto yeast extract	8 g	16 g
Difco Bacto peptone	16 g	32 g
Glucose	16 g	32 g
Adenine hemisulfate	80 mg	80 mg
Difco Bacto agar	12 g	—
Distilled/deionized water	800 mL	800 mL

7. Microcentrifuge.
8. Water bath at 42°C to heat-shock cells in transformation mix.
9. Supply of cartridge-purified or double-distilled water to make medium and solutions.

## 2.2. Yeast Growth Media

### 2.2.1. Yeast Extract–Peptone–Adenine–Dextrose Medium

Yeast extract–peptone–adenine–dextrose (YPAD) medium is used for routine growth of yeast strains. The adenine is added to decrease the selective advantage of *ade2* to *ADE2* reversions. Double strength YPAD, 2X YPAD broth, can be used to regrow cultures to log phase before transformation. This medium reduces the doubling time of yeast strains and increases transformation efficiency (see **Table 1**).

Volumes of 800 mL are easier to handle than 1000 mL and can be made up and autoclaved in 1.0-L Pyrex® medium bottles or other suitable containers. The powdered ingredients for YPAD agar medium are added to the water in a 1.0-L Pyrex medium bottle and stirred with a magnetic stir bar on a stirring hot plate. Continue stirring and boil for 1 min to ensure that the agar is dissolved. The medium is sterilized by autoclaving for 15 min and allowed to equilibrate to 55°C in a water bath before it is poured it into Petri dishes. This volume of medium is sufficient for about 30 plates. YPAD and 2X YPAD broth are dissolved on a stirring hot plate, dispensed in 100- or 200-mL aliquots, and autoclaved. YEPD agar and broth media can be purchased from Becton Dickinson Microbiology Systems (Cockeysville, MD) (BBL YEPD agar and YEPD broth).

### 2.2.2. Synthetic Complete Selection Medium

The plasmids used in two-hybrid systems generally carry one or more of the following selectable markers: *URA3* (uracil requirement), *TRP1* (tryptophan requirement), *HIS3* (histidine requirement), *LEU2* (leucine requirement), and

**Table 2**  
**SC Selection Medium**

Ingredient	SC selection medium
Difco Yeast Nitrogen Base without amino acids	5.4 g
Amino acid mix	1.6 g
Glucose	16.0 g
Difco Bacto agar <sup>a</sup>	12.0 g
Distilled/deionized water	800.0 mL

<sup>a</sup>The agar is omitted to make liquid SC selection medium.

*ADE2* (adenine requirement). Synthetic complete (SC) selection medium (*see Table 2*) is made by adding a mixture of amino acids, purines, pyrimidines, and vitamins to Difco Yeast Nitrogen Base; specific components of the mixture are omitted in order to select for the genetic marker carried by the plasmid (*see Subheading 2.2.3.* below). Thus, SC-Ade lacks adenine and selects for the presence of a plasmid carrying the wild-type gene *ADE2*.

Add the ingredients to the water in a 1.0-L Pyrex medium bottle and stir with a magnetic stir bar on a stirring hot plate at room temperature. Adjust the pH to 5.6 with 1.0 *N* NaOH. Continue stirring and boil for 1 min to ensure that the agar is dissolved. Sterilize the medium by autoclaving for 15 min and allow it to equilibrate to 56°C in a water bath before pouring it into Petri dishes. You will need to use 150-mm Petri plates for the two-hybrid screen transformation (*see Subheading 3.3., step 15*). SC selection medium is light sensitive. The plates should be dried in the dark at room temperature for 1 or 2 d and then stored in sealed bags in the dark at 4°C.

### 2.2.3. Amino Acid Mix

The amino acid mix (**15**) is made by mixing the following ingredients in a plastic container and shaking thoroughly with two or three glass marbles. The compounds omitted in specific SC selection medium are indicated in bold: **0.5 g of adenine** SO<sub>4</sub>, 2.0 g of arginine, 2.0 g of aspartic acid, 2.0 g of glutamic acid, **2.0 g of histidine HCl**, 2.0 g of inositol, 2.0 g of isoleucine, **4.0 g of leucine**, 2.0 g of lysine HCl, 2.0 g of methionine, 2.0 g of phenylalanine, 2.0 g of serine, 2.0 g of threonine, **2.0 g of tryptophan**, 2.0 g of tyrosine, **2.0 g of uracil**, 2.0 g of valine, and 0.2 g of *p*-aminobenzoic acid.

### 2.3. Solutions

1. 1.0 *M* LiAc: Dissolve 5.1 g of LiAc dihydrate (cat. no. L-6883; Sigma St Louis, MO) in 50 mL of water in a 100-mL Pyrex medium bottle; autoclave for 15 min and store at room temperature.

2. PEG MW 3350 (50% [w/v]): Dissolve 50 g of PEG 3350 (cat. no. P-3640; Sigma) in 30 mL of distilled/deionized water in a 150-mL beaker on a stirring hot plate with medium heat. Allow the solution to cool to room temperature and make up the volume to 100 mL in a 100-mL measuring cylinder. Seal the cylinder with Parafilm® and mix thoroughly by inversion. Transfer the solution to a glass bottle and autoclave for 15 min. Store at room temperature. It is essential that the bottle be securely capped; evaporation of the solution will increase the concentration of PEG in the transformation reaction and severely reduce the yield.
3. Single-stranded carrier DNA (2.0 mg/mL): Dissolve 200 mg of salmon sperm DNA (cat. no. D-1626; Sigma) in 100 mL of TE buffer (10 mM Tris-HCl, 1 mM Na<sub>2</sub> EDTA, pH 8.0) with constant stirring at 4°C overnight. Store at -20°C in 16 lots of 5 mL and the remainder in 1.0-mL or 500-μL samples. Before use, the carrier DNA must be denatured in a boiling water bath for 5 min and immediately chilled in an ice and water mixture. It can be boiled three or four times without loss of activity.

### 3. Methods

#### 3.1. General Considerations of the Three Protocols

1. Growth of the yeast culture: Cells for the rapid transformation protocol can be grown overnight on YPAD agar or in YPAD broth. Cultures for the high-efficiency and two-hybrid screen protocols are grown overnight in 2X YPAD or SC selection medium and then regrown for two cell divisions in 2X YPAD.
2. Preparation for transformation: The cells must be washed in sterile water to remove traces of the growth medium.
3. Denaturation of carrier DNA: The carrier DNA must be incubated in a boiling water bath for 5 min and then chilled in ice water.
4. Resuspension of the cell pellet in transformation mix (*see Note 2*): A unit transformation of 10<sup>8</sup> cells is incubated in a total of 360 μL of transformation mix: 240 μL of PEG (50% [w/v]), 36 μL of 1.0 M LiAc, 50 μL of boiled carrier DNA (2.0 mg/mL), and 34 μL of plasmid DNA plus water. The transformation mix for several transformations can be made up in bulk and stored until use in ice water. It is best to make up one more unit of mix than the number of transformations required.
5. Incubation at 42°C: The duration of incubation differs for the three protocols. Cells grown overnight for the rapid transformation protocol can be incubated at 42°C for up to 3 h. Cells regrown for two divisions for the high-efficiency protocol and two-hybrid screen protocol should be incubated at 42°C for 40 and 60 min, respectively.
6. Resuspension of the transformed cells in sterile water and plating onto appropriate SC selection medium.

#### 3.2. Rapid Transformation

##### 3.2.1. Generating Primary Transformants

This protocol is used when large numbers of transformants are not required, e.g., when transforming your two-hybrid bait plasmid (*GAL4<sub>BD</sub>:YFG*) into a

yeast strain in preparation for a two-hybrid screen. Purified plasmid DNA is not essential; you can use plasmid DNA isolated by a miniprep method. The yield of transformants is affected by the genotype of the yeast strain, age of the culture, concentration of plasmid DNA, and duration of the incubation at 42°C. Cultures grown overnight on YPAD agar or in YPAD broth give about  $4 \times 10^4$  transformants/ $\mu\text{g}$  of plasmid/ $10^8$  cells using this protocol. If the cells have been stored at room temperature or in the refrigerator for several days, the yields will be lower. The highest transformation efficiencies are obtained when 100 ng of plasmid DNA is used to transform  $10^8$  cells. Higher plasmid concentrations will give more transformants but at a reduced efficiency. Extending the duration of incubation at 42°C from 40 to 180 min increases the number of transformants to  $>1 \times 10^6/\mu\text{g}$  of plasmid/ $10^8$  cells with some strains.

#### 3.2.1.1. DAY 1

1. Inoculate the yeast strains in 2-cm<sup>2</sup> patches on plates of YPAD agar and incubate overnight at 30°C.

#### 3.2.1.2. DAY 2

1. Boil a sample of carrier DNA for 5 min and chill in an ice-water bath. It is best to do this before starting the transformation in order to avoid having to wait for the water to boil if you forget this step.
2. Transfer approx 10–20  $\mu\text{L}$  of each culture (about  $10^8$  cells) into 1.0 mL of sterile water in sterile 1.5-mL microfuge tubes and wash the cells by vortexing vigorously.
3. Microcentrifuge the suspensions for 15 s at top speed, and remove the water with a micropipet or by aspiration with a micropipet tip attached to a vacuum line.
4. Make up an appropriate volume of transformation mix and pipet 360  $\mu\text{L}$  onto each cell pellet. Vortex vigorously to resuspend the cells. If you are carrying out a single transformation, add the PEG to the cell pellet first, followed by the LiAc, plasmid DNA, and water, and then vortex to resuspend the cells.
5. Incubate the transformation tubes at 42°C for 40 min. A longer incubation, up to 180 min, will increase the yield of transformants with most strains.
6. Microcentrifuge the tubes at top speed for 30 s and remove the transformation mix with a micropipet or by aspiration. The cell pellet adheres strongly to the walls of the tube.
7. Pipet 1.0 mL of sterile water into each tube, and resuspend the cell pellet by stirring with a micropipet tip to loosen the pellet and then by vortexing vigorously (*see Note 3*).
8. Plate two 10- $\mu\text{L}$  and two 100- $\mu\text{L}$  samples onto plates of appropriate SC selection medium (*see Note 4*). The 10- $\mu\text{L}$  samples should be pipetted into 100- $\mu\text{L}$  puddles of sterile water and then spread over the agar surface.
9. Incubate the plates at 30°C for 3 to 4 d and isolate the transformants.

### 3.2.2. Testing and Storing Primary Transformants

You should now have a number of primary transformants containing your two-hybrid bait plasmid ( $GAL4_{BD}:YFG$ ). These transformants should be tested to confirm that they do not autoactivate the reporter gene in your yeast strain. You may also wish to test for expression of the fusion protein (**13,16,17**). These primary transformants must be grown on or in SC selection medium. Make stock cultures by suspending about 50  $\mu$ L of cells from an overnight culture on SC selection agar in 500  $\mu$ L of 20% glycerol and store them at  $-70^{\circ}\text{C}$ . In addition you should compare the growth of these primary transformants with that of the parent strain in SC selection medium with appropriate supplements. If the expression of the fusion construct has a deleterious effect on the growth of the primary transformants, it may be necessary to cotransform the yeast strain with the bait plasmid ( $GAL4_{BD}:YFG$ ) and prey (library) plasmid ( $GAL4_{AD}:cDNA$ ).

## 3.3. High-Efficiency Transformation

### 3.3.1. Transformation Protocol

The high-efficiency transformation protocol is used to determine the quantity of library plasmid ( $GAL4_{AD}:cDNA$ ) that is required to generate the desired number of transformants for a successful two-hybrid screen. We suggest that you test 100 ng and 1, 2, 5, 10, and 20  $\mu$ g. If the bait plasmid ( $GAL4_{BD}:YFG$ ) adversely affects yeast growth, then the amounts of both plasmids will have to be optimized before attempting cotransformation by the two-hybrid screen protocol. The highest transformation efficiencies are obtained when the yeast strain is grown overnight and then regrown in 2X YPAD for at least two cell divisions.

#### 3.3.1.1. DAY 1

1. Inoculate the yeast strain carrying the bait plasmid ( $GAL4_{BD}:YFG$ ) into 25 mL of SC selection medium, and incubate at  $30^{\circ}\text{C}$  on a rotary shaker at 200 rpm overnight. If you are preparing for cotransformation with both plasmids, the yeast strain is grown in 5 mL of 2X YPAD on a rotary shaker. Cultures grown overnight in SC selection medium should reach a titer of about  $2 \times 10^7$  cells/mL. In 2X YPAD the titer should be about  $2 \times 10^8$  cells/mL.
2. Incubate a bottle of 2X YPAD broth and a 250-mL flask overnight at  $30^{\circ}\text{C}$ .

#### 3.3.1.2. DAY 2

1. Determine the titer of the overnight culture. This can be done by measuring the optical density (OD) at 545 or 600 nm of a  $10^{-2}$  dilution of the culture in sterile water. For most yeast strains, a suspension containing  $10^6$  cells/mL has an  $\text{OD}_{545}$



- of 0.1. Alternatively, you can count the number of cells with a hemocytometer. To accurately determine the cell titer, you should determine the relationship between OD or hemocytometer count and colony counts for your yeast strain.
2. Dispense 50 mL of prewarmed 2X YPAD into the prewarmed 250-mL flask and return it to the 30°C incubator.
  3. Calculate the volume of suspension that contains  $2.5 \times 10^8$  cells, and pipet this volume into the 50 mL of 2X YPAD in the flask. The starting titer will be  $5 \times 10^6$  cells/mL.
  4. Incubate the culture at 30°C on a rotary shaker at 200 rpm for 4 to 5 h and determine the cell titer. When the cells have divided at least twice (cell titer  $\geq 2 \times 10^7$ /mL), harvest the cells by centrifugation at 3000g for 5 min in a 50-mL centrifuge tube.
  5. Boil a sample of carrier DNA for 5 min and chill in an ice-water bath.
  6. Wash the cells in 25 mL of sterile water and resuspend them in 1 mL of sterile water.
  7. Transfer the suspension to a 1.5-mL microcentrifuge tube, centrifuge again, and discard the supernatant.
  8. Add water to a final volume of 1 mL and vortex vigorously to resuspend the cells.
  9. Pipet 100-μL samples (about  $10^8$  cells) into individual 1.5-mL microfuge tubes, centrifuge at top speed for 20 s, and remove the supernatant.
  10. Make up sufficient transformation mix lacking plasmid DNA and water for the planned number of transformations plus one extra. For 10 transformations, make enough for 11: 2640 μL of PEG, 396 μL of 1 M LiAc, and 550 μL of boiled carrier DNA. Keep the transformation mix minus DNA in ice water.
  11. Add 326 μL of transformation mix minus DNA to each transformation tube.
  12. Add 100 ng to 20 mg of the *GAL4<sub>AD</sub>:cDNA* plasmid DNA plus water, to a total volume of 34 μL, to each transformation tube and resuspend the cells by vortexing vigorously. If you are testing in preparation for cotransformation, we suggest that you optimize the bait plasmid first and then test the library plasmid DNA with the best bait concentration.
  13. Incubate the transformation tubes at 42°C for 40 min.
  14. Microcentrifuge at top speed for 20 s and remove the transformation mix.
  15. Pipet 1 mL of sterile water into each tube. Loosen the pellet by stirring with a micropipet tip and then vortex vigorously.
  16. Dilute the suspensions  $10^{-2}$  (10 μL into 1 mL), vortex thoroughly, and plate duplicate 10- and 100-μL samples onto plates of appropriate SC selection medium. The 10-μL samples should be pipetted into 100-μL puddles of sterile water.
  17. Incubate the plates at 30°C for 3 to 4 d and count the number of transformants on the plates.

### 3.3.2. Determination of Transformation Efficiency, Transformation Yield, and Scale-up Factor

You can now determine the concentration of plasmid DNA that will give the most efficient use of your *GAL4<sub>AD</sub>:cDNA* plasmid library. The results of a typical experiment are given in **Table 3**. The data clearly demonstrate that



**Table 3**  
**Transformation Efficiency Vs Transformation Yield**

Plasmid DNA	Transformation efficiency	Transformant yield
100 ng	$2.55 \times 10^6$	$0.25 \times 10^6$
1 g	$1.55 \times 10^6$	$1.55 \times 10^6$
5 g	$0.89 \times 10^6$	$1.77 \times 10^6$
10 g	$0.19 \times 10^6$	$1.89 \times 10^6$
20 g	$0.11 \times 10^6$	$2.21 \times 10^6$

increasing the amount of plasmid per transformation increases the yield but reduces the efficiency. Calculate the transformation efficiency (transformants/ $1 \mu\text{g}$  of plasmid/ $10^8$  cells) and transformant yield (total number of transformants/transformation). The transformation reaction containing  $1.0 \mu\text{g}$  of plasmid gave an average of 155 colonies on the two  $10\text{-}\mu\text{L}$  plates. The calculation is as follows:

$$155 \times 100 (10^{-2} \text{ dilution factor}) \times 100 (10\text{-}\mu\text{L plating factor}) \times 1 (\text{plasmid factor})$$

$$\text{Transformation efficiency} = 1.55 \times 10^6 / 1 \mu\text{g plasmid DNA} / 10^8 \text{ cells}$$

Scaling up the volumes of transformation mix 60-fold and using  $1 \mu\text{g}$  of plasmid DNA per unit transformation should result in an overall yield of  $93 \times 10^6$  transformants ( $1.55 \times 10^6 \times 60$ )—sufficient for the most demanding two-hybrid screen.

### 3.4. Two-Hybrid Screen Transformation

#### 3.4.1. Library Screen Transformation Protocol

The high-efficiency transformation protocol can be scaled up 30-, 60-, or 120-fold to ensure that an adequate number of transformants are screened for protein-protein interactions. The two-hybrid transformation protocol is used for both sequential and cotransformation of the bait ( $GAL4_{BD}:YFG$ ) and library ( $GAL4_{AD}:cDNA$ ) plasmids. Scaling up the transformation volumes by 30-, 60-, and 120-fold will require cell numbers, after regrowth, of  $3 \times 10^9$ ,  $6 \times 10^9$ , and  $12 \times 10^9$ , respectively. The protocol indicates the required volumes of medium and culture flasks.

##### 3.4.1.1. DAY 1

1. Inoculate the yeast strain carrying the bait plasmid ( $GAL4_{BD}:YFG$ ) into SC selection medium. If you are cotransforming the bait and library plasmids, grow the yeast strain in 2X YPAD. The volumes of medium and flask sizes are given in **Table 4**.

**Table 4**  
**Volumes of Medium and Flask Sizes**

	30X	60X	120X
SC selection medium	50 mL in 250 mL	100 mL in 500 mL	200 mL in 1 L
2X YPAD	50 mL in 250 mL	50 mL in 250 mL	50 mL in 250 mL

**Table 5**  
**Transformation Scale and Volumes**

	30X	60X	120X
Cells required	$7.5 \times 10^8$	$1.5 \times 10^9$	$3.0 \times 10^9$
Volume of SC culture (approximate)	40 mL	80 mL	160 mL
Volume of 2X YPAD culture (approximate)	5 mL	10 mL	20 mL
2X YPAD for regrowth	150 mL	300 mL	600 mL
Flask size for regrowth <sup>a</sup>	1000 mL	$2 \times 1000$ mL	$3 \times 1000$ mL

<sup>a</sup>A larger number of smaller flasks can be used. The volume of the medium should be one-fifth of the flask volume.

- Incubate at 30°C on a rotary shaker at 200 rpm overnight.
- Warm an appropriate volume of 2X YPAD and culture flasks at 30°C overnight (see **Subheading 3.3.1.2., step 2**).

#### 3.4.1.2. DAY 2

- Determine the titer of the overnight culture and calculate the volume required for regrowth. Cultures grown in SC selection medium should reach a titer of about  $2 \times 10^7$  cells/mL. In 2X YPAD, the titer should be between  $1.5$  and  $2 \times 10^8$  cells/mL. The numbers of cells, volumes of overnight culture, volumes of 2X YPAD, and flask sizes for regrowth from a starting titer of  $5 \times 10^6$  cells/mL are given in **Table 5**.
- Use a sterile pipet or measuring cylinder to measure the appropriate volume of overnight culture and transfer it to an appropriate number of sterile centrifuge tubes.
- Pellet the cells at 3000g for 5 min and discard the supernatant.
- Resuspend the pellet in warm 2X YPAD and dispense into the flasks for regrowth. Make up to the volumes indicated in **step 2**.
- Incubate the flasks at 30°C on a rotary shaker at 200 rpm until the cell titer reaches  $2 \times 10^7$ /mL. This may take 4 to 5 h.
- Boil sufficient carrier DNA (see **step 10**) for 5 min and chill in ice water until required.

**Table 6**  
**Transformation Mix Volumes**

Scale-up	30X	60X	120X
50% PEG	7.20 mL	14.40 mL	28.40 mL
1 M LiAc	1.08 mL	2.16 mL	4.32 mL
Carrier DNA (2 mg/mL)	1.50 mL	3.00 mL	6.00 mL
Plasmid DNA + sterile water	1.02 mL	2.04 mL	4.08 mL
Total volume	10.80 mL	21.60 mL	42.80 mL

7. Harvest the cells by centrifugation at 3000g and discard the supernatant.
8. Wash the cells twice in half the regrowth culture volume of sterile water, and transfer the suspension to a single 50-mL centrifuge tube (30X and 60X scale-up) or divide it between two tubes (120X scale-up).
9. Centrifuge and discard the supernatant.
10. Prepare the transformation mix for the appropriate scale-up (*see Table 6*).
11. Add the transformation mix to the cell pellet and vortex vigorously until the pellet is completely resuspended.
12. Incubate the tubes at 42°C for 45 min. Mix by inversion at 5-min intervals to ensure temperature equilibration.
13. Centrifuge at 3000g for 5 min and remove the supernatant.
14. Add sterile water to the cell pellet (30X = 20 mL, 60X + 40 mL, 120X = 40 mL), and resuspend the cells by pipetting up and down and then vortexing vigorously.
15. Spread 400-μL samples of the cell suspension onto 150-mm plates of appropriate SC selection medium. You will need 50 plates for a 30X scale-up and 100 plates for 60X and 120X.
16. Incubate the plates at 30°C for 3–7 d until colonies have grown.
17. Score the plates for colonies that show an interaction between the proteins specified by the *GAL4<sub>BD</sub>:YFG* and *GAL4<sub>AD</sub>:cDNA* plasmids.

### 3.4.2. Characterization and Confirmation of Two-Hybrid Positives

The putative positives isolated in a two-hybrid screen must now be confirmed and characterized. Details of the procedures for these tests can be found in Chapters 6–10 and in Gietz et al. (13) and Parchaliuk et al. (18,19).

## 4. Notes

1. The transformation efficiencies for commonly used yeast strains using the high-efficiency protocol range from  $5 \times 10^5$  to  $5 \times 10^6$  (12). These differences are probably owing to differences in genetic background. If your strain transforms poorly, we suggest that you vary (1) the duration of incubation at 42°C and (2) the concentrations of plasmid and carrier DNA (11). You should also test for

sensitivity to LiAc. If this proves to be the problem, you can transform either by the spheroplast procedure (20) or by electroporation (12).

2. Some protocols suggest the addition of dimethyl sulfoxide,  $\beta$ -mercaptoethanol, or ethanol to the transformation mix. We have found that these compounds reduce the required exposure to 42°C but do not have much effect on transformation efficiency.
3. Some protocols suggest resuspension and plating in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Transformation yield is affected by the pH of the SC selection medium. The optimum pH is 5.6; higher and lower values reduce efficiency. We have found that the resuspension of transformed cells in TE buffer reduces the recovery of transformants and also affects the growth of the colonies.
4. If you obtain no transformants, it is possible you are using the wrong medium (amino acid mix) for your particular yeast strain/plasmid combination or that your yeast strain requires a nutrient that is absent from the amino acid mix. Similarly, if it appears that all the cells have been transformed, then your yeast strain may not require the component missing from the amino acid mix.

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## Qualitative and Quantitative Assessment of Interactions

Monica M. Montano

### 1. Introduction

Interactions between proteins in the yeast two-hybrid system are scored by testing for expression of the reporter genes. In the protocols presented, the reporter genes are *LacZ* and *HIS3*, but other types are also available. The expression of the reporter genes is under the control of the upstream activating sequence for GAL4. The interacting pair is expressed as fusion proteins with the DNA-binding domain (DBD) or activation domain (AD) of GAL4. In a “hunt” for novel or unknown proteins from a cDNA library that specifically interact with a protein of interest, cDNA library proteins are expressed as fusion proteins with GAL4 AD (referred to as target or prey), and the protein of interest is expressed as a fusion protein with the GAL4 DBD (referred to as bait). Interaction between bait and target fusion proteins results in activation of the *LacZ* and *HIS3* reporter genes resulting in (1) growth of the strain on medium lacking histidine and (2) transcriptional activation of the *LacZ* reporter leading to production of  $\beta$ -galactosidase. The use of two different reporter genes under the control of different promoters eliminates many false positive AD/library fusion proteins, particularly those that do not bind to the bait fusion proteins but instead interact directly with promoter sequences flanking the GAL4-binding sites or with proteins bound to the flanking sequences. Putative true positive clones should be tested further to determine whether they can activate the reporter genes only in the presence of the GAL4 DBD bait protein. Yang et al. (1) determined that interactions with affinity above 70  $\mu M$  can be detected with the yeast two-hybrid system. However, one must use caution in comparing relative affinities of different bait and target pairs based on

$\beta$ -galactosidase activity. Several factors can affect the interaction phenotypes, such as the stability of the fusion proteins, transport of the fusions proteins into the nucleus, and the ability of the bait to bind DNA. These factors limit one's ability to use two-hybrid data to compare affinities between sets of unrelated proteins (*see Note 1*).

## 2. Materials

### 2.1. Screen for Expression of Reporter Genes Containing Auxotrophic Markers

1. Plates containing synthetic defined (SD) minimal medium, and appropriate dropout solution, and appropriate carbon source are required (*see Note 2* and Chapter 3). After autoclaving solutions, allow to cool to about 55°C before adding 3-amino-1,2,4-triazole (3-AT) or 5-Bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal).
2. 3-AT: Make 1 M solution by dissolving 8.4 g of 3-AT (A-8056; Sigma, St. Louis, MO) in 100 mL of water. Sterilize by filtration.

### 2.2. Screen for Expression of LacZ Reporter Gene

1. Z buffer: Dissolve 16.1 g of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 5.5 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.75 g of KCl, and 0.246 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 900 mL  $\text{H}_2\text{O}$ . Adjust the pH to 7.0 and add water to a final volume of 1 L. Autoclave or filter sterilize and store at 4°C.
2. Z buffer with  $\beta$ -mercaptoethanol: Add 0.27 mL of  $\beta$ -mercaptoethanol to 100 mL of Z buffer.
3. X-gal: Dissolve X-gal in *N,N*-dimethylformamide (DMF) at a concentration of 20 mg/mL. Store in the dark at -20°C.
4. Z buffer with X-gal: Add 1.67 mL of X-gal stock solution to 98 mL of Z buffer with  $\beta$ -mercaptoethanol. Prepare fresh each time.
5. Filters: Whatman no. 5 paper filters (9 or 12.5 cm) or VWR 413 filters (7.5 or 12.5 cm). Nitrocellulose filters are not recommended because they crack when frozen.
6. X-gal in agar plates: To prepare 1 L, dissolve components of SD medium in 760 mL of  $\text{H}_2\text{O}$ , add agar (20 g/L), autoclave, and cool to 55°C. Add appropriate dropout solution (100 mL of 10X stock), dextrose (40 mL of 50% stock), 100 mL of 0.7 M potassium phosphate (pH 7.0), and 4 mL of X-gal (20 mg/mL in DMF). Pour plates, allow to solidify, and store at 4°C in the dark for up to 2 mo.
7. 1 M  $\text{Na}_2\text{CO}_3$ : Dissolve 10.6 g of  $\text{Na}_2\text{CO}_3$  in 100 mL of  $\text{H}_2\text{O}$ .
8. *o*-Nitrophenyl- $\beta$ -D-galactopyranoside (ONPG): Dissolve 4 mg of ONPG/mL of Z buffer. Adjust to pH 7.0. ONPG may require 1 to 2 h to dissolve completely.

## 3. Methods

### 3.1. Screen for Expression of Reporter Genes Containing Auxotrophic Markers

The *HIS3* reporter can be scored by plating yeast cell onto plates that lack histidine. Interactions are then identified by growth of colonies on minus histi-

dine SD plates. Colonies that grow the fastest are frequently the strongest interactors. Most *HIS3* reporter plasmids have a significant amount of constitutive, leaky expression. This leaky expression may be controlled by including 3-AT, a chemical inhibitor that suppresses the basal level of *HIS3* expression, in the plates (2). To determine the appropriate concentration of 3-AT to use, plate yeast strain expressing the *HIS3* reporter onto SD plates minus histidine and containing various concentrations of 3-AT (try 3, 6.25, 12.5, 25, and 50 mM to start). Use the lowest concentration of 3-AT that allows only small (<1-mm) colonies to grow after 1 wk.

### 3.2. Screen for Expression of *LacZ* Reporter Gene

The activity of the *LacZ* reporter is measured using X-gal, a chromogenic substrate for the *LacZ* gene product in a filter lift assay (3).  $\beta$ -Galactosidase cleaves the X-gal substrate, producing a product that turns the yeast colony blue. The amount of color allows an estimate of the level of *LacZ* expression in a strain. An appropriate negative control is a yeast strain transformed only with the empty target or GAL4-AD vector (for other recommended controls see **Subheading 3.3.**). The colonies to be assayed for  $\beta$ -galactosidase activity should be growing on SD minimal medium with the appropriate dropout solution and carbon source. The SD medium will keep selective pressure on the hybrid plasmids, and in certain cases the reporter plasmid, before the cells are lysed for the assay.

Using X-gal in a filter lift assay is more sensitive than using X-gal in agar plates. X-gal is the  $\beta$ -galactosidase substrate of choice for use in the filter assay and in agar plates because of its relatively higher degree of sensitivity. Filter lift assays can be used to screen large numbers of cotransformants that survive histidine growth selection. Thus, this type of assay is particularly useful in a selection for novel or unknown library proteins that interact with a protein of interest. It can also be used to test for an interaction between two known proteins in a GAL4 two-hybrid system. The whole-plate assay incorporates the X-gal substrate directly into the plating medium, and the subsequent development of blue colonies is used as the screen. This is used to screen large numbers of cotransformants for the expression of the *LacZ* reporter gene in a LexA two-hybrid library screening. In this case, the reporter gene is maintained on an autonomously replicating plasmid. The whole-plate assay works for the LexA transformants because of the high copy number of the *LacZ* reporter plasmid and the preamplification step that usually precedes the  $\beta$ -galactosidase assay in this system. However, because of its relatively low sensitivity, the whole-plate assay is not suitable for screening transformants in a GAL4-based two-hybrid assay, or in a LexA-based two-hybrid assay when the reporter gene has been integrated into the host genome.



Note that there is no direct correlation between  $\beta$ -galactosidase activity and the  $K_d$  of an interaction (4). In addition, promoter strength can vary among different *LacZ* reporter genes. Thus, quantitative data cannot be compared between host strains having different *LacZ* reporter constructs.

### 3.2.1. Filter Lift Assay

1. Grow transformants until the colonies are 1 to 2 mm in diameter. Use only fresh transformants (2- to 4-d-old) colonies.
2. Prepare Z buffer containing X-gal.
3. Pipet 4.5 mL of the Z buffer with X-gal to a sterile, empty 150-mm Petri dish or add 1.75 mL to a 100-mm Petri dish.
4. Place a sterile filter paper into the dish containing X-gal solution, making sure that the filter paper is completely soaked.
5. Place a separate sterile filter paper on the surface of the plate containing the colonies. Remove any air bubbles and press the filter paper firmly onto the surface of the plate. Mark the orientation.
6. Using forceps lift the filter paper from the plate.
7. To lyse the cells, place the filter paper in liquid nitrogen for 5–10 s.
8. Carefully remove the filter paper from the liquid nitrogen (the filter paper is very fragile at this point and can crack). Place the filter paper colony side up on a new filter paper to thaw.
9. Place the thawed filter paper colony side up onto the filter paper soaked in Z buffer with X-gal being careful to remove any air bubbles between the two pieces of filter paper.
10. Wrap the Petri dish containing the filter paper in plastic, and incubate the plates at room temperature or at 30°C.
11. Monitor the plates for production of the blue color indicating  $\beta$ -galactosidase activity (*see Note 3*).
12. Isolate the colonies with high  $\beta$ -galactosidase activity by aligning the filter paper onto the plate. Colonies should be streaked again on a new plate with selective medium. It is recommended that this assay be repeated to verify  $\beta$ -galactosidase activity.

### 3.2.2. Whole-Plate assays

1. Streak, replicate plate, or spread transformants to be assayed on selection medium containing X-gal (*see Subheading 2.2.*).
2. Incubate the plates at 30°C for 4–6 d (*see Note 4*).
3. Check the plates every 12–16 h (up to 96 h) for the development of blue colonies.

### 3.2.3. Liquid $\beta$ -Galactosidase Activity

Because of their quantitative nature, liquid assays can be used to compare the relative strengths of protein-protein interactions (5,6). Assay three to five separate transformant colonies and perform each assay in triplicate.

1. Grow the colony in 2 mL of selective medium to stationary phase.
2. Prepare a 1 : 10 dilution of the stationary phase culture in yeast extract, peptone, dextrose (YPD) medium.
3. Incubate the culture at 30°C for 3–5 h with shaking (225–250 rpm) until the cells are in mid log phase ( $OD_{600}$  of 1 mL = 0.5–0.8). Record  $OD_{600}$  at the time the cells are harvested.
4. Aliquot 1.5 mL of culture into a microfuge tube and centrifuge at 16,000g for 30 s.
5. Remove the supernatant and resuspend the cell pellet in 300  $\mu$ L of Z buffer.
6. Dilute cells in 1 mL of Z buffer.
7. Lyse the cells by three freeze/thaw cycles. Freeze by placing the tubes in a dry ice/ethanol bath for 1 min and thaw at 37°C for 1 min in a heat block.
8. Set up a blank tube with 1 mL of Z buffer.
9. Add 200  $\mu$ L of ONPG in Z buffer to the cell lysis reaction or the blank tube.
10. Incubate the tubes at 30°C until a yellow color develops (record reaction time).
11. Add 0.5 mL of 1 M  $Na_2CO_3$  to the reaction and blank tubes to stop the reaction.
12. Centrifuge the reaction tubes for 10 min at 16,000g to pellet cell debris.
13. Transfer the supernatant to a clean microcentrifuge tube.
14. Obtain the  $A_{420}$  and  $A_{550}$  of the supernatant. The  $A_{420}$  should be between 0.1 and 1 to be within the linear range of the assay. The  $A_{550}$ , which is a measure of light scattering by cell debris, should be near 0.
15. Calculate units of  $\beta$ -galactosidase activity as follows:

$$\beta\text{-galactosidase units} = \frac{1000 \times [A_{420} - (1.75)A_{550}]}{(t_{\min})(\text{vol})(OD_{600})}$$

in which  $t$  is the elapsed time (minutes) of incubation, and vol is the volume of culture used in the assay (milliliters),

### 3.3. Verification of Interactions

#### 3.3.1. Confirmation of *His3* and *LacZ* Phenotypes

Individual  $His3^+$  and  $LacZ^+$  positive clones are streaked on SD with appropriate dropout solution, plated, and retested for their  $His3^+$  and  $LacZ^+$  phenotypes. This is important because some of the initial transformants will contain more than one AD/library plasmid. Additional methods of verifying interactions and recommended controls are described next.

#### 3.3.2. Yeast Matings

In the yeast-mating method, the GAL4 DBD bait protein and the GAL4 AD target protein are expressed in two different haploid yeast strains of opposite mating type, and the strains are mated to determine whether the two proteins interact (7–9). Mating occurs when haploid yeast strains of opposite mating type come into contact, and it results in fusion of the two haploids to form a

diploid yeast strain. Thus, an interaction can be determined by measuring activation of the two-hybrid reporter gene in the diploid strain.

Yeast matings can also be used to eliminate false positives. For example, the GAL4 AD cDNA library clone can be mated with a yeast strain expressing one of three plasmids: the GAL4 DBD plasmid lacking an insert, the GAL4 DBD bait protein, or GAL4 DBD plasmid that expresses an unrelated fusion protein. True positives are those GAL4 AD cDNA library clones exhibiting reporter gene expression only when the GAL4 AD cDNA is introduced by mating with yeast strain expressing plasmid encoding the GAL4 BD bait protein. Yeast matings can reduce the amount of time and the number of transformations required to demonstrate specificity of the interaction when many clones are being analyzed.

1. Generate the yeast strain containing GAL4 DBD bait plasmid (or GAL4 DBD parent plasmid) by transformation and selection.
2. Transform the mating partner with the GAL4 AD cDNA library clone of interest.
3. Pick one colony of each type to use in the mating.
4. Place both colonies in a 1.5-mL sterile microcentrifuge tube containing 0.5 mL of YPD medium.
5. Vortex and incubate at 30°C with shaking at 250 rpm for 8–15 h.
6. Spread an aliquot of the mating culture on 100-mm plates containing the appropriate SD minimal medium. For cultures incubated for  $\leq 8$  h, spread 100  $\mu$ L; for cultures grown for  $> 8$  h, spread 10  $\mu$ L.
7. Incubate the plates at 30°C for 3–5 d to allow diploid cells to form visible colonies.
8. Assess reporter activity in the diploid strain by plating on His-deficient medium and by  $\beta$ -galactosidase assays.

### 3.3.3. Recommended Controls and Further Tests

One can eliminate false positives by including several controls in the yeast genetic screens. Every potential interacting or target protein should be tested to determine whether it activates the reporter gene in the absence of the bait plasmid (i.e., in yeast strains expressing target plasmid with the empty bait vector). The activation of reporter genes by an interacting pair of interest should also be compared with the reporter activity in a strain that expresses a well-characterized interacting pair. The following are additional tests to verify positive interactions:

1. Switch vectors by moving the library insert from the GAL AD to GAL DBD vectors and vice versa. Then repeat the two-hybrid assay.
2. Test the library and bait inserts using a different two-hybrid system.
3. Create a mutant form of the interacting protein and repeat the two-hybrid assay.
4. Interactions observed in yeast genetic screenings should be verified in vitro

using glutathione-*S*-transferase pull-down assays (Chapter 9) or coimmunoprecipitation assays (Chapter 10).

#### 4. Notes

1. Note that strong interactions do not necessarily translate into biologic relevance. There are many weak interactions that are biologically relevant and strong interactions that are not. Sequences of the interacting proteins may yield helpful information. We are less confident of interactions with proteins that are ubiquitously expressed in cells. We tend to pursue interactions between our bait protein and a set of highly related proteins, whereas we are more cautious about interactions between our bait protein and a set of unrelated proteins. False positive classification is discussed in Chapter 8, and a list of common false positives in a collection of two-hybrid screens can be obtained from <http://www.fccc.edu/research/labs/golemis/intro.html>.
2. Plates should be prepared in advance to allow them to dry at room temperature. Excess moisture can lead to uneven spreading of cells and contamination. We routinely air-dry uncovered plates in a sterile hood for approx 1 h or covered plates on a benchtop for 2 to 3 d. Plates are then stored covered, in the dark, at 4°C for several months.
3. When using filter lift assays to test for  $\beta$ -galactosidase activity, blue color should appear within 3 to 4 h. Longer incubations (>24 h) may lead to false positives. After a colony turns blue, one needs to be cautious of nearby colonies that turn blue because this may be attributed to “smearing.” This is especially true if an excessive volume of Z buffer with X-gal is used.
4. Cells grown on medium containing X-gal tend to be smaller than colonies grown in the absence of X-gal.

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## Strategies for Rescuing Plasmid DNA from Yeast Two-Hybrid Colonies

Alyson Byrd and René St-Arnaud

### 1. Introduction

Once a yeast colony has been identified as containing a target plasmid insert of interest (as determined by growth on His<sup>-</sup> medium and a positive [blue]  $\beta$ -galactosidase assay; *see* Chapter 6), it becomes necessary to isolate the correct insert-containing plasmid. Isolating the plasmid DNA from yeast is not a trivial task, for several reasons. First, there is always contamination of the plasmid DNA by yeast genomic DNA since the isolation method breaks the yeast chromosomes. Second, most plasmids used tend to be large (>6 kb) and have a low copy number (~50/cell), frequently resulting in low plasmid yields. And, finally, unlike bacteria, yeast are capable of replicating more than one plasmid at a time, making it difficult to identify the one containing the relevant insert. Thus, multiple steps are necessary to isolate the single insert-containing plasmid responsible for the interaction and activation and then to prepare it for analysis.

Several strategies can be used to isolate and analyze positive inserts. Prior to isolating the yeast plasmid DNA, it is possible and desirable to generate segregants that contain only the activation domain/target protein plasmid (i.e., that have been cured of bait plasmid) in order to minimize the number of clones to analyze. This can be achieved simply by removing the Trp selection on the yeast culture for several days, isolating the yeast plasmid DNA from this, and then transforming it into *Escherichia coli* to obtain transformants containing individual activation domain (AD) plasmids ready for analysis. Alternatively, nutritional selection of *E. coli* transformants containing the relevant plasmid of interest is another method to rescue one type of plasmid (e.g., the AD plasmid) from a pool of plasmids (e.g. DNA-binding domain [DBD] plasmids) carrying

different nutritional transformation markers. Finally, polymerase chain reaction (PCR) can be used to amplify the insert in an AD plasmid directly from yeast plasmid DNA isolated from a positive culture using specific primers flanking the multiple cloning site. Regardless of the procedure used to identify the desired plasmid insert, a quick and easy yeast plasmid isolation procedure is performed that yields DNA of sufficient quality and quantity to use as a PCR template or to transform *E. coli*. The fact that most yeast plasmid vectors have been designed to replicate in both yeast and *E. coli* renders the latter possible.

This chapter discusses various options for isolating plasmid DNA from yeast colonies, eliminating the undesired bait plasmid to decrease the number of clones screened, identifying the correct insert responsible for the observed interaction, and analyzing the product obtained.

## 2. Materials

### 2.1. Manipulation in Yeast: Conventional Method

1. Synthetic defined (SD) medium and plates lacking leucine ( $L^-$ ) and lacking leucine + tryptophan ( $LT^-$ ): 6.7 g of Difco yeast nitrogen base without amino acids, 182.2 g of D-sorbitol (*see Note 1*), and  $H_2O$  to 850 mL (for plates add 20 g of agar). Adjust the pH to 5.8, autoclave, and cool to  $<65^\circ C$ . Then add 100 mL of appropriate 10X drop-out solution ( $L^-$  or  $LT^-$ , respectively) and 50 mL of 40% D-glucose.
2.  $\beta$ -Galactosidase assay:
  - a. X-Gal stock solution: Dissolve X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in *N,N*-dimethylformamide (DMF) at a concentration of 20 mg/mL; store at  $-2^\circ C$ .
  - b. Z buffer stock solution (per liter): 16.1 g  $Na_2HPO_4 \cdot 7H_2O$ , 5.5 g  $NaH_2PO_4 \cdot H_2O$ , 0.75 g KCl, 0.246 g  $MgSO_4 \cdot 7H_2O$ , add  $dH_2O$  to one liter, adjust pH to 7.0, autoclave or filter sterilize; store at  $4^\circ C$ .
  - c. Z buffer with X-Gal (100 mL): 98 mL Z buffer, 0.27 mL of  $\beta$ -mercaptoethanol, 1.67 mL of X-Gal stock solution.
  - d. Sterile qualitative filter paper.
  - e. Liquid nitrogen.
3. Liquid broth (LB)/Amp plates: 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract, 20 g of agar, and  $H_2O$  to 1 L. Adjust the pH to 7.0 and autoclave. Cool to  $\sim 55^\circ C$  and add ampicillin to a final concentration of 100  $\mu g/mL$ .

### 2.2. Manipulation in Yeast: Shortcut Method

The materials used are the same as those in **Subheading 2.1**.

### 2.3. Manipulation in *leuB E. coli* Using Nutritional Selection

1. SD( $L^-$ ) medium: *See Subheading 2.1., item 1*.
2. Isolation of yeast plasmid DNA: The materials used are the same as those in **Subheading 2.5**.

3. Electroporation of *E. coli*: The materials used are the same as those in **Subheading 2.6**.
4. LeuB *E. coli* cells: HB101 or KC8 (**1**) (see **Note 2**.)
5. M9 minimal medium/plates: 790 mL of sterile deionized H<sub>2</sub>O (cooled to ~50°C), 200 mL of sterile 5X M9 salts (64 g/L of Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 15 g/L of KH<sub>2</sub>PO<sub>4</sub>, 2.5 g/L of NaCl, 5.0 g/L of NH<sub>4</sub>Cl; autoclave), 10 mL of 40% glucose. For culture plates, use 20 g of agar/L of medium. After autoclaving, allow the medium to cool to ~55°C, and then to 900 mL of M9 medium, add 1 mL of 100 mg/mL ampicillin stock, 1 mL of 1 M thiamine-HCl stock, and 100 mL of the appropriate 10X dropout stock solution used in yeast SD medium. For only HB101 cells, add 4 mL of a 10 mg/mL proline stock.

#### 2.4. Alternative Method Using PCR

1. SD(L<sup>-</sup>) medium: See **Subheading 2.1., item 1**.
2. Isolation of yeast plasmid DNA: The materials used are the same as those in **Subheading 2.5**.
3. 10X PCR buffer: 10 mM dNTP.s, 5' and 3' primers (50 pmol/μL; see **Note 3**), dH<sub>2</sub>O, *Pfu* DNA polymerase enzyme (or *Taq* polymerase enzyme).
4. Agarose gel (0.8 and 1.5%).
5. Gel extraction kit (we use the QIAquick Gel Extraction Kit [Qiagen, Mississauga, Ontario]).

#### 2.5. Isolation of Plasmid DNA from Yeast

1. SD(L<sup>-</sup>) medium: See **Subheading 2.1., item 1**.
2. Acid-washed glass beads.
3. Yeast lysis solution: 2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, in dH<sub>2</sub>O. Store at room temperature.
4. Chloropane (phenol-chloroform-isoamyl alcohol, 25:24:1 [v/v/v]): 50 mL of phenol (neutralized with Tris-HCl), 48 mL of chloroform, 2 mL of isoamyl alcohol. Store at 4°C.
5. 3 M sodium acetate, pH 5.2.
6. Absolute ethanol or isopropanol, 70% ethanol.
7. TE buffer, pH 7.5: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

#### 2.6. Transformation of Yeast Plasmid DNA into *E. coli*

1. Electrocompetent *E. coli* (we have used both house-made electrocompetent XL-1s and *Epicurian coli* SURE<sup>®</sup> electroporation competent cells [Stratagene, La Jolla, CA]).
2. Electroporation cuvettes (0.1-cm gap).
3. Electroporation apparatus.
4. SOC medium (this should be prepared just before use): 99 mL of SOB medium (20.0 g of tryptone, 5.0 g of yeast extract, 0.5 g of NaCl, H<sub>2</sub>O to 980 mL; auto-



clave and add 10 mL of 1 M sterile MgCl<sub>2</sub> and 10 mL of sterile 1 M MgSO<sub>4</sub> prior to use), 1 mL of 40% (w/v) sterile glucose.

5. LB plates: *See Subheading 2.1., item 3.*

## 2.7. Analyzing Potential Target Inserts

1. LB medium: *See Subheading 2.1., item 3.*
2. Miniprep plasmid DNA isolation: Materials are as specified in the protocol used.
3. Diagnostic restriction enzymes: These vary depending on the site used to clone the library inserts into the AD vector (we use *EcoRI* and *XhoI*).
4. Agarose gel (1.2–1.8%).
5. DNA sequencing: We use the ThermoSequenase radiolabeled terminator cycle sequencing kit from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec). *See Note 3* for primer description.
6. Sequence analysis: We subject the DNA sequences obtained to a National Center for Biotechnology Information BLAST search to compare with known sequences.
7. Retransformation of yeast with potential candidates:
  - a. 2<sup>-</sup> SD Agar plates (*see* recipe in **Subheading 2.1.**).
  - b. 3<sup>-</sup> SD Agar plates: prepare as 2<sup>-</sup> plates, except use 3<sup>-</sup> 10X dropout solution (without L-histidine Hcl monohydrate, L-tryptophan, and L-leucine).

## 3. Methods

### 3.1. Manipulation in Yeast: Conventional Method

For many situations, it is desirable to obtain a segregant that contains only the target AD plasmid, not the bait DBD plasmid. This can be achieved through manipulation either in yeast or *E. coli*. The conventional method described subsequently theoretically decreases the number of unwanted plasmids analyzed later on. In practice, however, since loss of the unselected plasmid does not occur in 100% of the transformants, both DBD and AD plasmids are often still represented in the *E. coli* transformants obtained in a later step, requiring the screening of many more transformant colonies for the presence of the desired plasmid. We rarely felt the time and effort spent in performing the extra steps of the conventional method were worthwhile and, thus, developed the shortcut method. Furthermore, when using target and bait plasmids that contain different drug selection markers (as is the case for Stratagene's [2] new kit, which has the Cam<sup>r</sup> gene on the DBD plasmid and the Amp<sup>r</sup> gene on the AD plasmid), the conventional method becomes unnecessary because selection for the AD plasmid no longer occurs during yeast culture, but, rather, only after transformation into *E. coli*.

Because yeast are able to replicate more than one plasmid at once, multiple AD/cDNA fusion plasmids will often be present in each  $\beta$ -galactosidase-positive colony. It may therefore be helpful, before beginning, to restreak each

positive colony onto a new SD(LT<sup>-</sup>) selection plate to allow segregation of the cDNA fusion plasmids to occur (**I**). These individual colonies should then be reassayed and blue ones kept for further analysis. This step is optional and one that we did not find necessary to perform because we preferred to separate the multiple AD plasmids during the miniprep analysis step (*see Subheading 3.7.*).

1. For each  $\beta$ -galactosidase-positive transformant, inoculate 2 to 3 mL of SD(L<sup>-</sup>) medium (lacking Leu but containing Trp) with a well-isolated colony and grow at 30°C with shaking at 250–300 rpm for 2 to 3 d. This culture allows all AD plasmids (carrying the *Leu2* gene) present in the yeast colony to be maintained, while allowing the bait DBD plasmid (carrying the *Trp1* gene) to be randomly lost from some of the transformants at a rate of 10–20% per generation.
2. Plate 25–100  $\mu$ L of a 1/1000 dilution of this culture onto SD(L<sup>-</sup>) plates and grow at 30°C for 2 to 3 d until individual colonies form.
3. Using sterile toothpicks, pick 20–30 colonies from the SD(L<sup>-</sup>) plate in a grid pattern onto both an SD(LT<sup>-</sup>) and SD(L<sup>-</sup>) plate. Incubate these another 2 to 3 d at 30°C, until growth is observed.
4. Colonies that grow on SD(L<sup>-</sup>) but not on SD(LT<sup>-</sup>) medium are cured of the bait DBD plasmid carrying the *Trp1* gene but still contain the AD plasmid(s) carrying the *Leu2* gene. These colonies are Trp auxotrophs. Retest the Trp auxotrophs for  $\beta$ -galactosidase activity. Colonies that turn blue should be discarded since this indicates that they do not require an interaction with the bait and, therefore, probably contain an AD/protein Y hybrid encoding a transcriptional activator that recognizes the Gal1 promoter by itself. Colonies that are negative (white) should be saved for further verification, since they potentially contain a plasmid encoding an interacting protein of interest.
5. Grow these negative colonies in 2 mL of SD(L<sup>-</sup>) medium at 30°C until saturated (2 to 3 d). Then isolate the yeast plasmid DNA as in **Subheading 3.5.**, electroporate into *E. coli*, and analyze the insert DNA as in **Subheadings 3.6.** and **3.7** (*see Note 4*).

### 3.2. Manipulation in Yeast: Shortcut Method

This is by far the most economical strategy for analyzing yeast plasmid DNA, particularly if the two plasmids being used carry different drug selection markers. If this is the case, then the shortcut essentially leaves the generation of Leu<sup>+</sup>Trp<sup>-</sup> segregants to drug selection when the yeast plasmid DNA is transformed into *E. coli* (*see Subheading 3.6.*). This saves a good week of work at this stage.

1. If the bait and target plasmid contain the same drug selection marker, inoculate 3 mL of SD(L<sup>-</sup>) medium with a well-isolated colony for each  $\beta$ -galactosidase-positive colony. Grow at 30°C for 2 to 3 d, shaking at 250–300 rpm. This extra culture time allows for random loss of bait from some transformants. If bait and

target plasmids have different drug selection markers, use 2 mL of SD(L<sup>-</sup>) medium and grow only overnight, since there is no need to try to eliminate the bait plasmid at this step.

2. Isolate the yeast plasmid DNA as in **Subheading 3.5.** and transform into *E. coli* as in **Subheading 3.6.**, plating on LB/Amp plates. If the bait and target plasmids have different drug selection markers, all colonies that grow on the LB/Amp plates will contain an AD plasmid (which carries the Amp<sup>r</sup> gene). Of course, not all of these plasmids will have the insert encoding the interacting partner, because a single yeast colony can replicate more than one plasmid. If both plasmids have the same drug resistance gene, some of the resulting *E. coli* transformants may contain the bait plasmid. These can be identified and eliminated in the insert analysis step (see **Subheading 3.7.**).

### 3.3. Manipulation in *leuB* *E. coli* Using Nutritional Selection

An alternate method to isolate the plasmid containing the target insert of interest is through nutritional selection in *E. coli*. This eliminates the need for multiday selection cultures of the yeast colony because selection occurs after the yeast plasmid DNA has been isolated and transformed into the bacteria. As with the conventional method, this procedure is not necessary when the DBD and AD plasmids carry different drug-resistance markers.

An *E. coli* strain carrying a *leuB* mutation (e.g., HB101) should be used for this procedure (see **Note 2**). The *leuB* mutation prevents the bacteria from growing on medium lacking leucine. The yeast *LEU2*, *TRP1*, and *HIS3* genes are expressed well enough in *E. coli* to allow complementation of the *E. coli* *leuB*, *trpC*, or *hisB* mutation present in certain bacterial strains. Thus, bacteria with a *leuB* mutation that take up a yeast AD plasmid (carrying the *LEU2* gene) can be selected for by plating transformed *E. coli* on minimal medium lacking leucine. Bacteria that take up the DBD plasmid (containing the *TRP3* gene, but not the *LEU2* gene) will not survive the selection. As with the shortcut method, the resulting bacterial transformants will all necessarily carry an AD plasmid and never the DBD plasmid. However, the inserts in the AD plasmids must be tested to determine which one encodes the interacting partner of interest.

1. Pick individual  $\beta$ -galactosidase-positive colonies into 5 mL of SD(L<sup>-</sup>) medium each and grow at 30°C with shaking at 250–300 rpm overnight.
2. Isolate the yeast plasmid DNA from each of these liquid cultures (see **Subheading 3.5.**), resuspending the DNA in 10–20  $\mu$ L of TE buffer.
3. Transform 1 to 2  $\mu$ L of the plasmid DNA into electrocompetent *leuB* *E. coli* cells (see **Subheading 3.6.**) with the following changes after the transformation step:
  - a. Spin the cells at 2500 rpm for 5 min.
  - b. Wash the cells twice with M9 minimal medium.
  - c. Resuspend the cells in 100 mL M9 minimal media

- d) Plate 25 and 75 mL of the cell suspension on M9 agar plates containing 50 mg/mL of ampicillin (or appropriate antibiotic), 40 mg/mL of proline, and 1 mM thiamine-HCl. For best recovery of transformants, also add 1X L<sup>-</sup> drop-out solution (same as that used for the yeast minimal medium) to the bacterial minimal medium. Grow for 36–48 h at 37°C.
4. Analyze the resulting Leu<sup>+</sup>Amp<sup>r</sup> *leuB* transformants as in **Subheading 3.7**.

### 3.4. Alternative Method Using PCR

Occasionally we have encountered, for unknown reasons, a problem in eliminating the bait plasmid from the yeast colony while performing selection in yeast using both the shortcut and conventional methods. This resulted in the frustrating screening of infinite numbers of bacterial minipreps, all of which ended up containing the bait, not the target plasmid of interest. To overcome this problem, we used a PCR-based strategy to isolate the target insert. Another situation in which this PCR-based approach is beneficial is when the yeast two-hybrid screen yields very high numbers of His<sup>+</sup>LacZ<sup>+</sup> candidate clones, rendering transformation of each of the yeast plasmid DNAs into *E. coli* extremely cumbersome. In this situation, analysis of the yeast plasmid inserts by PCR can help sort the colonies and eliminate duplicates, thereby decreasing the workload (*see Note 5*). More detail can be found in **ref. 1**.

1. Inoculate 3–5 mL of SD(L<sup>-</sup>) medium with a single, well-isolated yeast colony for each individual His<sup>+</sup>LacZ<sup>+</sup> clone. Grow at 30°C until saturated (~3 d) with shaking at 250–300 rpm.
2. Isolate the yeast plasmid DNA of each culture (*see Subheading 3.5*).
3. Use 1 to 2 µL of each yeast plasmid DNA preparation to perform the PCR amplification as follows (*see Note 3*):
  - a. Mix well, avoiding air bubbles, the following ingredients: 1 µL of yeast plasmid DNA, 5 µL of 10X PCR buffer, 1 µL of 10 mM dNTPs, 1 µL of 5' primer (50 pmol/µL), 1 µL of 3' primer (50 pmol/µL), 40 µL of dH<sub>2</sub>O, and 1 µL of *Pfu* DNA polymerase (or *Taq* polymerase) for a total of 50 µL. Add 15–30 µL of light mineral oil.
  - b. Use PCR cycling parameters suitable to the primers, templates, tubes, and cycler (*see Ref. 1* for more details). We used the following: 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 68°C for 1 min, 72°C for 2 min; and 1 cycle of 72°C for 10 min.
4. This step is optional. To eliminate duplicate clones, digest 10 µL of PCR product with a frequent-cutter restriction enzyme (e.g., *AluI* or *HaeIII*) and run on a >1.5% agarose gel. Analyze the resultant restriction pattern and keep one representative clone from each group.
5. Run each entire uncut product on a 0.8% gel, cut out the bands, and isolate the DNA using a gel extraction kit. The presence of more than one band when the product is uncut indicates that there is more than one plasmid in the yeast colony

isolated. Each band should be isolated and analyzed further to determine which is the interacting partner of interest.

6. Sequence and analyze each product as in **Subheading 3.7**.

### 3.5. Isolation of Plasmid DNA from Yeast

As discussed in **Subheading 1.**, it is useful to be able to isolate plasmid DNA from yeast. Although the plasmid DNA obtained is heavily contaminated with genomic DNA and, thus, is not pure enough for such procedures as sequencing or restriction enzyme digestion, it is of sufficient quality and quantity to transform *E. coli* (see **Subheading 3.6.**) or to use as a PCR template for amplifying the insert (see **Subheading 3.4.**).

Several protocols are available, varying mainly in the method used to break the cells and in the time required. The procedure we use and describe here is an ~30-min protocol based on the method of Hoffman and Winston (**3**). An alternative procedure, adapted from Ling et al. (**4**), is described by Clontech in **ref. 1**. However, this method requires closer to 2 h to perform and we have not tried it.

1. Inoculate 3 mL of SD(L<sup>-</sup>) medium with a single, well-isolated His<sup>+</sup>LacZ<sup>+</sup> colony. To ensure good growth, vortex to disperse the cells. Incubate with vigorous shaking (250–300 rpm) at 30°C until saturated (2 to 3 d).
2. Fill a 1.5-mL microcentrifuge tube with the yeast culture and spin at 10,000g for 10 s. Decant the supernatant. Repeat until the 3-mL culture is pelleted.
3. Add ~0.3 g (or reasonable estimate) of acid-washed glass beads, 200 µL of yeast lysis solution, and 200 µL of chloroform.
4. Vortex at least 2 min, ensuring that the cell pellet has completely resuspended to a single cell mixture.
5. Spin at 16,000g (14,000 rpm) for 5 min at room temperature. Transfer the top aqueous layer (containing the DNA) to a new microcentrifuge.
6. This step is optional. Add another 200 µL of chloroform to this aqueous phase, vortex briefly, and spin again as in **step 5**. Transfer the top aqueous, DNA-containing phase to a new microcentrifuge tube. We find that this extra chloroform extraction helps clean up the DNA and thus improves the transformation efficiency into bacteria.
7. Precipitate the DNA by adding 1/10 vol of 3 M NaOAc (pH 5.2) and 2.5 vol of ethanol (or 0.7 vol of isopropanol). Vortex and then spin at 10,000g for 10–30 min at 4°C. Decant the supernatant.
8. Wash the DNA pellet with 750 µL of 70% ethanol and respin at 10,000g for 5–10 min. Decant the supernatant. Extra washes can also be performed to help improve transformation efficiency into bacteria.
9. Dry the pellet for 5 min in a vacuum centrifuge.
10. Resuspend the DNA in 10–20 µL TE buffer or H<sub>2</sub>O.

### 3.6. Transformation of Yeast Plasmid DNA into *E. coli*

In general, electroporation is the method of choice for transforming *E. coli* with yeast plasmid DNA. This is mainly because the contaminating genomic DNA that is always present in the plasmid preparation isolated from yeast greatly decreases the transformation efficiency obtained. It is possible to perform chemical transformation, but it is imperative that the chemically competent cells be able to yield a high transformation efficiency. A protocol for this is detailed in **ref. 1**, but we have never tried it. We successfully perform electroporation using the following procedure adapted from Seidman et al. (5). A procedure for direct electroporetic transfer of plasmid DNA from a yeast colony into *E. coli* is also contained in **ref. 5**. The protocol given next involves the transfer of plasmid DNA from a “shuttle vector” (which can grow in both yeast and bacteria) directly from the yeast colony into the *E. coli* without the need to first isolate the yeast plasmid DNA.

1. Thaw a 50- $\mu$ L aliquot of electrocompetent bacteria (we use house-made electrocompetent XL-1s or Stratagene’s SURE electroporation-competent cells) (see **ref. 5** for preparation of electrocompetent *E. coli*) for each clone to be transformed (in 1.5-mL microcentrifuge tubes).
2. On ice, add 1 to 2  $\mu$ L of the yeast plasmid DNA prepared in **Subheading 3.5**. Mix gently by stirring the contents of the tube with the pipettor.
3. Working quickly, transfer the cell/DNA mixture (avoid forming air bubbles) into a cuvet (0.1-cm gap) that has been on ice at least 5 min. Tap the cuvet gently to settle the cells to the bottom and wipe dry with a Kimwipe.
4. Place the cuvet in a sample holder and provide pulse as per the instructions on the pulser (we set the pulser at 1.8 kV).
5. Still working quickly, remove the cuvet and immediately add 500  $\mu$ L of freshly prepared room temperature SOC medium, pipet up and down, and transfer back into the original microcentrifuge tube. Wash the cuvet with another 500  $\mu$ L of SOC medium and add to the microcentrifuge tube.
6. Incubate the tubes at 37°C with shaking at 225–250 rpm for 1 h.
7. Spin the tubes at 10,000g at room temperature for 10 s, decant the supernatant, and resuspend the pellet in the remaining liquid.
8. Plate all on a 100-mm LB plate containing the appropriate antibiotic (e.g., ampicillin) and grow overnight at 37°C.

### 3.7. Analyzing Potential Target Inserts

To identify the plasmid encoding the interacting partner, plasmid DNA must be isolated from the *E. coli* transformants. If the yeast colonies were segregated by nutritional selection in *E. coli* (see **Subheading 3.3**), or if the bait DBD plasmid and target AD plasmids used in the screen had different drug selection markers, all *E. coli* transformant colonies obtained (in **Subheading**

3.6.) should contain an AD plasmid. However, given that yeast colonies can replicate multiple plasmids at once but bacteria cannot, it is possible, indeed probable, that not all the transformants will contain the same AD plasmid encoding the true, desired insert responsible for the observed interaction. Thus, it becomes necessary to analyze each AD plasmid present to identify the true one.

In the situation in which segregation is carried out in yeast (by either the conventional or shortcut method) and both plasmids used carry identical drug selection markers, it is probable that some of the *E. coli* transformants would contain the undesired bait DBD plasmid, while the rest would contain AD plasmids. This is owing to the fact that random loss of the bait plasmid does not occur in 100% of the yeast transformants during selection in SD(L<sup>-</sup>) medium. In this case, more bacterial transformants may need to be analyzed to identify the clone of interest.

In either case, miniprep DNA is isolated from *E. coli* transformant clones and analyzed by restriction enzyme digest and sequencing. The resulting sequences of representative clones are then compared to known sequences in a database. Potential candidate interacting target inserts are then retransformed with the bait and tested again to confirm the interaction. Retransformations in various other combinations are performed to eliminate false positives. The elimination of false positives is discussed in detail in Chapter 8.

1. Pick 12 *E. coli* transformants each into 3 mL of LB medium containing appropriate antibiotics. Grow overnight at 37°C with shaking at 300 rpm (if there is a possibility of bait DBD plasmids being present, pick 24 colonies at this stage to increase the chances of isolating the correct AD plasmid).
2. Isolate the miniprep DNA from these cultures using a standard miniprep procedure, such as that found in Sambrook et al. (6) or Qiagen's plasmid isolation kit. We have found that even when used for sequencing, there is no need for phenol-chloroform extraction or for column purification to obtain good results; ethanol extraction is sufficient (see **Note 6**).
3. Cut 1 to 2 µL of miniprep plasmid DNA with diagnostic restriction enzymes (these will vary depending on the sites used to clone the library inserts into the AD vector). Run on a 1.2–1.8% agarose gel.
4. Eliminate clones whose restriction pattern indicates them to be the bait (if necessary). Select representatives of each different pattern obtained to sequence.
5. Sequence these plasmids using primers flanking the multiple cloning site as in **Subheading 3.4.** (see **Note 3** for primer description).
6. Subject these sequences to a National Center for Biotechnology Information BLAST search to compare with known proteins and to eliminate false positives.



7. Retransform the remaining potential candidates back into yeast with and without the bait and reassay for  $\beta$ -galactosidase activity to ensure that the observed interaction is indeed a true one.
8. Eliminate false positives and confirm interactions as described in **step 3**.

### **3.8. Potential Solutions to Common Problems in Plasmid Rescue**

#### **3.8.1. Inability to Rid Yeast Colony of Bait Plasmid**

When using the conventional or shortcut methods to analyze LacZ<sup>+</sup>His<sup>+</sup> colonies, on occasion the bait plasmid is never lost, resulting in all *E. coli* transformants obtained in **Subheading 3.6** containing the bait. The reasons for this remain unclear, but it is best overcome by using the PCR method described in **Subheading 3.4**.

#### **3.8.2. Amp<sup>r</sup> Colonies Are Not Observed When *E. coli* Is Transformed with DNA Isolated from Yeast**

1. If nutritional selection on M9 minimal medium, using HB101 or KC8 *E. coli* strains, was used (*see Subheading 3.3*), repeat the transformation plating the cells on LB/Amp plates instead of on M9 minimal medium plates. Generally, recovery of new transformants is better on LB/Amp plates than on M9 minimal medium. After colonies have formed on LB/Amp plates, replica plate them onto the appropriate M9 plates in order to select for the desired plasmid and to ensure that any undesired plasmid (i.e., containing the bait) has been lost. In addition, leave the M9 plates to grow longer (36–48 h) because growth of colonies is slower on this medium than on LB.
2. If chemical transformation was performed in **Subheading 3.6**, switch to electroporation, because the transformation efficiency obtained is usually higher. In addition, ensure that the competent cells being used are known to be transformed with very high efficiency.
3. Verify that the yeast plasmid preparation in fact has plasmid DNA in it. Ensure that the working stock plate of the yeast colony used as the inoculum source and the medium used for overnight culture maintain selection on the desired plasmid. Furthermore, during plasmid isolation, ensure that the yeast cell pellet is completely resuspended during the 2-min vortexing step in order to obtain complete cell lysis. It is possible to check the concentration of the total DNA in the plasmid preparation using absorbance at 260 nm or by running 10  $\mu$ L on a 1% agarose gel. This can confirm that there is DNA in the preparation but cannot quantify the plasmid DNA because it makes up only a small fraction of the total DNA.
4. There is a small possibility that the plasmid of interest integrated into the yeast chromosome. Undoubtedly, this is unlikely because yeast plasmids do not efficiently integrate into the yeast genome if they carry a yeast origin of replication and are used uncut. However, if this did occur, the plasmid would not be able to



replicate autonomously when transformed into *E. coli*. In such a situation, it is best to try to isolate the plasmid following the alternate method using PCR described in **Subheading 3.4**.

5. Another possible reason for not obtaining *E. coli* transformants is if the plasmid encodes a protein that is toxic to the bacteria. Once again, it would be best to attempt to isolate the plasmid by amplifying the insert using PCR (*see Subheading 3.4*.) and subcloning it.

#### 4. Notes

1. D-Sorbitol is recommended for SD medium by Stratagene (2) protocols but is not used by Clontech (1). We have used SD medium both with and without D-sorbitol and have not noticed a significant difference with respect to colony growth or background.
2. The KC8 *E. coli* strain could also be used for the transformation. It carries the *trpC*, *leuB*, and *hisB* mutations and can therefore be used to select for transformants carrying only the bait plasmid, only the library (AD) plasmid, or both, depending on the nutrients specifically omitted for the selection.
3. Clontech provides primers that are specifically designed for amplifying inserts in AD plasmid vectors. However, it is possible to construct primers to use for this procedure. In doing this, it is essential that the primers flank the multiple cloning site. More details concerning primer design can be found in **ref. 1**. The primers we used are as follows:
  - a. 5' primer: 5'>GAT ACC CCA CCA AAC CCA AAA<3' (pAD-Gal4 5' position = 803).
  - b. 3' primer: 5'>TAA TAC GAC TCA CTA TAG GGC T<3' (T7 primer from Stratagene).
4. An alternative method for the curing of the bait plasmid by manipulation in yeast, developed by Harper et al. (7), involves cycloheximide counterselection. Some yeast host strains (e.g., CG-1945 and Y190) (7,8) are cycloheximide resistant (because they carry the *CYH<sup>R</sup>2* mutant allele) (7). In addition, the DBD vector pAS2 for the bait construct carries the wild-type *CYH<sup>S</sup>2* gene that is dominant to its mutant counterpart and confers cycloheximide sensitivity (7). Using this bait plasmid and one of these yeast strains for the original transformation, it is then possible to select for segregants that have lost the pAS2 bait by plating Leu<sup>+</sup>Trp<sup>+</sup>His<sup>+</sup>LacZ<sup>+</sup> colonies on SD(L<sup>-</sup>) plates containing cycloheximide at 1 µg/mL (6). The resulting colonies (which are now necessarily Trp<sup>-</sup>Leu<sup>+</sup>) are then retested for LacZ expression, and any that are negative (white) are kept for further analysis. Positive (blue) colonies are discarded because they probably contain a target insert that alone is capable of activating the LacZ gene, without interaction with the bait protein.
5. It is also possible, if desired, to eliminate duplicate colonies carrying an abundant library plasmid by yeast colony hybridization (9), but we have never performed this. It is done by screening the library transformants using a vector-free oligonucleotide probe designed from the sequence of the most abundant insert. A clas-

sic protocol for this procedure can be found in **ref. 2** and a modification is described in **ref. 1**.

6. If manipulation in *leuB E. coli* was performed (see **Subheading 3.3.**), it is important to use extra care when isolating plasmid DNA, because such strains as HB101 and KC8 are *endA*<sup>+</sup>, and, thus, the presence of endonuclease A becomes an issue. To this end, boiling lysis is not recommended for these strains. More detail can be found in Sambrook et al. (6).

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II \_\_\_\_\_

**FALSE POSITIVES**

## Two-Hybrid System and False Positives

### *Approaches to Detection and Elimination*

Ilya G. Serebriiskii and Erica A. Golemis

#### 1. Introduction

The two-hybrid system has gained wide popularity in the last decade because of the relative ease and speed with which novel interacting proteins can be isolated. Because the two-hybrid approach is based on the interaction of hybrid proteins in a living yeast cell, it offers numerous advantages in comparison to traditional biochemical methods, including the avoidance of costly protein purifications or development of antibodies, and, consequently, great reduction in time required to identify novel protein partners. On the other hand, because two-hybrid screens rely on the indirect readout of transcriptional activation of reporter genes, they are intrinsically prone to distinct classes of artifacts related to the biasing of the transcriptional response. While two-hybrid assays have been quite successful at identifying real interactions (one estimate of success rate is ~70% for usable baits; [1]), the vast majority of library screens also identify at least one protein that is presumed to be a false positive. The first part of this chapter discusses what classes of protein tend to isolate or be isolated as false positives, and the second part presents simple approaches to limit the occurrence of false positive.

#### 1.1. Definition of a False Positive

Although the term *false positive* is frequently used, in fact somewhat arbitrary criteria have been used to define false positives in the two-hybrid system. One trend is to label as false positive any genes that are obtained as primary yeast isolates (e.g., activate *LacZ* and auxotrophic reporters on initial plating)

but that then are sifted out using the internal gage of the two-hybrid system itself, either by being subsequently shown to interact with multiple unrelated baits, or by failing to recapitulate the initial interaction on retransformation. In many cases, the isolation of many of this class of false positive reflects properties intrinsic to the bait or its interaction with the screening strain of yeast, issues covered in **Subheadings 3** and **4**. A second is to define a false positive as a protein that reproducibly interacts in a two-hybrid setting but appears to be implausible as a partner based on the known physiology of bait and prey (i.e., the proteins are not known to be expressed in the same cellular or subcellular compartment at the same time). Of course, even if interaction does not make immediate sense to the investigator, it may still occur inside living cells and contribute to regulatory pathways yet to be discovered; exclusion based on this criterion is a judgment call. A third trend is to consider clones that reproducibly interact by two hybrid, are plausible partners, but for which the interaction cannot be readily confirmed by other means (glutathione-S-transferase pulldowns, coimmunoprecipitations, and so on) as at least suspicious: this third definition is also somewhat of a judgment call, because such interactions may still be real but be below the sensitivity threshold of non-two-hybrid methods.

It is relatively easy to exclude clones as false positives based on the first definition. However, for genes that pass initial two-hybrid specificity and reproducibility criteria, unless the biology of the isolated prey is well defined, researchers generally must resort to extensive biochemical and biologic characterizations before being able to exclude the protein in question as irrelevant to the starting bait or, alternatively, relevant to the bait but of such general function that it does not make sense to devote limited resources to its characterization when the main interest is bait biology. To try to limit the waste of time and resources that such characterization entails, considerable effort has been devoted to devising simple methods and controls to reduce the numbers of false positives obtained in two-hybrid screens, several of which are summarized in **Subheading 6**.

## **2. Web Resources to Detect False Positives**

In the 8 yr since the first reported two-hybrid library screen, the results of more than a thousand library screens have been published, with many more in progress. Furthermore, a number of companies, consortia, and individual laboratories are embarking on genomic analyses using the two-hybrid system, employing arrayed libraries representing the complete proteomes of budding yeast (laboratory of Stan Fields), *Candida elegans* (laboratory of M. Vidal), *Drosophila melanogaster* (laboratory of R. L. Finley), and other higher eukaryotes (several groups; see *ref. 2*). Analysis of published data, survey of

investigators' unpublished results (*1*), and compilation of genomic analyses (e.g., Fields lab: [*3*]) together are leading to the development of lists and resources describing proteins that have been multiply isolated and are unlikely to be of biologic significance, as well as describing classes of bait associated with poor library-screening outcomes. These resources are likely to be greatly enhanced over the next several years. Data from the currently available Web sites are incorporated into the following discussion of false positives.

We note that there are a number of independently developed variants of two-hybrid system utilizing different sets of bait and prey vectors based on LexA (*4,5*) or GAL4 (*6,7*) DNA-binding domains (DBD) being utilized in these projects. To date, there is little reason to believe that these systems have strikingly different properties in regard to isolating false positives in two-hybrid screens; hence, for now the points discussed can be regarded as general.

### **3. Parameters Affecting Isolation of False Positives in Two-Hybrid Screens**

It is perhaps a truism to note that the best way to eliminate false positives from an interactor set is to avoid obtaining them in the first place. Although this cannot be done with complete efficiency, there are particular bait characteristics that are associated with more problematic outcomes to library screens. If such characteristics are detected in a bait prior to initiation of a library screen, the bait can frequently be modified to eliminate the undesired properties. Alternatively, if the characteristics cannot be removed without destroying the structural integrity of the bait, the investigator may either decide against two-hybrid screening or at least be prealerted to the likelihood of a number of artifactual clones among library isolates. Some of the bait properties that should be monitored are discussed next.

#### **3.1. Weak Transcriptional Activation by Bait**

Positive clones in a two-hybrid screen are detected by transcriptional activation of reporter genes above a background level. Baits that activate transcription strongly cannot be used in standard two-hybrid screens because this background level is too high. In many cases, a bait will possess an intrinsic weak transcriptional activation activity, and investigators may use such a bait with the intention of screening, e.g., for dark blue colonies that grow well on histidine—medium vs pale blue colonies that grow slowly. This approach has been known to work well; however, it is associated with a higher incidence of false positive isolation than that associated with baits with no ability to activate transcription.

### **3.2. Low Level of Bait Expression, Large Bait, and Nuclear Excluded Bait**

In a related situation, a bait may initially appear to possess no intrinsic background ability to activate transcription but be particularly poised to develop a higher background when used under screening conditions. This property is in part likely to be related to the issue of bait occupancy of its DNA-binding sites upstream of the reporter gene. If initial circumstances of expression allow only a small fraction of the operator-binding sites to be occupied, a transcriptionally active bait may bind infrequently enough that activation of the reporters may not be observed: during the course of a screen, under selective pressure for growth, binding of the bait may be increased, resulting in a high background activity. If a bait is expressed initially at very low levels (as gaged by Western blot analysis) or is nuclear excluded (this class is rare), protein levels may be amplified. If a bait is very large ( $\geq 60$  kDa, inclusive of DBD), it may be cleaved by yeast peptidases to produce short forms that bind better to the DNA-binding sites and can activate transcription. Finally, in one rare incarnation of the same problem, multiple deletional variants of a bait (8) were selected following library transformation. All these variants possessed a strong activation ability, absent in the original bait, that behaved well through all characterization steps.

Because this is more a bait-specific rather than prey-specific problem, it is generally easy to detect this class of artifact in a conditional two-hybrid system (e.g., when the library is under control of an inducible promoter; [4]); it can be a significant problem when bait and prey are constitutively expressed.

### **3.3. Toxic Bait**

Constitutive expression of some baits is deleterious to the viability of the host yeast. Baits that noticeably make yeast sick almost invariably yield a high frequency of false positive clones in screens (*see Subheading 5.3.*). Toxicity can be detected in several ways. Significantly fewer clones obtained in transformation of a toxic bait vs comparable quantities of DNA for a nontoxic control bait, slow growth of colonies expressing the bait, and development of a dimorphic population of yeast containing the bait (small colonies that express the bait by Western blot analysis, and large colonies that fail to express the bait) are generally indicative. This problem can, in some cases, be ameliorated by placing the toxic bait under control of an inducible promoter (pGilda for the Interaction Trap [9]).

### **3.4. Bait on False Positive List**

A bait may be a potential source of problems because the intrinsic nature of the protein fusion makes it likely to be broadly interactive. This interactivity could result from the inclusion of proteins that are unfolded or otherwise denatured (e.g.,

because they represent a small region cleaved from a larger protein that lacks an intrinsic domain structure or possesses large clusters of surface-exposed hydrophobic amino acids). It also could be attributed to the inclusion of protein domains notable for multiple low-affinity protein-protein interactions (e.g., segments of coiled-coil domain). Overlapping and extending this set, proteins noted themselves as being frequently isolated as false positives in two-hybrid screens could be involved (**1**). Finally, early data from the genomic analysis projects have allowed the identification of specific proteins in *Saccharomyces cerevisiae* that yield irreproducible results and many false positives when used in two-hybrid screens (**3**). Intriguingly, distinct classes of proteins, such as those involved in metabolic functions, tend to be particularly bad baits as a group, whereas others, such as those involved in splicing, are characteristically well behaved (**13**; Uetz, P., personal communication). Caution would be advisable, in all of these cases.

#### 4. Yeast Mutations

An additional cause of misleading transcriptional activation of the reporter genes in a two-hybrid screen is changes in the host yeast strain that promote more robust growth. Such changes, whether mutational or epigenetic, are particularly notable in their effects on the auxotrophic selections utilized in library screening (e.g., growth on histidine- or leucine- selective medium); characteristically, only minor activation of the *LacZ* reporter would accompany apparent strong activation of the auxotrophic reporter. There is clearly some component of bait specificity to this phenomenon, because screens with particular baits result in the isolation of many clones with *LEU+LacZ*-reporter profiles, whereas other screens with different baits, using the same library and host strain combination, identify only biologically relevant *LEU+LacZ*+ interactors; however, to date the nature of this specificity is unknown. Although there are clearly cases in which specific interaction pairs activate one reporter much more strongly than the other (**10**), identification in a screen of a large number of colonies activating only the auxotrophic reporter should generally be regarded with suspicion.

#### 5. Classes of Library-Encoded False Positives

At the dawn of the two-hybrid era, a pessimistic prediction was that two-hybrid systems would produce an unworkably high background of “positives” owing to nonspecific interaction of library-encoded proteins with the DBD of the bait fusion. In fact, this has been one of the rarest classes of false positives, in either the GAL4- or LexA-based systems. Nevertheless, a number of different classes of false positive have been noted. The most common of these are discussed in the following sections.



### **5.1. Proteins That Directly Bind and Activate Reporter Promoters**

One of the first classes of false positives to be identified was proteins that interacted directly with promoter sequences, or in general with DNA upstream of the reporter genes (**11**). These false positives may bind DNA by themselves or depend on additional interaction with other DNA-binding proteins recruited to the particular promoter. This class of false positives was much reduced with the widespread adaptation of dual reporter systems (rather than just *LacZ*; [**12**]) with minimal sequence overlap in their promoter regions; nevertheless, proteins that appear to be operating at the level of chromatin and transcription still are occasionally isolated with apparently biologically irrelevant baits.

### **5.2. “Sticky” Proteins**

Common false positives obtained with many diverse baits (e.g., ribosomal subunits, heat-shock proteins, proteasome subunits, and cytoskeletal components [**11**]), are sometimes hypothesized to possess their false positive character because they are intrinsically “sticky.” As noted in the previous discussion (*see Subheading 3.4.*), broadly interactive proteins may have intrinsic secondary structure properties (exposed hydrophobics or charged patches on ribosomal subunits, coiled-coil regions on cytoskeletal proteins) that lead to nonspecific frequent interactions. Alternatively, the natural biologic function of a protein may involve binding a large number of different proteins. This would potentially include heat-shock proteins, which bind denatured proteins; proteasomal subunits involved in targeting substrates for degradation; and proteins involved in transport functions. Note, however, that even the most common false positives such as ribosomal subunits and heat-shock proteins will not bind every single bait against which they are tested, might appear specific enough to pass the initial specificity tests with one or two nonspecific baits, but be detected when compared against 5–10 nonspecific baits ([**13**]; unpublished observations).

### **5.3. False Positives Inducing Indirect Effects on Reporter Systems**

Accumulated observations suggest that the idea that two-hybrid false positives mostly represent sticky proteins cannot explain the action of all these proteins. In a specific investigation of a set of false positives to identify potential indirect consequences of their expression that might bias transcriptional activation of two-hybrid reporter genes, it was determined that some of these clones induce an array of biologic effects in yeast, including altered growth rate, viability, and cell permeability (**13**). In some cases, the apparent enhanced activation of *LacZ* reporters occurred in the absence of a transcriptional activa-

tion motif on the false positive. In general, these false positives had stronger effects on the *LacZ* reporter than the auxotrophic reporter, although weak enhancement of the auxotrophic reporter's activity could be observed. Further, the false positives possessed greater activity in assays of *LacZ* activity performed by growth on plates containing 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) than in assays performed by filter or overlay (14). As with the sticky false positives, these false positives showed partial specificity in their activity, not being reactive with every bait tested. Speculatively, if this class of false positives functions indirectly by regulating yeast cell metabolism and substrate (e.g., X-gal)-enzyme access, then yeast with a higher basal level of enzyme to modulate might be expected to register variance in these properties more strongly. It is suggestive that the general survey of parameters affecting two-hybrid library screening success rates reveals a somewhat higher incidence of false positives with baits that possess intrinsic weak transcriptional activation activity.

## **6. Methods to Rapidly Assess Specificity of Two-Hybrid Clones in Yeast**

As two-hybrid systems and practice have been refined over the last decade, a number of incorporated elements and strategies have been developed to limit the occurrence of false positives. Some of these strategies emerged early (*see Subheadings 6.1–6.3.*); others have been more recently developed.

### **6.1. Utilization of Multiple Reporters**

As already mentioned, the frequency of isolation of false positives directly interacting with sequences upstream of the reporter gene is reduced by the use of the second, unrelated reporter gene under the control of a largely different promoter. All extant two-hybrid systems now offer dual selection, most often including one auxotrophic reporter (*HIS3*, *LEU2*) and one colorimetric reporter (*lacZ*, *GFP*). To further winnow out promoter-specific false positives, a host strain was recently created that contains three reporter genes (*HIS3*, *ADE2*, *lacZ*), each under the control of a different inducible promoter (15). Theoretically, since these promoters share only minimum sequence identity, this should eliminate virtually all false positives specific to the promoter element in the GAL4 system.

Note, however, that deliberate use of different promoters for reporter genes also leads (at least in some cases) to the differential access of bait proteins to reporter promoters and may result in altered perception of activation strength of a given reporter. It is conceivable that some proteins that appear to be false positives because they strongly activate the auxotrophic reporter but only

weakly activate even the most sensitive *lacZ* reporter may still be valid (10). Finally, because the reporter elements used in different variants of the two-hybrid system are different from each other, it is also possible that some interactions might be detected differently in these systems.

### **6.2. Inducible Expression of Libraries**

One two-hybrid system variant, the interaction trap, expresses activation domain (AD)-fusion components under control of the GAL1 promoter, so that AD-fused proteins are expressed in the presence of galactose but not glucose (4). This conditional expression is helpful in reducing background owing to mutations in yeast strains leading to more robust growth (apparent activation of the auxotrophic reporter), or changes in the bait protein expression contributing to greater transcriptional activation potential, because these do not generally demonstrate a galactose-dependent phenotype.

### **6.3. Segregation Analysis**

Another way to check for dependence of two-hybrid interaction on the presence of plasmid-encoded proteins is to check it for plasmid dependence itself. In the segregation analysis, most often performed in the GAL4-based system, cells from positive colonies are induced to selectively lose the bait or prey plasmids. This is accomplished using a negative selectable marker incorporated into the plasmids, e.g., cycloheximide sensitivity marker *CYH2* (16). Selection on the cycloheximide medium would favor plasmid loss, since the yeast strain itself carries a recessive cycloheximide resistance marker. Clones that lose either bait or prey plasmid but still activate reporters would be considered false positives and should be discarded. An additional advantage of this technique is that if the bait is counterselected in a haploid strain background, this allows generation of a population of cells containing only prey and reporters; these can be utilized as in **Subheading 6.4**.

### **6.4. Specificity Testing**

The built-in controls noted above tended to reduce much spurious background owing to changes in baits and yeast strains. Nevertheless, library-encoded false positives, as noted in **Subheadings 5.2 and 5.3**, in some cases pass these initial weeding out steps. To date, the best way to confirm that a particular library plasmid encodes a true interactor is to test its ability to activate reporters in the presence of the original bait and other related or unrelated bait plasmids as negative control. This can be achieved by the recovery of library plasmids into *Escherichia coli*, followed by the retransformation into “virgin” bait-containing yeast strains that have not been subjected to aux-

otrophic selection. Ideally, interaction-dependent phenotypes still will be observed and be specific to the starting pBait. This test will eliminate false positives including mutations in the initial yeast that favor growth or transcriptional activation on selection medium, library-encoded cDNAs that interact with the DBD, or library-encoded proteins that are sticky, and interact with multiple biologically unrelated fusion domains. Initial recommendations with two-hybrid systems suggested that testing a library clone against one or two nonspecific baits would be adequate to control for false positives: in fact, many appear to show some degree of specificity, as noted in **Subheading 5.2.**, and, therefore, it is a good idea to test for interaction against a number of different nonspecific baits. Because the numbers involved can be quite large (e.g., if ~20 library clones are to be tested against ~10 baits), one desirable approach is to use interaction mating (*17*).

Ideally, one would assemble a panel of baits with different degree of relationship to the bait used in the screening, from completely unrelated, to belonging to the same protein family, to the original bait with a mutation that should abolish the interaction of interest. These baits are transformed into haploid yeast of one of the two mating types and the potential interactors transformed into a strain of the opposite mating type (or isolated in such a strain [*see Subheading 6.3.*]; although, arguably, it may be preferable to conduct the experiment in virgin strains). This set of strains is then mated on the grid and the resulting diploids are analyzed on indicator plates. An interaction matrix constructed this way would facilitate the identification of false positives; it would additionally yield functional information about the isolated proteins (*18*). Candidates that interact with the original bait but not with other members of the same protein family would be considered most specific; candidates that interact with other members of a protein family but not with unrelated baits may or may not gain the researchers' attention, whereas candidates interacting with unrelated baits of the panel would be considered false positives and usually discarded.

Recently, a quick recombination-based technique to rapidly characterize specificity of two-hybrid clones in yeast was developed that does not require plasmid isolation and intermediate hosts (*19*) and can be used to facilitate interaction mating and direct retest of interactions by retransformation. This method makes use of polymerase chain reaction (PCR)-amplified library inserts, typically obtained from primary positive transformants to quickly characterize and sequence library insert DNAs. Since oligonucleotide primers used for such amplification are complementary to flanking plasmid sequences, vector sequence tags are generated at both ends of the product. Following retransformation into yeast, homologous recombination between linear library

vector and plasmid sequences in the PCR product efficiently reconstructs circular, recombinant plasmids. Using this approach, yeast reporter strains, pretransformed with original and alternative baits, are then transformed with PCR products along with linearized AD hybrid vector; alternatively, a haploid strain can be transformed with just the PCR products/linearized AD vector, then mated to a panel of interactors.

### **6.5. Assessment of Indirect Biologic Effects**

Some classes of false positives exert indirect effects on cell permeability and viability. Although it is not clear that the identification of these properties is in itself sufficient to exclude interactors as false positives, it seems valid to consider such behavior as a warning sign. These properties can be simply and readily assayed. As a first measure of differences in yeast permeability, yeast expressing primary isolates can be replica plated or streaked onto normal yeast growth plates incorporating ~0.1% bromophenol blue, in parallel with replica plating on selective medium. Enhanced uptake of the bromophenol blue from the growth medium over the first 24 h of growth on the medium (development of a dark green color in colonies vs a light green for control colonies, has been observed in a number of false positives (13). Alternatively, growth rate of yeast expressing positive clones can be compared under nonselective conditions; notably, slow growth may indicate toxicity associated with overexpression of prey.

### **6.6. Double Bait Systems**

Over the last few years, a number of two-hybrid systems have been developed that utilize two different baits with separate reporter gene readout for each bait (20–23). Although most of these so far have only a single reporter for each specific bait, one (23) utilizes two for each bait (with a LexA fusion activating *LacZ* and *LEU2*, and a cI fusion activating *GusA* and *LYS2*), making it useful for library screening applications. Although not all false positives interact with all baits, these reagents still seem likely to be able to reduce false positive isolation by particular problematic baits by allowing comparison of interactive properties with a specific and carefully chosen nonspecific bait. For instance, if screening with a LexA-fused bait known to possess coiled-coil tendencies, the use of a cI-fused alternative coiled-coil domain should allow a single step discrimination of nonspecific interactors. Nevertheless, clones obtained in such systems should still be used in tests against a significant number of other baits, to obtain greater confidence in specificity.

## **7. Conclusion**

Complexity is inherent and unavoidable in assays taking place within a living organism, but as two-hybrid systems move toward genomic-scale applica-

tions, the ability to determine whether apparent interactions are likely to be physiologically relevant has become one of the key issues for their effective use. While two-hybrid systems are not perfect, and this chapter, by its nature, has necessarily assumed a somewhat alarmist tone, it is nevertheless clear that the systems are extremely useful and become better controlled with each passing year. As the genomic era arrives, it is hoped that much information generated in large-scale screens will appear in public databases, reducing further the need for individual investigators to devote extensive efforts to false positive elimination, and allowing them more time to follow up the exciting leads provided by true positive clones.

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## Confirming Yeast Two-Hybrid Protein Interactions Using In Vitro Glutathione-S-Transferase Pulldowns

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### 1. Introduction

Protein interactions play a vital physiologic role in mediating many important aspects of cell biology including cell division, transcriptional regulation, and signal transduction. The biologic significance of protein interactions is highlighted by the important role that they play in nuclear receptor-mediated transcription. Nuclear hormone receptors, which include the receptors for thyroid hormone, vitamin D<sub>3</sub>, estrogen, and progesterone, are ligand-inducible transcription factors that activate and repress transcription through a complex array of protein interactions (1–4). In an effort to understand the mechanism by which nuclear receptors regulate the transcription of specific target genes, many recent studies have focused on the identification of proteins that bind nuclear receptors. The concerted efforts of a number of investigators have led to the isolation and characterization of a growing list of proteins that interact with nuclear hormone receptors. The majority of these proteins were identified through the yeast two-hybrid system, while others were identified by Far-Western blotting or conventional chromatography.

The yeast two-hybrid system has proven to be a powerful genetic technique for the characterization of protein interactions between nuclear hormone receptors and components of the transcription preinitiation complex, such as basal transcription factor IIB (TFIIB) (5). More recently, it has been utilized in the isolation of novel factors involved in hormone-dependent transcriptional regulation. For example, using the ligand-binding domain (LBD) of the human progesterone receptor (PR) as bait to screen a human  $\beta$ -lymphocyte cDNA library, Oñate et al. isolated a novel protein designated steroid receptor



coactivator-1 (SRC-1) (**6**). The term *coactivator* given to SRC-1 indicates one of its key functional properties of enhancing ligand-activated transcription without affecting basal or general transcription. The interaction between PR and SRC-1 occurs in a strictly agonist-dependent manner both in yeast and in vitro, with strong interaction in the presence of progesterone but no interaction with unliganded or RU-486 antagonist-occupied PR. Since the discovery of SRC-1, many other coactivators or transcriptional inhibitory proteins (corepressors) have been isolated in yeast two-hybrid screens of cDNA libraries using the LBD of nuclear receptors in the presence of their respective agonists or antagonists (reviewed in **refs. 7–13**). Because of an inherent property of the yeast two-hybrid system to occasionally yield false positives (*see* Chapter 8), it is important that each of these protein interactions identified in yeast were confirmed using in vitro methods with purified proteins in their initial characterization.

One of the most widely used in vitro methods for confirming protein interactions identified in the yeast two-hybrid system is the glutathione-S-transferase (GST) pulldown assay. The GST pulldown assay is a quick and easy to perform in vitro technique for characterizing protein interactions. In this assay, a protein of interest (e.g., the human vitamin D receptor [hVDR] LBD) is expressed as a fusion protein with GST. This fusion protein is used to test for protein interaction with a [<sup>35</sup>S]-methionine-labeled target protein (e.g., SRC-1) prepared by in vitro transcription and translation. Following the addition of glutathione agarose and extensive rinses, protein-protein complexes of GST-VDR and SRC-1 are visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The usefulness of this method for confirming yeast two-hybrid protein interactions stems largely from how quickly it can be performed. Within 1 to 2 d, the GST pulldown method can determine whether a test protein binds the GST fusion protein and whether this interaction is ligand dependent. This chapter discusses several procedures and strategies used to establish a GST pulldown assay to examine proteins that interact with the VDR or any protein of interest that is expressed as a GST fusion.

## **2. Materials**

### **2.1. Plasmids**

#### **2.1.1. Yeast Two-Hybrid Activation Vector (the prey or target)**

These yeast expression vectors commonly contain the transcription activation domain (AD) of either GAL4 (AD-GAL4) (**14,15**), the nuclear localized VP16 acidic AD (VP-16<sub>ACT</sub>) (**16**), or the B42 transcription AD (**17,18**). Our

laboratory generally uses either pAD-GAL4 (Stratagene, La Jolla, CA), which contains amino acids 761–881 of the yeast GAL4 AD, or pGAD.GH (**19**), which contains amino acids 786–881 of the GAL4 AD. Both vectors have a nuclear localization signal and a *LEU2* selectable marker.

### 2.1.2. AD cDNA Library

Using an appropriate tissue or cell line as a source of mRNA, an AD cDNA library can readily be prepared by carefully following previously described protocols (**20**) or by using a cDNA synthesis kit (Stratagene; or Gibco-BRL, Gaithersburg, MD). Alternatively, many two-hybrid cDNA-AD libraries are now commercially available (Invitrogen, Carlsbad, CA, or Clontech, Palo Alto, CA).

In our laboratory, we have identified several VDR-interacting proteins from at least two different cDNA libraries. Using a fusion protein consisting of the GAL4-DNA-binding domain (DBD) and the hVDR LBD as bait, we screened a GAD.GH-HeLa cell cDNA library (prepared by Drs. Gregory J. Hannon and David Beach) and isolated TFIIB (**5**), as well as a novel coactivator protein, NCoA-62 (**21**). Another fusion library, consisting of cDNA sequences from mouse osteoblastic MC3T3 cells, was constructed by Dr. Rene St.-Arnaud in the *EcoRI/XhoI* restriction endonuclease sites of the yeast multicopy expression vector pAD-GAL4. Through a similar screening strategy (again using the GAL4 DBD–hVDR LBD fusion protein as bait), we isolated the putative coactivators SRC-1 and 140-kDa receptor interacting protein (**22**), as well as a number of other potentially interesting proteins.

### 2.1.3. In Vitro Transcription/Translation Vector Containing an Initiator Methionine

Plasmid vectors that have a promoter for T7 RNA polymerase are able to transcribe downstream DNA sequences producing messenger RNA (mRNA) (see **Note 1**). The mRNA generated can then be translated in vitro, resulting in the synthesis of the desired protein. Many commercially available vectors contain T7 promoters and have been successfully utilized for the in vitro transcription and translation of cloned genes. Our laboratory has found that cDNA sequences cloned into the pSG5 (Stratagene), pCR 3.1 (Invitrogen), and pTZ18U (U.S. Biochemical, Cleveland, OH) vectors reliably produce high levels of desired proteins.

This technique is particularly useful for the in vitro synthesis of proteins obtained from a yeast two-hybrid screen of a cDNA library. Depending on the size of the cDNA insert obtained and the size of the mRNA transcript, a particular clone may encode a full-length protein, although more likely it will encode a fragment of a protein. Efficient translation requires the presence of an

initiator methionine (AUG) upstream of the mRNA to be synthesized. However, if only a fragment of a protein is obtained from the yeast two-hybrid screen, it may or may not contain an appropriate initiation codon. Therefore, we modified the pTZ18U vector to incorporate an initiator methionine with a strong Kozak sequence (23) and engineered a convenient *Eco*RI restriction site (pTZ18U-Met) that is in the same reading frame as the yeast two-hybrid prey vector AD-GAL4. Thus, any clone obtained from a cDNA library in the AD-GAL4 vector can be readily transferred to this vector, and protein may be generated and tested for protein interaction in the GST pulldown assay.

#### 2.1.4. GST Fusion Protein Expression Vector

A wide variety of pGEX vectors containing unique restriction endonuclease sites in all three translational reading frames are now commercially available (Pharmacia, Piscataway, NJ). These prokaryotic plasmid vectors are useful for the high-level bacterial expression of a cloned gene as a fusion protein with GST. pGEX vectors contain the *lacI* gene for the *lac* repressor, which binds the *tac* promoter and represses the expression of the GST fusion protein. These vectors also contain the  $\beta$ -lactamase gene, which confers resistance to 100  $\mu$ g/mL of ampicillin. Using the pGEX-KT vector (24), a modified vector that contains a glycine kinker for more efficient thrombin cleavage of the GST fusion (see Note 2), our laboratory has successfully generated many proteins as GST fusions.

### 2.2. Expression and Purification of a GST Fusion Protein

High-quality reagents are essential for the preparation of media to support the growth and maintenance of GST fusion protein expression vectors in *Escherichia coli*. Bacto brand tryptone (0123-17-3) and yeast extract (0127-17-9) are obtained from Difco (Detroit, MI). All media and solutions are prepared with reagent grade, distilled, deionized water and are autoclaved at 15 lb/in<sup>2</sup> at 140°C (15 min for 1 L of medium) or are sterile filtered with a 0.2- $\mu$ m filter.

#### 2.2.1. *E. coli*

Several strains of bacteria can be used for the expression of a GST fusion protein. The subcloning grade strain DH5 $\alpha$  (F<sup>-</sup>  $\phi$ 80d*lacZ* $\Delta$ M15  $\Delta$  [*lacZYA-argF*] U169 *deoR* *recA1* *endA1* *hsd* R17 [r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>] *phoA* *supE*44  $\lambda^-$  *thi-1* *gyrA*96 *relA*1) and the BL21 (DE3) strain (F<sup>-</sup> *ompT* *hsdS*<sub>B</sub> [r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>] *gal* *dcm* [DE3]), which contain a single copy of the gene for T7 RNA polymerase under the control of the inducible *lac* promoter, have been used successfully for the high-level expression of proteins as GST fusions.

### 2.2.2. Luria Broth (LB)

Luria-Bertani is a general purpose medium for the routine propagation of bacterial strains that harbor a plasmid of interest. LB medium is prepared by combining 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride in 1 L of distilled water, and autoclaving for 15 min at 15 lb/in<sup>2</sup>. A stock solution of ampicillin (100 mg/mL) is prepared in distilled water and filter sterilized. Once the medium has cooled, 500  $\mu$ L of the ampicillin stock solution is added to 500 mL of LB (LB/Amp).

### 2.2.3. Isopropyl- $\beta$ -D-thiogalactopyranoside

Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) is an inhibitor of  $\beta$ -galactosidase activity in many strains of bacteria, including DH5 $\alpha$  and BL21 (DE3). In the absence of IPTG, the *lac* repressor constrains the expression of the GST fusion protein. On induction with IPTG, which functions as a *lac* analog, derepression occurs allowing GST fusion protein expression. A 100 mM stock solution of IPTG (I-5502; Sigma, St. Louis, MO) is prepared in distilled water and sterile filtered with a 0.2- $\mu$ m filter. The sterile stock solution is stored at -20°C and is used at a final concentration of 0.1 mM.

### 2.2.4. Phosphate-Buffered Saline with Triton X-100 Detergent

For 10X phosphate-buffered saline (PBS), the stock buffer consists of 1.37 M NaCl, 27 mM KCl, 43 mM Na<sub>2</sub>HPO<sub>4</sub>, and 14 mM KH<sub>2</sub>PO<sub>4</sub>. A working solution of PBS with Triton X-100 detergent (PBST) (pH ~ 7.3) is prepared fresh for each experiment using the 10X PBS stock along with 2 mM EDTA, 14 mM  $\beta$ -mercaptoethanol, and Triton X-100 at a final concentration of 0.05%. The following protease inhibitors are added just prior to use: 4.0  $\mu$ g/mL of aprotinin, 2.0  $\mu$ g/mL of leupeptin, 1.0  $\mu$ g/mL of pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (final concentrations).

### 2.2.5. Glutathione Agarose Bead Slurry

Glutathione agarose beads have a high affinity for binding proteins that are expressed as fusions with GST. Glutathione agarose (G4510; Sigma) is obtained as glutathione immobilized on cross linked 4% beaded agarose (a lyophilized powder) and must be swelled in distilled water at 200 mL/g for 2 h and washed with 10 column volumes of distilled water prior to use. The glutathione-agarose beads are then resuspended in distilled water, generating a 50% slurry. The glutathione agarose bead slurry can be stored for up to 6 mo at 4°C.

### **2.2.6. Purification of Bound GST Fusion Protein**

Following the binding of a GST fusion to the glutathione agarose beads, interfering contaminating proteins are removed by successive washes, three times with ice-cold PBST and twice with 50 mM Tris-HCl, pH 8.0. The bound, purified GST fusion protein is then eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione (GSH).

### **2.2.7. Dialysis of Purified GST Fusion Protein**

The purified GST fusion protein is dialyzed three times against 300 vol of dialysis buffer consisting of 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol (DTT).

### **2.2.8. SDS-PAGE Final Sample Buffer (2X FSB)**

The purified, dialyzed GST fusion protein is tested for purity by separation on SDS-PAGE followed by Coomassie blue staining. The final purified, dialyzed protein sample (10  $\mu$ L) is combined with 50  $\mu$ L of 1X FSB (1:1 dilution of 2X FSB in water) and run on an SDS-PAGE gel. The 2X FSB consists of 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.28 mM  $\beta$ -mercaptoethanol, and ~20  $\mu$ g/mL of bromophenol blue.

## **2.3. Protein Synthesis by In Vitro Transcription and Translation**

In vitro coupled transcription and translation from a DNA template is a rapid and efficient method of protein synthesis. Once a standard protocol is established, desired proteins can be generated in vitro in as little as 2 h. Another advantage of this method is that by performing the reaction in the presence of L-[<sup>35</sup>S]-methionine, the protein can be synthesized as a radiolabeled species and can then be visualized by autoradiography.

### **2.3.1. DNA Template**

The quality of the DNA and the proper cloning of the protein-coding DNA sequence used as the template in the in vitro coupled transcription and translation reaction are the most important determinants of successful protein synthesis. High-quality DNA (Midi or Maxi plasmid kits; Qiagen, Valencia, CA) is essential for efficient in vitro protein synthesis. DNA prepared by standard miniprep procedures (e.g., alkaline lysis) is generally not suitable for in vitro protein synthesis because high concentrations of RNA can inhibit the synthesis of mRNA. Furthermore, miniprep DNA is prone to contaminating ribonucleases that can rapidly degrade transcription products.

The successful in vitro synthesis of a protein also requires that the uninterrupted DNA sequence that encodes the protein be cloned in the proper orienta-

tion downstream of a bacteriophage promoter (e.g., T7, T3, or SP6). This sequence must contain an initiator methionine (AUG) at the 5' end of the mRNA. The position of this initiator codon is critical for efficient in vitro synthesis, because having the AUG too close to the promoter sequence (<20 bases) may affect the ability of the RNA polymerase to transcribe the entire protein-coding sequence. Likewise, having too much 5' untranslated sequence (>100 bases) may potentially lead to problems if the upstream RNA folds in such a way that the secondary structure makes the AUG initiation codon inaccessible. We generally clone our protein-coding sequence ~20–30 bases downstream of the promoter and have had little trouble generating proteins in vitro.

### 2.3.2. In Vitro Transcription and Translation Mix

Many “master mixes” that contain all the reaction components needed for the in vitro transcription and translation of a protein from a plasmid DNA template are commercially available. These convenient solutions allow one to generate a protein of interest by simply combining the master mix with a plasmid DNA template in the presence of exogenous methionine and incubation of this reaction mix for 90 min at 30°C. These transcription and translation kits contain RNA polymerase, nucleotides, salts, and a ribonuclease inhibitor in a single reaction mix. The most common kits for mRNA synthesis and translation are a TNT-coupled reticulocyte lysate system (L-1170) or a TNT-coupled wheat germ extract (L-4140), both of which are available from Promega (Madison, WI) (*see Note 3*).

### 2.3.3. L-[<sup>35</sup>S]-Methionine

When the transcription and translation reaction, described in **Subheading 2.3, item 3**, is carried out in the presence of L-[<sup>35</sup>S]-methionine, the protein synthesized will be isotopically labeled. We have found that translation grade [<sup>35</sup>S]-methionine (specific activity of >1000 Ci/mmol) (obtained from either New England Nuclear [Boston, MA] or ICN [Costa Mesa, CA]) works well for the in vitro synthesis of many radiolabeled proteins.

### 2.3.4. L-Methionine and Cycloheximide

To halt protein synthesis, following the reaction, excess unlabeled (cold) methionine and the protein synthesis inhibitor cycloheximide are added to the reaction mix. L-methionine (M-6039) and cycloheximide (C-7698) are obtained from Sigma (St. Louis, MO). Stock solutions of 10 mM cold methionine in sterile water and 25 mM cycloheximide in ethanol are prepared and stored at –20°C.

## **2.4. In Vitro Protein Interaction Assay**

### **2.4.1. GST Binding Buffer**

GST binding buffer (GBB) is prepared as a 2X stock solution just prior to use (*see Note 4*). 2X GBB consists of 40 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.4% Nonidet P-40, 2 mM DTT, and the following protease inhibitors: 8.0 µg/mL of aprotinin, 4.0 µg/mL of leupeptin, 2.0 µg/mL of pepstatin A, and 0.4 mM PMSF.

### **2.4.2. Purified, Dialyzed GST Fusion Protein**

The preparation, purification, and dialysis of a GST fusion protein is described in **Subheading 2.2**.

### **2.4.3. [<sup>35</sup>S]-Methionine-Labeled Protein**

Protein synthesis by in vitro transcription and translation is described in **Subheading 2.3**.

### **2.4.4. Ligand**

The GST pulldown assay is well-suited for rapidly testing the ligand dependence of a protein interaction. This assay can quickly test the interaction between two proteins over a wide range of concentrations of ligand. In binding assays, we typically add 1 µL of a 1 µM stock solution of 1,25-(OH)<sub>2</sub>D<sub>3</sub> dissolved in ethanol to a volume of 100 µL, resulting in a final concentration of 10 nM.

## **3. Methods**

### **3.1. Analytical Scale Test Expression of a GST Fusion Protein**

Following the subcloning of a protein coding cDNA sequence into a pGEX expression vector (*see Subheading 2.1.4.*), those transformed bacterial clones that appear to harbor the plasmid of interest as determined by restriction endonuclease analyses are tested for the ability to express a GST fusion protein of the predicted molecular mass.

1. For each bacterial clone that appears to be correct, grow a 2-mL culture in LB-Amp medium overnight at 37°C with vigorous shaking.
2. Use 100 µL of each overnight culture to seed 2 mL of LB/Amp (1 : 20 dilution). These cultures are grown at 37°C with shaking, until the OD<sub>600</sub> = 1.00 U (~2.5 h).
3. Induce the expression of the GST fusion proteins by adding 4 µL of 100 mM IPTG (final concentration 0.2 mM) to each culture for 2 h at 30°C with vigorous shaking.



4. After the 2-h induction is complete, transfer the entire 2-mL culture to individual microcentrifuge tubes and centrifuge for 15 s at 14,000g at 4°C.
5. Discard the supernatant and resuspend each of the bacterial pellets in 300 µL of ice-cold PBST and transfer 10 µL of each to separate tubes. Add 50 µL of 1X FSB to each of these 10-µL samples and boil for 5 min (these are whole-cell extracts for SDS-PAGE analysis).
6. Sonicate the remaining 290 µL of samples using three 20-s pulses with a microtip probe sonicator. Store on ice between sonications.
7. Centrifuge for 5 min at 14,000g at 4°C.
8. Transfer the supernatant to fresh microcentrifuge tubes and add 100 µL of a 50% slurry of glutathione agarose. Place on a rocking plate for 30 min at 4°C.
9. Centrifuge for 15 s at 14,000g at 4°C.
10. Discard the supernatant and wash the beads twice with 0.5 mL of ice-cold PBST.
11. Remove the last wash and add 50 µL of 2X FSB directly to the glutathione agarose beads. Boil the samples for 5 min and analyze the entire sample by SDS-PAGE.

### **3.2. Preparative Scale Expression and Purification of a GST Fusion Protein**

Following the successful analytical test expression of a GST fusion protein in a 2-mL culture, the GST fusion protein is expressed in a 250-mL culture and purified over a glutathione agarose column. This preparative scale expression and purification protocol is a modification of that described by Smith and Corcoran (25).

1. Grow a 2-mL overnight culture from a glycerol stock or isolated colony at 37°C in LB-Amp.
2. Dilute the entire overnight 2-mL culture into 250 mL of LB/Amp in a 1-L flask. Grow at 37°C with vigorous shaking until the OD<sub>600</sub> of the large culture is near 1.00 U. This will require approx 4 to 5 h but should be monitored closely in initial trials.
3. Add IPTG to a final concentration of 0.2 mM (500 µL of a 100 mM sterile stock solution dissolved in water). Grow at room temperature with shaking for 2 h (*see Note 5*).
4. Pellet the bacterial cells by centrifuging for 15 min at 5000g and discard the supernatant (*see Note 6*).
5. Resuspend the bacterial pellet in 2.5 mL of PBST and transfer to an ice-cold 15-mL conical tube. Transfer 25 µL of the resuspended cells to a microcentrifuge tube for SDS-PAGE analysis (crude).
6. Sonicate the resuspended cells with three 20-s pulses with storage on ice between sonications. It is critical to keep this preparation as cold as possible to slow protease activity.
7. Transfer the lysate to prechilled microcentrifuge tubes and centrifuge for 10 min at 14,000g at 4°C.



8. Transfer the supernatant to a fresh, prechilled 15-mL conical tube. Save 25  $\mu$ L for SDS-PAGE analysis (lysate).
9. Add 650  $\mu$ L of a 50% slurry of glutathione agarose beads. Incubate for 30 min at 4°C with gentle rocking.
10. Pellet the glutathione agarose beads and save 25  $\mu$ L of the supernatant for SDS-PAGE analysis (supernatant).
11. Wash the glutathione agarose pellet three times with 2 mL of PBST followed by two times with 2 mL of 50 mM Tris-HCl, pH 8.0.
12. Elute the GST fusion protein with two 0.5-mL, 5 min washes with 50 mM Tris-HCl, pH 8.0, containing 10 mM GSH. Combine elutions and analyze 10  $\mu$ L of this preparation by SDS-PAGE (predialysis).
13. Dialyze this preparation into dialysis buffer overnight against three 300 vol. Analyze 10  $\mu$ L of this final preparation by SDS-PAGE (final) (*see* **Note 7**).

The purified, dialyzed final preparation of the GST fusion protein is analyzed on an SDS-PAGE gel along with several protein samples that were collected during the purification. The Coomassie blue-stained gel should yield a highly enriched band corresponding to the predicted molecular mass of the GST fusion protein in the crude and lysate sample lanes, an absence of this band in the supernatant lane, and a single stained protein band for the purified GST fusion protein in the predialysis and final preparation lanes.

Any standard protein assay is used to determine the protein concentration of the purified, dialyzed final preparation of the GST fusion protein. We routinely use the Coomassie Protein Assay Reagent (23200ZZ; Pierce, Rockford, IL). Once a standard curve is generated, 20  $\mu$ L of the purified, dialyzed GST fusion protein final preparation is combined with 1 mL of the assay reagent to determine the protein concentration (*see* **Note 8**). Once the protein concentration has been determined, the GST fusion protein is divided into aliquots in microcentrifuge tubes, snap-frozen in a dry ice/ethanol bath, and stored at -80°C.

### **3.3. Protein Synthesis by In Vitro Transcription and Translation**

High-quality DNA is used as the template in the in vitro-coupled transcription and translation reaction. The protocol is essentially that recommended by the manufacturer (Promega).

1. Rapidly thaw the reticulocyte lysate master mix.
2. Combine 2  $\mu$ g of the template DNA, 6  $\mu$ L of nuclease-free water (supplied in the TNT-coupled reticulocyte lysate system), 44  $\mu$ L of the TNT T7 Quick Coupled Master Mix, and 2  $\mu$ L of translation grade L-[<sup>35</sup>S]-methionine in a microcentrifuge tube.
3. Mix the contents of the tube and incubate the mixture for 90 min at 30°C.

4. Add 3  $\mu\text{L}$  of 10 mM unlabeled (cold) methionine.
5. Add 6  $\mu\text{L}$  of 25 mM cycloheximide.
6. Place the reaction mix on ice.

### **3.4. In Vitro Protein Interaction Assay**

#### **3.4.1. Binding of Purified, Dialyzed GST Fusion Protein to Glutathione Agarose Beads**

1. Prepare 2X GST binding buffer (2X GBB) and place on ice.
2. Thaw an aliquot of a purified, dialyzed GST fusion protein and transfer 5  $\mu\text{g}$  to a microcentrifuge tube.
3. Add 50  $\mu\text{L}$  of 2X GBB.
4. Add 25  $\mu\text{L}$  of a 50% slurry of glutathione agarose beads.
5. Bring the final volume to 100  $\mu\text{L}$  by adding sterile water.
6. If appropriate, add 1  $\mu\text{L}$  of a 1 mM stock solution of ligand, resulting in a final ligand concentration of 10 nM (*see Note 9*).
7. Mix the contents of the tube and incubate the mixture on a rocking plate for 1 h at 4°C (*see Note 10*).

#### **3.4.2. Combining GST Fusion Protein Bound to Glutathione Agarose Beads with [ $^{35}\text{S}$ ]-Methionine-Labeled Protein**

1. Centrifuge the GST fusion protein and glutathione agarose beads mixture for 1 min at 14,000g at 4°C to pellet the glutathione agarose beads with the bound GST fusion protein.
2. Remove the supernatant and add 50  $\mu\text{L}$  of 2X GBB.
3. Add 35  $\mu\text{L}$  of sterile water.
4. Add 15  $\mu\text{L}$  of the [ $^{35}\text{S}$ ]-methionine-labeled protein that was generated in the in vitro transcription and translation reaction.
5. If appropriate, add 1  $\mu\text{L}$  of a 1  $\mu\text{M}$  stock solution of ligand, resulting in a final ligand concentration of 10 nM (*see Note 9*).
6. Mix the contents of the tube and incubate the mixture on a rocking plate for 1 h at 4°C.

#### **3.4.3. Washing Complex of GST Fusion Protein, [ $^{35}\text{S}$ ]-Methionine-Labeled Protein, and Glutathione Agarose Beads to Remove Any Nonspecific Binding Proteins**

1. Centrifuge the mixture for 1 min at 14,000g at 4°C to pellet the glutathione agarose beads with the bound GST fusion protein and the [ $^{35}\text{S}$ ]-methionine-labeled protein.
2. Remove the radioactive supernatant and handle as radioactive liquid waste (*see Note 11*).
3. Wash the glutathione agarose beads with 0.5 mL of ice-cold 1X GBB (a 1:1 dilution of 2X GBB in water) and centrifuge for 1 min at 14,000g at 4°C.

Remove the radioactive supernatant using the [<sup>35</sup>S] liquid waste aspirator. Follow this wash protocol twice more with 1X GBB (*see Note 12*).

4. Repeat the wash protocol with 0.5 mL of ice-cold 50 mM Tris-HCl, pH 8.0.
5. Elute the GST fusion protein complex from the glutathione agarose beads using 35  $\mu$ L of 50 mM Tris-HCl, pH 8.0, containing 10 mM GSH and incubate for 15 min at 4°C on a rocking plate (*see Note 13*).
6. Combine the GST fusion protein complex eluent (35  $\mu$ L) with 35  $\mu$ L of 2X FSB and boil for 5 min. Then load the entire sample onto an SDS-PAGE gel.
7. Once the proteins are sufficiently resolved (the bromophenol blue dye has reached the bottom of the gel), dry the gel and visualize by autoradiography the [<sup>35</sup>S]-methionine-labeled protein that was pulled down by the GST fusion protein.

#### 4. Notes

1. T7 RNA polymerase, which does not occur naturally in *E. coli*, is highly selective for its own promoter. In fact, not only is T7 RNA polymerase very selective, but it is also very active. T7 RNA polymerase initiates transcription quite efficiently, nearly five times faster than does *E. coli* RNA polymerase. In some cases, it can be so active that transcription by *E. coli* RNA polymerase is greatly decreased. T7 RNA polymerase is able to transcribe almost any DNA linked to a T7 promoter; thus, the T7 expression system is capable of transcribing almost any gene in *E. coli* (26).
2. For some purposes, it may be desirable to remove the GST portion of a fusion protein. Many GST fusion protein expression vectors contain a short amino acid sequence (L V P R ↓ G S) encoding a thrombin protease recognition site for cleaving the desired protein from the GST. The following protocol for the thrombin cleavage of a GST fusion protein is a modification of that described by Smith (27). Briefly, a cleavage buffer consisting of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2.5 mM CaCl<sub>2</sub>, and 0.1%  $\beta$ -mercaptoethanol is prepared. Thrombin protease (27-0846-01), obtained from Pharmacia, is dissolved in sterile water at a concentration of 0.1  $\mu$ g/ $\mu$ L. The GST fusion protein (15  $\mu$ g) and the thrombin protease (0.3  $\mu$ g) are combined in the cleavage buffer at a final volume of 50  $\mu$ L and incubated for 40 min at room temperature. Following cleavage, the proteins are separated by SDS-PAGE and stained with Coomassie blue to monitor the efficiency of cleavage. Using these conditions, we typically observe the complete cleavage of GST without negatively affecting our protein of interest.
3. Many mRNAs can be translated efficiently in both reticulocyte lysates and wheat germ extracts. However, the *in vitro* synthesis of a desired protein may be more efficient in either one of these systems. Generally, wheat germ extracts initiate translation better than reticulocyte lysates but are more prone to premature termination or degradation of the mRNA template. Thus, wheat germ extracts are preferred for shorter proteins (<20 kDa) fused to the 26-kDa GST protein, whereas reticulocyte lysates should be used for longer proteins (>20 kDa).

4. GBB is prepared as a 2X GBB stock solution, because when it is combined with the purified GST fusion protein and glutathione agarose beads, the final concentrations of the buffer components will be diluted 1 : 1. For example, we typically use a final volume of 100  $\mu\text{L}$  for binding the purified GST fusion protein to the glutathione agarose beads. Thus, if the protein concentration of the GST fusion protein is 0.5  $\mu\text{g}/\mu\text{L}$ , then 10  $\mu\text{L}$  is needed for 5  $\mu\text{g}$  of the GST fusion. To this we add 25  $\mu\text{L}$  of the 50% slurry of glutathione-agarose beads and 15  $\mu\text{L}$  of sterile water followed by 50  $\mu\text{L}$  of 2X GBB.
5. In general, we have found that the best results for our proteins are obtained when the GST fusion protein induction occurs at room temperature. In some cases, protein expression in bacteria varies greatly depending on specific characteristics of individual proteins. Initial studies that examine time and temperature dependence of the induction are essential.
6. After centrifugation, it is permissible to freeze the bacterial cells at  $-80^{\circ}\text{C}$  overnight. This step is not required, but after the 4 to 5 h needed to grow the bacterial culture to an  $\text{OD}_{600}$  of 1.00 U and the 2-h IPTG induction, we have found it to be a convenient stopping point prior to the purification. Freezing the bacterial cells will not harm the GST fusion protein, in fact, the freeze/thaw may actually be beneficial and may promote cell lysis.
7. We have found that the use of Slide-A-Lyzer dialysis cassettes (10,000 mol wt cutoff, 0.5 to 3.0-mL capacity) (66425ZZ Pierce) are convenient for the dialysis of a GST fusion protein into a more stable storage buffer. Alternatively, buffer exchange may be performed with precalibrated Sephadex G-50 columns (Pharmacia).
8. The expression of GST alone (~26 kDa) yields very high levels of the purified, dialyzed protein. From a 250-mL culture we typically obtain ~3.10 mg of GST at a concentration of ~2.3 mg/mL. However, as proteins are fused to GST the yield decreases quite considerably. As the size of the desired fusion protein increases, the yield usually decreases. In our laboratory, the majority of proteins that we express are less than 60 kDa (e.g., hVDR LBD [116–427] fused to GST). For proteins of this size we typically obtain yields that range from 350  $\mu\text{g}$  at 310  $\mu\text{g}/\text{mL}$  to 725  $\mu\text{g}$  at 655  $\mu\text{g}/\text{mL}$ , depending on the solubility of a particular protein. One can increase the solubility and yield of a difficult-to-express GST fusion protein by coexpression of the GST fusion protein of interest with one of the highly soluble proteins groE or thioredoxin (Trx) (Takeshita, A., personal communication). For example, when the plasmid for GST NCoA-62 (ampicillin resistant) and the plasmid for pET-thioredoxin (chloramphenicol resistant) are cotransformed into the same bacterial host, the very high solubility of thioredoxin significantly increases both the solubility and purity of GST NCoA-62. By using glutathione agarose beads, the GST NCoA-62 can be purified and the thioredoxin protein is washed away during the purification.
9. In the case in which the GST fusion protein binds a ligand, the ligand may be added to the initial binding mixture of the purified, dialyzed GST fusion protein

bound to the glutathione-agarose beads as described in **Subheading 3.4.1**. Alternatively, ligand may be added directly to the binding assay consisting of the GST fusion protein bound to the glutathione agarose beads and the [<sup>35</sup>S]-methionine labeled protein as described in **Subheading 3.4.2**. We have found that the ligand-induced GST pulldown assay works well under both conditions.

10. To make the most efficient use of time, we have found it convenient to start the in vitro protein synthesis reaction (~90 min), and then while the in vitro transcription and translation reaction is running, we simultaneously bind the GST fusion protein to the glutathione agarose beads (~1 h). When multiple protein interactions are being examined (we generally do no more than 40 tubes per assay), the setup time usually delays the start of the 1-h incubation so that both the in vitro transcription and translation reaction and the binding of the GST fusion protein to the glutathione agarose beads finish at roughly the same time.
11. For the successive washes of the [<sup>35</sup>S]-methionine-labeled protein bound to the GST fusion protein and glutathione agarose beads, we have found it useful to set up a dedicated vacuum aspirator for the removal of the [<sup>35</sup>S] liquid waste.
12. We have found that the use of an Eppendorf Repeater Pipetter (21-380-8; available through Fisher, Pittsburgh, PA) expedites the wash process. However, one needs to be extra careful not to spray the radioactive beads out of the microcentrifuge tube. Furthermore, we have found that the use of a microcentrifuge tube rack (05-561-10; Fisher), which contains a lower tray to hold ice, makes the organization and handling of the samples throughout the washes much easier while maintaining the samples near 0°C to slow protease activity.
13. During our initial attempts to establish a GST pulldown assay protocol, we made several important modifications that optimized our results. The most noteworthy change was that instead of adding 2X FSB directly to the glutathione agarose beads, we chose to elute the GST fusion protein complex from the glutathione agarose beads using 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione prior to separation by SDS-PAGE. By using this modification described by Lee et al. (28), the background that we had observed in our assays was essentially eliminated. Using these conditions, we routinely observe a single band by autoradiography for the [<sup>35</sup>S]-methionine-labeled protein that is pulled down by the GST fusion protein.

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## Two-Hybrid Interactions Confirmed by Coimmunoprecipitation of Epitope-Tagged Clones

Louie Naumovski

### 1. Introduction

The yeast two-hybrid system is a powerful genetic assay for detecting interacting proteins in yeast. The system is based on the ability of a pair of hybrid proteins, one containing a DNA-binding domain (DBD) and the other a transcriptional activation domain (AD), to interact and activate transcription of a reporter gene (1). One of its most useful applications is that of screening expression cDNA libraries to identify proteins that interact with a bait protein (2). In one widely used system, a yeast strain expressing a bait protein fused to a DBD is transformed with a library engineered to express cDNA fragments fused to an AD. The transformants are first selected based on expression of a *HIS3* reporter gene and subsequently screened for expression of a *lacZ* reporter (2). A library screen generally yields many clones that need to be evaluated for specificity of interaction with the bait protein. Some false positives activate transcription when coexpressed in yeast with fusions unrelated to the target protein and can be eliminated by various methods (3,4).

Clones that are not false positives are then further evaluated. In the traditional approach, the difficult decision of which clones to pursue is made based on sequence homology or other parameters that are generally ill defined. A review of the literature on two-hybrid screening often fails to show why certain clones were further investigated in preference to others. The full-length genes are isolated and either epitope tagged or an antibody is raised against fusion proteins to monitor protein expression. Biologic or biochemical assays are then performed to determine whether the clone affects the function of the



bait protein. These manipulations can take weeks to months (or longer) for each individual clone.

To determine quickly which clones to pursue further, we have devised an approach based on epitope tagging the yeast two-hybrid-derived fragments that interact with a bait protein. These epitope-tagged fragments (ETFs) are then coexpressed in mammalian cells with the bait protein, and *in vivo* interaction is documented by coimmunoprecipitation studies. We have successfully applied this approach to Bcl-xL, an antiapoptotic protein homolog of Bcl-2 (5). We screened two libraries for proteins that interact with Bcl-xL and identified Bad and Bax, which were previously known to interact with Bcl-xL and inhibit its antiapoptotic properties (6,7). Using the epitope tagging assay, we demonstrated strong coimmunoprecipitation of Bcl-xL with two-hybrid-derived fragments of Bax and Bad. We also identified clones that had not been previously implicated in interacting with Bcl-xL. The clones we identified fell into three groups: clones that strongly coimmunoprecipitate, clones that weakly coimmunoprecipitate, and clones that do not coimmunoprecipitate with Bcl-xL (8). Since Bax and Bad strongly coimmunoprecipitate with Bcl-xL and inhibit its function, it is likely that the clones we identified that strongly immunoprecipitate with Bcl-xL will also affect its function.

Our approach simplifies the process of choosing clones for an extensive workup by allowing rapid, single-step epitope tagging of two-hybrid-derived fragments in an expression vector useful for coimmunoprecipitation studies. This strategy allows researchers to quickly and objectively characterize ETFs on the basis of coimmunoprecipitation with the bait protein in mammalian cells. Clones that are positive in these assays can then be analyzed in a more traditional manner involving cloning of the full-length gene (or obtaining clones from repositories) and determining biologic function. One obvious caveat is that only the fragment of the cDNA that was isolated in the screen is tagged (although at times the full-length cDNA is isolated in the two-hybrid screen). Moreover, the fragment may not contain other sequences that are critical to the localization of the full-length protein in mammalian cells. However, at a minimum, the tagged fragment carries the determinants that are necessary for interaction with the bait in yeast.

Rapid tagging with the CEP4FLAG vector described herein is feasible when pACT1, pACT2, and GAD vectors are used for two-hybrid screening. Tagging vectors with appropriate reading frames can be designed to accommodate fragments derived from other screening vectors. In addition, using the same strategy, tagging vectors can be designed with different epitope tags (HA, MYC and so on). Furthermore, fusions between two-hybrid-derived fragments and green fluorescent protein can be similarly constructed, although a large tag could potentially alter the distribution or binding affinity of the protein fragment. Finally, the

CEP4FLAG tagging vector carries a selectable marker and an origin of replication and has been used to construct stable mammalian cell lines expressing ETFs.

## 2. Materials

### 2.1. Preparing Tagged Clones

1. Restriction enzymes.
2. Calf intestinal alkaline phosphatase (CIP). The CIP must be titrated to obtain maximal cloning efficiency (*see Note 1*).
3. T4 DNA ligase (Roche).
4. Agarose (usually 0.8% prepared in TAE buffer).
5. TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0.
6. Ethidium bromide (10 mg/mL stock prepared in water). Ethidium bromide is a powerful mutagen, and gloves and mask should be worn when preparing stock. Gloves should be worn when working with solutions. Add ethidium bromide (0.5 µg/mL) to the agarose solution just before pouring the gel and to the electrophoresis buffer.
7. DNA fragment isolation: We use the Gene Clean kit (Bio101), but any similar technique for isolating DNA from gels is sufficient.
8. TE buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
9. Competent *Escherichia coli*: Prepare or purchase competent *E. coli* to use for transformation using electroporation or other methods. Select for marker on plasmid using appropriate antibiotic. For CEP4FLAG, we use ampicillin selection in plates and broth at 100 µg/mL.
10. Plasmid isolation: Potential clones are screened using miniprep techniques. DNA used for transfection of mammalian cells is isolated using commercial DNA purification columns (Qiagen).

### 2.2. Transfection of Mammalian Cells

#### 2.2.1. Cell Culture Reagents

1. DMEM (Cellgro) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL of penicillin, 290 mg/mL of L-glutamine, and 100 mg/mL of streptomycin (Irvine Scientific).
2. Trypsin/EDTA in normal saline (0.5% trypsin and 0.2 g of EDTA/L) (Irvine Scientific).
3. Incubator (5% CO<sub>2</sub>/95% air): Cells are maintained at 37°C.

#### 2.2.2. Transfection Reagents

All reagents are sterilized by passing through 0.22-µ filters. Reagents can be purchased commercially in a transfection reagent kit (Clontech).

1. 2X HBS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM HEPES. In separate 100-mL aliquots, adjust the pH to 7.00, 7.05, 7.10, or 7.15 with 5 M NaOH. The 2X HBS must be tested to determine which pH solution is optimum for transfection (*see Note 2*).

2. 2 M Calcium chloride dissolved in H<sub>2</sub>O.
3. Chloroquine (5 mg/mL), dissolved in H<sub>2</sub>O.

### **2.2.3. Other Reagents**

1. Phosphate-buffered saline (PBS) (autoclaved).
2. 100 mM EDTA, pH 7.4.
3. Cell scraper.

### **2.3. Coimmunoprecipitation of Tagged Clones and Bait**

1. Cell lysis and immunoprecipitation buffer: 10 mM HEPES pH 7.2, 142.5 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2% NP-40. Store at room temperature and chill on ice prior to the addition of protease inhibitors to final concentrations of 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg/mL of leupeptin, 0.5 µg/mL of pepstatin, 0.5 µg/mL of aprotinin, and 5 µg/mL of antipain. PMSF is prepared as 100 mM stock dissolved in isopropanol and kept at -20°C.
2. Antibodies: Mouse monoclonal anti-Flag M2 (Eastman Kodak) if clones are tagged with a FLAG epitope, a rabbit antimouse IgG secondary antibody (Jackson), and antibait antibody.
3. Protein A agarose (Roche).
4. 2X Sample buffer: 100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 20% glycerol.

### **2.4. Western Blot Analysis of Coimmunoprecipitation**

1. SDS-polyacrylamide gels: Polyacrylamide concentration will vary depending on the size of expected products, but usually 10–15% will suffice.
2. Ponceau stain (Sigma, St. Louis, MO): Prepare by diluting stock solution 1:10 into sterile PBS.
3. Blocking reagent: 5% nonfat dry milk (NFDM) prepared in sterile PBS. Do not autoclave or filter.
4. PBS (sterile) and PBS/bovine serum albumin [BSA]/azide (prepared by adding BSA to 2% and azide to 0.01% in PBS).
5. Primary and secondary antibodies. Prepare primary antibodies or purchase from various suppliers. Secondary antibodies are conjugated to horseradish peroxidase (Accurate Antibodies).
6. Methanol.
7. Transfer buffer: 39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol.
8. Polyvinylidene difluoride (PVDF) membranes (Roche).
9. Chemiluminescent substrate kit (Roche or similar supplier).

## **3. Methods**

### **3.1. Preparing Tagged Clones**

Clones identified in a yeast two-hybrid screen are subcloned in the proper orientation into an epitope-tagging vector such as CEP4FLAG (8). The

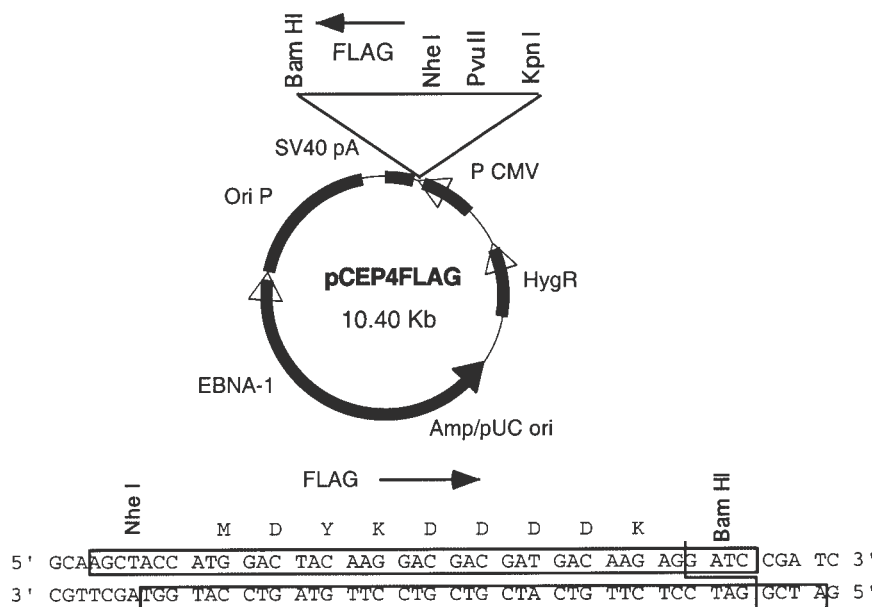


Fig. 1. Schematic diagram of CEP4FLAG tagging vector. The DNA and amino acid sequence of the FLAG tag and reading frame of the cloning site are shown. Boxes indicate FLAG tag oligos used to construct the vector. pCMV, CMV promoter; HygR, hygromycin resistance; Amp/pUC ori, ampicillin resistance gene and origin of replication in *E. coli*; EBNA-1, Epstein-Barr virus nuclear antigen-1; Ori P, origin of replication in mammalian cells; SV40 pA, Simian virus 40 polyadenylation site.

CEP4FLAG vector is designed to accept *Bgl*II fragments from pACT1 and other two-hybrid screening vectors in the proper reading frame so that two-hybrid-derived clones can be epitope tagged in one step (**Fig. 1**). If the two-hybrid vector you use does not generate inserts compatible with CEP4FLAG, then obtain a commercial tagging vector or prepare one using an oligonucleotide strategy (*see Note 3*).

1. Cut 1  $\mu$ g of the CEP4FLAG vector with 5 U of *Bam*HI, and 2 to 3  $\mu$ g of the clone to be tagged with 10 U of *Bgl*II, each in the manufacturer's suggested buffer for 1 h at 37°C.
2. Treat the vector with CIP for the period of time determined by titration (*see Note 1*).
3. Load the cut and phosphatased vector and cut two-hybrid clone onto a 0.8% agarose gel (you may need to vary the gel percentage depending on the size of the insert) prepared in TAE buffer and run to separate fragments (for a standard Bio-Rad minigel, this is usually 100 V for 1 h).

4. Isolate the fragments using the Gene Clean (Bio101) or similar technique and dissolve each DNA in 20  $\mu$ L of TE. Ligate 2  $\mu$ L of vector and 5  $\mu$ L of insert using standard cloning techniques per ligase manufacturer's recommendation.
5. Transform *E. coli* (DH10b or other strain) selecting for ampicillin resistance and analyze colonies by restriction mapping of minipreps. Identify clones in proper orientation to use for coimmunoprecipitation analysis. Prepare DNA from 100–200 mL of *E. coli* using a Qiagen column or other technique to yield mammalian transfection-quality DNA.

### 3.2. Transfection of Mammalian Cells

COS-7 monkey kidney cells are cotransfected by standard calcium phosphate precipitation techniques with a vector expressing a tagged clone and a vector expressing the bait protein. It is important to include one transfection with a control plasmid expressing an epitope-tagged protein that is not expected to interact with the bait. If available as a positive control, include a transfection with a vector producing an epitope-tagged protein that interacts with the bait. The COS-7 cell line was utilized because of its high transfection efficiencies but other cell lines can also be used. The pH of the 2X HBS is very important for high-efficiency transfection (*see Note 2*). Three days after transfection, the cells are harvested and protein interaction is determined by analysis of immunoprecipitates.

1. Grow COS-7 cells in 10 mL of complete DMEM in a T-75 flask to near confluence. Six to 24 h prior to transfection, aspirate the medium and rinse with 10 mL of PBS. Add 5 mL of trypsin/EDTA solution and incubate at 37°C for about 5 min or until the cells detach. Add 5 mL of fresh medium and count the cells using a hemocytometer or automated counter. Plate 200,000 cells in 5 mL into 60-mm dishes. The cells must not be confluent prior to transfection (approx 30–50% confluent works well for us).
2. For each transfection, mix 3  $\mu$ g of ETF-expressing plasmid with 3  $\mu$ g of bait-expressing plasmid in 90  $\mu$ L of H<sub>2</sub>O. Then add 12  $\mu$ L of CaCl<sub>2</sub> and 102  $\mu$ L of 2X HBS. Pipet the mixture up and down two times, and then pipet air into the mixture three times. Drip the mixture onto the cells. Add 10  $\mu$ L of chloroquine and swirl the plates. View the cells under a microscope to see that a precipitate has formed (this will usually happen within 30 min).
3. Incubate the cells with the precipitate overnight (about 10–15 hr). Then aspirate the medium and rinse the cells with 5 mL of PBS. Replace the medium (5 mL) and incubate the cells for an additional 2 d.
4. To harvest the cells, add 1/10 vol of 100 mM EDTA, pH 7.4 (0.5 mL), directly to the medium and return the plates to the tissue culture incubator at 37°C for 15 min. Monitor the cells by microscopy to confirm that they are detaching from the plate; gentle scraping with a cell scraper is sometimes necessary.

5. Pipet the cells into tubes and spin at room temperature in a tissue culture centrifuge (500–1000 rpm for 5 min). Pipet 1 mL of cold PBS into the tubes and disperse the pellet. Transfer to a microfuge tube and spin the cells at 850g (3000 rpm) for 5 min or at 21,000g (15,000 rpm) for 10 s. Aspirate the PBS and proceed to the immunoprecipitation step or freeze the pellets at  $-80^{\circ}\text{C}$ .

### 3.3. Coimmunoprecipitation of Tagged Clones and Bait

Cell lysis and immunoprecipitations are carried out using a previously published buffer that works well for demonstrating interactions between Bcl-2 family members (6,8). Buffer and detergent conditions may need to be varied to demonstrate optimal interactions for other proteins. We use the anti-FLAG M2 antibody, because it works well for immunoprecipitation.

1. Resuspend cell pellets in 500  $\mu\text{L}$  of ice-cold immunoprecipitation (IP) buffer, rotate in a cold room for 30 min, and spin at 15,000–21,000g (13,000–15,000 rpm) for 15 min in a microfuge. Distribute 400- and 50- $\mu\text{L}$  aliquots of supernatant into separate microfuge tubes.
2. To the 50- $\mu\text{L}$  aliquot add 50  $\mu\text{L}$  of 2X sample buffer, boil for 3 min, and freeze at  $-80^{\circ}\text{C}$ .
3. To the 400- $\mu\text{L}$  aliquot add 1  $\mu\text{g}$  of mouse anti-FLAG M2 antibody (Kodak) for a minimum of 1 h at  $4^{\circ}\text{C}$  (leave on ice or rotate in a cold room), followed by 1  $\mu\text{g}$  of rabbit-antimouse antibody (Jackson) for 30 min.
4. During the above incubation, prepare the protein A agarose (Roche). For each immunoprecipitation, remove 50  $\mu\text{L}$  of slurry from the bottle with a pipet tip that has had the end cut off to expand the opening. Mix the slurry with 5 mL of IP buffer and centrifuge at 1000 rpm for 5 min. Aspirate the supernatant and resuspend the protein A agarose in IP buffer to make a 50% slurry.
5. Add 50  $\mu\text{L}$  of the protein A agarose to the immunoprecipitation and rotate the tubes for a minimum of 1 h in a cold room (*see Note 4*).
6. Centrifuge the tubes for 3 min at 5000 rpm or 10 s at 15,000 rpm. Aspirate the supernatant.
7. Wash the beads with 1 mL of NP-40 containing lysis buffer three to four times. Then aspirate the lysis buffer, add 50  $\mu\text{L}$  of 2X Laemmli Sample Buffer, and boil for 3 min. Freeze the samples or proceed with Western blotting (*see Subheading 3.4*).

### 3.4. Western Blot Analysis of Coimmunoprecipitation

We analyze for expression of the ETFs and bait in the total lysate and immunoprecipitates with the anti-FLAG and antibait antibodies by Western blotting. Most, but not all, ETFs will be detected by Western blotting of the total lysate (*see Note 5*).

1. Run 20  $\mu\text{L}$  of each sample on the appropriate percentage polyacrylamide gels. Run duplicate gels for the supernatants and immunoprecipitates. Prepare PVDF

membranes by wetting in methanol and then placing in transfer buffer. Transfer the gels to PVDF membranes (Roche) using a semidry transfer apparatus (Bio-Rad) per the manufacturer's directions.

2. After completion of the transfer, rinse the membranes in sterile PBS. Incubate with Ponceau S prepared in sterile PBS for 1 min to obtain qualitative data on the efficiency of the transfer. Rinse the membranes in PBS until the red Ponceu stain is removed.
3. Block the membranes in 5% NFDM/PBS for 10–30 min.
4. Incubate one supernatant and one immunoprecipitate membrane in separate trays with anti-FLAG (1 : 2000 dilution of 1 mg/mL stock prepared in PBS/BSA/azide) for 1 to 2 h. Incubate one supernatant and one immunoprecipitate membrane in separate trays with antibait protein antibody.
5. Remove and save the dilutions of the primary antibodies, because they can be reused several times before signal intensity weakens. Rinse the membranes three times for 10 min each in PBS.
6. Incubate with secondary peroxidase-conjugated antibody (Accurate Antibodies) for 1 h and rinse with PBS three times for 10 min each.
7. Image bands using a peroxidase-specific chemiluminescent substrate per the manufacturer's recommendations (Roche). Expose the membranes to film for 5 s to 5 min depending on the strength of the signal (determined empirically).
8. Data interpretation: In the total lysate, you should see bands for most of the ETFs (*see Note 5*) and for the bait protein. In the immunoprecipitates, you should see bands for the ETFs if the immunoprecipitation worked and bands for the bait protein if it coimmunoprecipitated with some of the ETFs. The inclusion of an epitope-tagged ETF that is not expected to interact with the bait (as one of the transfections) is an important control to determine the background associated with the immunoprecipitation and other reagents (*see Subheading 3.2.*). The antibodies used for the immunoprecipitation will be detected on the Western blot at 50 (heavy chain) and 25 kDa (light chain) and may complicate analysis of the immunoprecipitation if the bait is a similar size.

#### 4. Notes

1. To titrate CIP, linearize 1 µg of Bluescript or similar plasmid with *Bam*HI for 1–2 hr. Add CIP buffer and 1 µL of CIP. Incubate at 37°C and remove four time points at 5-min intervals. After agarose gel electrophoresis, purify CIP-treated DNA to use for self-ligation and ligation with test insert. With the Roche product, 5 min of phosphatase treatment is usually sufficient to produce a vector with low self-ligation background and good cloning efficiency.
2. Test the various 2X HBS solutions by setting up transfections with a reporter construct. For example, transfection efficiency can be monitored using expression constructs for lacZ, green fluorescent protein, or luciferase.
3. Epitope-tagging vectors can be easily designed and constructed. To create pCEP4FLAG, pCEP4 (Invitrogen) was cut with both *Bam*HI and *Hind*III and



phosphatased. Two complementary oligonucleotides containing a FLAG epitope tag (amino acids DYKDDDDK) and a *Bam*HI site were kinased, annealed, and ligated to the vector (**Fig. 1**). Ligated products were transformed into *E. coli* and screened by restriction digestion followed by DNA sequencing to confirm the presence of the oligonucleotide. The pCEP4FLAG vector is available from the author.

4. Samples can be treated with the primary antibody or the protein A agarose for longer than 1 h without increasing the background.
5. Most clones can be expressed as ETFs detectable by Western blotting. In our original work, 4 of 13 clones could not be expressed presumably because the proteins were unstable or insoluble, although other reasons for lack of expression are also possible (8).

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### III \_\_\_\_\_

## APPLICATIONS

## Smad Interactors in Bone Morphogenetic Protein Signaling

Xiangli Yang and Xu Cao

### 1. Introduction

The yeast genetic-based two-hybrid system has been widely used in identifying a pair of interacting proteins. The basis for this technique is that eukaryotic transcription activators are modular with respect to their DNA-binding domain (DBD) and transcription activation domain (AD) (*1*). The two domains are required for activating gene expression, but they do not need to be covalently linked. Indeed, they may be located on entirely separate proteins. It is sufficient that the interaction between two (or more) proteins bring these domains into close proximity (*2*). Therefore, the transcriptional activity of the target gene can be used as a measure of the protein-protein interaction.

A variety of versions of the two-hybrid system are commercially available. The most commonly used are the yeast Gal4- (*3*) and the *Escherichia coli* LexA- (*4*) derived systems. Three components are essential for all of the two-hybrid systems. The first is a bait plasmid that directs the synthesis of the protein being studied that has been fused to a DBD, of Gal4 or LexA proteins. The second is a prey plasmid that directs the synthesis of a second protein fused to an AD such as those of Gal4 or herpesvirus VP16 (*5*). This second protein either can be one chosen specifically for study or can be an unknown protein derived from a cDNA library. Finally, a yeast reporter strain that contains one or more reporter genes, such as yeast *Leu2* or *His3* genes and *E. coli* *LacZ*, with upstream binding sites for the bait is also required. Productive interaction between the bait and prey proteins leads to expression of the reporter genes that can be determined by the ability of yeast strains to grow on a selective medium or to turn blue with the correct substrate.

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Smads are a family of newly discovered downstream mediators of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily signaling. In mammalian systems, eight members of Smads identified thus far can be categorized into three subgroups. The receptor-regulated Smads (R-Smads) can be phosphorylated by TGF- $\beta$ /activin receptors (Smad2 and Smad3) (6–8) or by bone morphogenetic protein (BMP) receptors (Smad1, Smad5, and Smad8) (9–11). Hence, they determine the specificity of the signaling pathways by interacting with different receptor molecules. The common Smads (Co-Smads) shared by both TGF- $\beta$  and BMP signaling pathways comprise solely Smad4 (8), which interacts with the R-Smads and translocates into the nucleus, where the complex activates gene transcription. The antagonist Smads (Anti-Smads), including Smad6 and Smad7 (12), inhibit the signaling by inhibiting the phosphorylation of R-Smads by their receptors (13,14), by competing with R-Smads for the Co-Smad4 (15), or by preventing the R-Smads from interacting with other DNA-binding molecules (unpublished data).

Similar to other signaling molecules, Smads transduce their signal by interacting other Smads or with other cellular proteins. In addition, Smad complexes also function as transcription activators, which bind directly to other nuclear DNA-binding proteins or to DNA (16). The two-hybrid system, as a tool for studying protein-protein interactions, is exceedingly helpful in hunting for Smad interactors in the TGF- $\beta$  superfamily signaling pathway, because it allows the building of individual binary links to more complex patterns of connections (17).

Another feature of Smads that makes the yeast two-hybrid system so useful is that Smads contain three distinct domains, called MH1, linker, and MH2 for amino-terminal, intervening region, and carboxyl-terminal domains, respectively. Each domain possesses characteristic structures that support the interaction with specific proteins. In particular, MH2 of all the R-Smads has an SSXS motif that is recognized and phosphorylated by TGF- $\beta$  or BMP type I receptors. The MH2 domain is also the transactivator, which interacts with other transcription factors. The linker of R-Smads can be phosphorylated by mitogen-activated protein kinases, which leads to an inhibition of nuclear translocation of Smad complexes. The MH1 domain associates with MH2 of the same R-Smads when the protein is in the inactive state (18). The MH1 domain of Smad1 and Smad4 is also found to bind other proteins, such as Hoxc-8 and Hoxa-9 (unpublished observation). Thus, different domains of Smads can be separately fused with DBDs and used as baits to pull out their specific interactors.

The yeast two-hybrid assay has advantages over crosslinking, coprecipitation (pulldown), coimmunoprecipitation, cochromatography, and

other approaches for the study of protein-protein interaction. It has a higher sensitivity, which supports identification of weak and transient interactions *in vivo*. Furthermore, it detects the interactions in yeast host cells, a physiologically relevant environment. Many groups have successfully identified Smad interactors by using either intact Smads (19) or truncated Smads (20) as baits in two-hybrid assays. The two-hybrid system is also used to study the interaction of individual domains of Smads (21,22) or of Smad and its interactor (12,20). Here, we describe detailed yeast two-hybrid protocols that have been successfully used to identify the Smad1 interactor, Hoxc-8, and to map the interaction domains between the two proteins.

## 2. Materials

### 2.1. Vectors, Yeast and Bacterial Strains, and Equipment

1. pGBT9 cloning vector (5.4 kb) (Clontech) for generating the fusion of the Smad1 bait protein with the GAL4 DBD. pGBT9 carries the *trp1* gene that confers a Trp<sup>+</sup> phenotype to yeast transformants.
2. Human osteosarcoma MATCHMAKER cDNA library (HL4026AH; Clontech). The cDNA library was fused with the GAL4 AD in pACT2 cloning vector (8.1 kb). pACT2 contains the *leu2* gene that gives a Leu<sup>+</sup> phenotype to its host yeast strains.
3. Y190 *Saccharomyces cerevisiae* yeast reporter strain that requires histidine (His), leucine (Leu), and tryptophan (Trp) in the medium to grow. Y190 also contains *E. coli lacZ* and yeast *HIS3* reporter genes.
4. Two primers for sequencing the fusion genes: GAL4 DBD primer TCATCGGA AGAGAGTAG, and GAL4 AD primer TACCACTACAATGGATG.
5. *E. coli* competent cells, such as DH5 $\alpha$  or HB101.
6. Spectrophotometer.
7. Incubators (30 and 37°C).
8. Water bath (42°C).
9. Electroporator.

### 2.2. Media and Plates

1. YPD medium: 2% Difco peptone (w/v), 1% yeast extracts (w/v), and 2% glucose (use separately, autoclaved 50% stock).
2. SD synthetic medium: 0.67% (w/v) Difco yeast nitrogen base without amino acids (cat. no. 0919-15; Difeo, Detroit, MI), 2% glucose (autoclave and add separately), and 1X dropout solution (*see Subheading 2.3.*; autoclave and add separately). Store at 4°C.
3. LB broth: 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 5 g of NaCl in 1 L of water. Autoclave and store at room temperature.
4. YPD/SD/LB plates: 15% agar in appropriate medium and autoclave. Pour the 100, 15-mm or 150, 15-mm plates and store at 4°C.

### 2.3. Stock Solutions and Chemicals

All chemicals are from Sigma (St. Louis, MO).

1. 10X Dropout solutions: Dissolve the following in water and autoclave. Store at 4°C up to 1 yr.
  - a. L-Isoleucine: 0.3 g/L (cat. no. I-7383).
  - b. L-Adenine hemisulfate salt: 0.2 g/L (cat. no. A-9126).
  - c. L-Lysine HCl: 0.3 g/L (cat. no. L-1262).
  - d. L-Phenylalanine: 0.5 g/L (cat. no. P-5030).
  - e. L-Tyrosine: 0.3 g/L (cat. no. T-3754).
  - f. L-Valie: 1.5 g/L (cat. no. V-0500).
  - g. L-Arginine HCl: 0.2 g/L (cat. no. A-5131).
  - h. L-Methionine: 0.2 g/L (cat. no. M-9625).
  - i. L-Threonine: 2.0 g/L (cat. no. T-8625).
  - j. L-Uracil: 0.2 g/L (cat. no. U-0750).
2. 200X L-Tryptophan (cat. no. T-0254) (0.4 g/100 mL of water) Autoclave and store at 4°C.
3. 100X L-Leucine (cat. no. L-1512) (1 g/100 mL of water) Autoclave and store at 4°C.
4. 1000X L-Histidine (cat. no. H-8511) (2 g/100 mL of water) Autoclave and store at 4°C.
5. 1 M 3-Amino-1, 2, 4-triazole (3-AT), a competitive inhibitor of the His3 protein.
6. 50% Polyethylene glycol (PEG) 4000 (average mol wt of 3350) (cat. no. P-3640). Autoclave and store at room temperature.
7. 100% Dimethyl sulfoxide (DMSO) (cat. no. D-8779).
8. 100% Glycerol.
9. 10X TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5. Store at room temperature after autoclaving.
10. 10X LiAc: 1 M lithium acetate (cat. no. L-6883). Autoclave and store at room temperature.
11. Salmon sperm single-stranded DNA (ssDNA) (10 mg/mL).
12. Z buffer: 16.1 g of Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 5.5 g of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.75 g of KCl, and 0.246 g of MgSO<sub>4</sub>·7H<sub>2</sub>O in a total volume of 1 L of distilled water. Autoclave and store at room temperature.
13. *o*-Nitrophenyl β-D-galactopyranoside (ONPG) (cat. no. N-1127).
14. 1 M Na<sub>2</sub>CO<sub>3</sub>.
15. Yeast lysis solution: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100, and 1% sodium dodecyl sulfate.
16. Phenol/chloroform/isoamyl alcohol (25:24:1).
17. Acid-washed glass beads (425–600 μm) (cat. no. G-8772).
18. 70% and 95% EtOH.
19. 1000X Ampicillin (100 mg/mL in H<sub>2</sub>O, filter sterilize and store at –20°C).

### 3. Methods

All yeast manipulations are based on the MATCHMAKER GAL4 two-hybrid system user manual (PT3061-1; Clontech) with modifications. Unless stated otherwise, any bacterial or yeast culture should be a freshly grown overnight culture that was started from a single colony. All the materials and solutions should be sterile by autoclaving or filtering.

#### 3.1. Construction of Bait Plasmid

We used an intact human Smad1 cDNA inserted between *SalI* and *PstI* sites in pGBT9 vector as a bait to screen a human osteoblast-like cell cDNA library constructed in pACT2 vector. The first step was to construct a bait plasmid that directs the expression of GAL4 DBD–Smad1 fusion. A multistep cloning strategy was used to obtain the bait plasmid because the *SalI/PstI* sites were too close to be used together.

1. Digest the pGBT9 vector with *PstI* and dephosphorylate with alkaline phosphatase.
2. Insert any DNA fragment in the *PstI* site for the purpose of providing some distance between the two intended cloning sites.
3. Sequentially digest the pGBT9/insert with *SalI* and then *PstI* to release the pGBT9 vector.
4. Digest pCMV5B/Smad1 (9) with *SalI* and *PstI* to release Smad1 cDNA.
5. Ligate pGBT9 vector and Smad1 cDNA insert and transform bacteria with the ligation mix.
6. Verify the hybrid construct by restriction digestion with *SalI/PstI* and sequencing using the GAL4 DBD primer to confirm the in-frame fusion of Smad1 cDNA and the GAL4 DBD.
7. This step is optional. Check expression of fusion protein of GAL4 DBD1–Smad1 by Western blot with Smad1 antibody.

#### 3.2. Generation of Bait Plasmid–Carrying Strain and Testing of Transactivation Activity of the Bait Protein

It is not uncommon that the target protein activates transcription when linked to a DBD. In this case, the yeast two-hybrid system is not suitable for a library search for the unknown target-interacting proteins. The pGBT9/Smad1 bait plasmid was transformed into Y190 reporter strain and plated onto SD/–Trp and SD/–Trp–His plates. The phenotype of the transformants should be Trp+His– if the bait alone does not act as a transactivator.

##### 3.2.1. Transformation of Y190 with Bait Plasmid (pGBT9/Smad1)

1. Inoculate Y190 cells from a single colony (2 to 3 mm in diameter) into 3 mL of YPD medium and incubate at 30°C with shaking at 200 rpm until OD<sub>600</sub> = 1.5 (1 to 2 d).

2. Transfer enough (1 to 2 mL) of the culture to 40 mL of YPD medium in a 250-mL flask to get an  $OD_{600} = 0.2$ . Grow this culture at 30°C with shaking at 200 rpm for 3 h.
3. Pour the culture into a 50-mL Falcon tube and pellet the cells by centrifuging at 1000g for 5 min at room temperature.
4. Discard the medium and wash the cells by resuspension in water and harvest the cells as in **step 3**.
5. Discard the supernatant and resuspend the cells in 0.2 mL of 1X TE/LiAc (made freshly by mixing 0.1 mL of each 10X TE and 10X LiAc and 0.8 mL of sterile water).
6. Transfer the cell suspension to a 1.5-mL microcentrifuge tube.
7. Boil 0.1 mL of ssDNA for 5 min and quickly chill in ice water.
8. To 0.1 mL of cell suspension, add the following components onto the top of the cells in the order given, and mix well by vortexing: 0.48 mL of PEG (50%), 60  $\mu$ L of 10X TE buffer, 60  $\mu$ L of 10X LiAc, 10  $\mu$ L of ssDNA (10 mg/mL), and 0.1  $\mu$ g of pGBT9/Smad1.
9. To the other 0.1-mL cell suspension, add the mix in **step 8** without omitting the bait plasmid (control).
10. Incubate the tubes at 30°C for 30 min with shaking or with occasional inversion.
11. Add 70  $\mu$ L of DMSO to each of the tubes and mix by vortexing gently.
12. Heat-shock for 15 min at 42°C and then chill on ice briefly.
13. Pellet the cells by centrifuging at 15,600g (14,000 rpm) in a microcentrifuge for 5 s.
14. Carefully aspirate the supernatant and resuspend the cells with 0.5-mL of 1X TE buffer.
15. Plate 0.1 and 0.4 mL of each transformation mixture onto a 100-mm SD/-Trp and an SD/-Trp-His+3-AT (25 mM) plate, respectively.
16. Incubate the plates at 30°C for 2–4 d.

### 3.2.2. Scoring Phenotype of Transformants

The following steps establish whether the bait can be used in the Smad1 interactor hunt. The bait-bearing strain can also be used in the two-step transformation for library screening (*see Subheading 3.4.*). In our practice, transformants of Y190/pGBT9/Smad1 failed to grow on SD/-His+3-AT plates, and they were also negative in  $\beta$ -gal activity, so the bait fusion is suitable for library screening (*see note 3*). It is advisable to include a control plasmid encoding a GAL4 DBD fused with an intrinsic transactivator, such as pVA3 (Clontech), which can grow on SD/-His+3-AT plates.

1. The Y190 strain harboring the pGBT9/Smad1 should grow on SD/-Trp plates but not on the SD/-Trp-His+3-AT plates. Y190 control cells should not grow on either of these selective media (*see note 1*).
2. If any visible colonies formed on the SD/-Trp-His+3-AT plates before 3 d, the bait cannot be used for the interactor hunt. In this case, remove the AD of the bait.

3. Pick one to five transformants from the SD/-Trp plate and inoculate 3 mL of SD/-Trp liquid medium and grow at 30°C for 1 to 2 d until the cell density is  $OD_{600} = 1.5$  (see **note 2**).
4. Prepare bait-bearing yeast (Y190/pGBT9/Smad1) stocks by mixing 0.75 mL of the liquid culture and 0.25 mL of glycerol. Store at -80°C.
5. This step is optional. Perform  $\beta$ -galactosidase liquid assay on the bait-carrying clones to verify the negative for transactivation activity (see **Subheading 3.4.4.2.**).

### 3.3. Amplification of cDNA Library

One of the main uses of the two-hybrid system is to screen a relevant cDNA library for genes encoding the bait interactors. Smad1 as a BMP downstream intracellular transducer may play a role in osteoinduction. To this end, we used the human osteoblast-like cDNA library in pACT2 (Clontech). The cDNA library plasmids, supplied as a population of bacterial carrying clones, were amplified by the method described next. Before the amplification, one should aliquot the library bacterial culture after the first thawing to avoid multiple freeze/thaw cycles and titer the library to estimate the colony-forming units (CFU) present per milliliter.

#### 3.3.1. Titering cDNA Library

We titered our library culture and obtained the same titer as Clontech expected ( $>10^8$  CFU/mL). Several factors reduce the titer of a library culture, such as repeated freeze/thaw cycles, prolonged storage, and storage of diluted culture.

1. Thaw one tube containing 1 mL of the library bacterial culture and chill nine 0.5-mL Eppendorf tubes on ice. Gently vortex the culture and then transfer 0.1-mL aliquots into each of the 0.5-mL Eppendorf tubes. Leave one tube on ice and store all the rest at -80°C.
2. Prepare three 1.5-mL Eppendorf tubes, each containing 990  $\mu$ L of LB/Amp medium.
3. Transfer 10  $\mu$ L of library culture to first tube to obtain a  $10^{-2}$  dilution. Mix well by vortexing.
4. Transfer 10  $\mu$ L of the  $10^{-2}$  dilution to the second tube and mix well to obtain a  $10^{-4}$  dilution. Do the same from the second to the third tube, which is the  $10^{-6}$  dilution.
5. Plate 0.1-mL aliquots from each of the second and third dilutions onto LB/Amp plates.
6. Incubate the plates at 30°C until colonies appear.
7. Count the colonies formed on each plate and calculate the colony-forming units as follows:  
2nd dilution = no. of colonies  $\times 10^5$  = CFU/mL.  
3rd dilution = no. of colonies  $\times 10^7$  = CFU/mL.



### 3.3.2. Amplifying the Human Osteosarcoma cDNA Library

This protocol was used to amplify the human osteoblast-like library, and it produced enough cDNA library plasmids for at least seven rounds of screening.

1. Prepare 250 LB/Amp plates (150 × 15 mm) and let dry at room temperature for 3 d.
2. Dilute the library with LB/Amp (100 µg/mL) to a concentration of  $1.5 \times 10^5$  CFU/mL to make a total of 50 mL of cell suspension.
3. Spread 0.2 mL of the cell suspension onto each LB/Amp plate with a sterile glass or metal spreading rod over agar surface until all the visible liquid has been absorbed.
4. Incubate the plates at 30°C until confluent (36–48 h).
5. Add 5 mL of LB containing 25% glycerol to each plate to resuspend the colonies.
6. Pool all the suspension into one flask and mix well.
7. Remove one-third of the library culture for plasmid DNA preparation using any standard Maxi-prep method.
8. Store the remainder of the library culture in 50-mL aliquots at –70°C for later use. The expected yield of plasmid DNA/ $1 \times 10^6$  CFU (colony-forming units as referred to in **step 2**) for pACT2 library is >0.25 mg.

### 3.4. Library Screening

Both bait and prey plasmids can be cotransformed into a single yeast cell, however, it is often more efficient to perform two-step (sequential) transformation. We describe here a sequential transformation procedure in which the cDNA library plasmids are transformed into a yeast strain already carrying the bait plasmid. The PEG/LiAc method described in **Subheadings 3.4.1.** and **3.4.2.** usually gives us a transformation efficiency of  $10^5$  transformants/µg of DNA. In theory, to screen 2 to  $3 \times 10^6$  independent clones in a library, 20–30 µg of cDNA and 1 to 2 mg of carrier ssDNA are required. We used 500 µg of cDNA library plasmid to ensure that proteins encoded by low-abundance transcripts are represented (*see note 4*).

#### 3.4.1. Preparation of Competent Y190 Carrying pGBT9/Smad1 Bait Plasmid

1. Grow a 3-mL overnight culture of the pGBT9/Smad1 bait-carrying Y190 from a single colony (2 to 3 mm in diameter) at 30°C.
2. Inoculate 100 mL of SD/–Trp with the 3-mL culture and incubate at 30°C overnight.
3. Remove 60–100 mL of the overnight cell culture to inoculate 1 L of YPD in a 2800-mL flask to obtain an  $OD_{600} = 0.4$ . Incubate at 30°C for 4 h with shaking at 200 rpm.
4. Pellet the cells by centrifuging at 2500 rpm for 5 min at room temperature.
5. Decant the supernatant and wash the cells in 500 mL of water. Pellet the cells as in **step 4**.

6. Discard the supernatant and resuspend the cells in 20 mL of 1X TE/LiAc solution.
7. Leave the cell suspension at room temperature for 10 min.

### 3.4.2. Transformation of Bait-Carrying Yeast Strain with cDNA Library Plasmids

1. To an autoclaved 2-L beaker, add the following components in the order given and mix well by vigorous vortexing: 112 mL of 50% PEG, 14 mL of 10X TE, 14 mL of 10X LiAc (10X), 2 mL of 10 mg/mL ssDNA, and 500 µg of cDNA library plasmid.
2. Add 20 mL of competent cells (from **step 7** in **Subheading 3.4.1.**) to the mix.
3. Mix well by swirling and incubate at room temperature for 30 min.
4. Add 17.6 mL of DMSO and mix well by swirling the beaker gently. Heat-shock in a 42°C water bath for 6 min with constant swirling to equilibrate the temperature.
5. Add 50 mL of sterile water to cool the cells to room temperature and transfer the transformation mix to five 50-mL conical tubes.
6. Pellet the cells by centrifuging at 1000g (2500 rpm) for 5 min at room temperature.
7. Aspirate the supernatant. Wash the cells by resuspending each pellet in 25 mL of TE buffer and collect the cells by centrifuging as in **step 6**.
8. Discard the supernatant and resuspend the cells in 1 L of YPD in a 2.8-L flask.
9. Incubate the cells at 30°C for 1 h with shaking at 200 rpm.
10. Harvest the cells by collecting cells in a 1-L centrifuge tube and spinning at 1000g (2500 rpm) for 5 min.
11. Plate 10 µL (dilute in 0.2 mL of TE) of the same transformation mix and its dilution onto separate SD/-Trp-Leu plates for estimation of the transformation efficiency.
12. Plate 0.2 mL of the cell suspension onto each of the 150 × 15 mm agar plates containing SD/-Trp-Leu-His+AT (45 mM) medium (50 plates).
13. Incubate all the plates at 30°C for 3–5 d until His+ colonies are visible. To search for weak interactions, the incubation time can be prolonged to 8 d.
14. On the next day, count the colonies formed on the SD/-Trp-Leu plates and calculate the transformation efficiency (no. of transformants/µg of DNA) (*see note 5*).

### 3.4.3. Preparation of Master Plate

Colonies formed within 1 wk are collected onto one or more master plates by streaking all the His+ colonies in a grid pattern to facilitate future identification of the colonies.

1. Mark the back of 100-mm SD/-Trp-Leu-His+3-AT plates with a grid pattern.
2. Streak single His+ colonies to each grid.
3. Incubate the master plates for 1 to 2 d until the colony size reaches 2 mm in diameter.

4. Seal the plates with Parafilm and store at 4°C if not used immediately. These are the master plates.
5. Restreak fresh plates every 3 to 4 wk until all the colonies are examined (*see Subheading 3.4.4.*).

#### 3.4.4. Elimination of False Positives

One of the disadvantages of the two-hybrid assays is that the His<sup>+</sup> transformants sometimes are false positives; that is, the His<sup>+</sup> clones contain no plasmids encoding hybrid proteins that directly interact with Smad1 target proteins. The true positives should be examined for the expression of the second reporter gene by the  $\beta$ -gal activity assays described next. Filter  $\beta$ -gal assay allows one to select positive clones quickly by blue/white screening. If one wishes to obtain quantitative  $\beta$ -gal activity data, the liquid assay can be performed to measure the production of a yellow compound, *o*-nitrophenol, from the substrate ONPG.

##### 3.4.4.1. FILTERING $\beta$ -GAL ASSAY

1. Prepare Z buffer/5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) solution: 10 mL of Z buffer; 27  $\mu$ L of  $\beta$ -mercaptoethanol, and 167  $\mu$ L of X-gal (20 mg/mL).
2. Prepare a 1-L liquid nitrogen bucket.
3. Place a sterile Whatman no. 1 filter paper (75 mm in diameter) over the surface of the master plate, and poke holes at the edge through the filter into the agar to orient the filter paper.
4. Lift the filter off the plate with forceps, drop the filter in the liquid nitrogen, and leave for 5 s to freeze the colonies.
5. Put the plate back in the incubator to allow yeast colonies to grow at 30°C for 1 to 2 d.
6. Remove the filter and place it in the 100-mm dish (colony side up) to allow it to thaw at room temperature for 2–5 min to permeabilize the cells.
7. Add 0.5 mL of the Z buffer/X-gal solution to the dish by touching the pipet tip to the edge of the dish. Allow the solution to migrate over the filter paper.
8. Incubate the filter at 37°C and check periodically for the appearance of blue colonies.
9. Identify the blue colonies by aligning the filter paper with the master plate using the orienting marks.
10. Grow liquid cultures by inoculating the corresponding positive colonies from the master plate to 3 mL of SD/-Trp-Leu-His medium to make stocks for further analysis.

##### 3.4.4.2. LIQUID $\beta$ -GAL ASSAY

The time it takes colonies or reactions to develop color varies from minutes to hours. Prolonged incubation (>24 h) may give false positives. We found that

fresh colonies take less time to turn blue or yellow. Thus, when one tries to make comparisons of the  $\beta$ -gal activity on candidate clones, the growth condition, age and size of the colonies, and efficiency with which the cells are permeabilized should be similar or identical. The liquid assay is less sensitive than the filter lift assay and the result varies from time to time. We recommend analyzing all the clones at once if one wishes to compare  $\beta$ -gal activities.

1. Inoculate individual yeast-positive colonies from the master plate to 3 mL of SD/-Trp-Leu-His medium and incubate at 30°C overnight.
2. Inoculate 2 mL of the overnight culture into 8 mL of YPD medium and incubate at 30°C for 3–5 h until the culture reaches OD<sub>600</sub> = 0.5–0.8 (mid-log phase).
3. Record the OD<sub>600</sub> value for each transformant culture.
4. Prepare ONPG solution by dissolving 40 mg of ONPG in 10 mL of Z buffer.
5. Transfer 1.5 mL of culture into a 1.5-mL microcentrifuge tube, and spin at 15,600g (14,000 rpm) for 30 s to pellet the cells.
6. Carefully remove the supernatant and wash the cells once with 1.5 mL of Z buffer.
7. Resuspend each pellet in 0.3 mL of Z buffer, which concentrates the cell culture fivefold.
8. Transfer 0.1 mL of the cell suspension to a fresh microcentrifuge tube.
9. Freeze (1 min in liquid nitrogen) and thaw (1 min at 37°C) the cells three times to permeabilize the cells.
10. Prepare Z buffer/ $\beta$ -mercaptoethanol by mixing 10 mL of Z buffer with 27  $\mu$ L of  $\beta$ -mercaptoethanol, and set up a blank tube with 0.1 mL of Z buffer.
11. Add 0.7 mL of the Z buffer/ $\beta$ -mercaptoethanol to each reaction and the blank tubes.
12. Add 0.16 mL of ONPG solution to each tube and record the start time for the reaction.
13. Incubate reactions at 37°C until the yellow color develops. Add 0.4 mL of 1 M Na<sub>2</sub>CO<sub>3</sub> to stop the reaction, and record the elapsed time in minutes for each reaction.
14. Centrifuge reaction tubes for 10 min at 15,600g (14,000 rpm) to pellet cell debris.
15. Calibrate the spectrophotometer with the blank tube at OD<sub>420</sub> and read OD<sub>420</sub> for all the reactions. The linear range of OD value is 0.02–1.0. Make dilutions and reread the OD if necessary.
16. Calculate the  $\beta$ -gal activity using Miller's equation:

$$\beta\text{-gal units} = 1000 \times (\text{OD}_{420}/T \times V \times \text{OD}_{600})$$

In which T = duration of the reaction in minutes, and

V = 0.1 mL  $\times$  concentration factor (5, in this case).

#### 3.4.4.3. ISOLATION OF LEU+TRP- CLONES

His/LacZ double-positive transformants need to be isolated and the prey plasmids sequenced for further characterization. The genetic manipulation

described next was used to isolate transformants that contain only prey plasmids (putative Smad1 interactors).

1. Restreak  $\beta$ -gal-positive clones on selection medium containing SD/+Trp+Leu to segregate the cDNA fusion plasmids.
2. Reassay for LacZ+ ( $\beta$ -gal active) to verify the positive clones.
3. Grow each transformant (Trp+Leu+LacZ+) in 3 mL of liquid SD/Leu- medium, which maintains only the prey plasmid (Leu+), until the culture is saturated (8–10 d) to allow random loss of the pGBT9/Smad1 plasmid.
4. Dilute the liquid culture and plate onto SD/Leu- plates. Incubate the plates at 30°C for 2 to 3 d.
5. Patch 30–50 colonies on the SD/-Leu and SD/-Leu-Trp agar plates in parallel (*see Note 6*).
6. Pick up the colonies that grow on SD/-Leu but not on SD/-Trp-Leu (Trp auxotrophs, presumably, have lost their bait plasmids but maintain the prey plasmid).
7. Assay for LacZ phenotype, and save the ones that are  $\beta$ -gal negative for isolation of pACT2/prey cDNA.

#### 3.4.4.4. ISOLATION OF PLASMIDS FROM Y190/PREY cDNA

1. Inoculate single colonies into 2 mL of YPD medium, and grow overnight at 30°C for 16–24 h.
2. Transfer 1.5 mL of the culture into a microcentrifuge tube and spin at 18,000g to pellet the cells.
3. Add 0.2 mL of yeast lysis solution, mix well, and then add 0.2 mL of phenol/chloroform/isoamyl alcohol.
4. Add 0.3 g of acid-washed glass beads and vortex for 2 min or longer to break the cell wall.
5. Centrifuge at 18,000g for 5 min at room temperature.
6. Transfer the supernatant to a clean Eppendorf tube and add 1/10 vol of 3 M NaAc and 2.5 vol of 95% ethanol to precipitate the DNA.
7. Wash the DNA once with 75% EtOH and dry the DNA at room temperature for 5 min.
8. Resuspend the plasmid DNA in 20  $\mu$ L of TE buffer.

#### 3.4.4.5. AMPLIFICATION OF PREY PLASMID IN BACTERIA AND cDNA ANALYSIS

Because of the low yield of yeast plasmid DNA, the isolated pACT2/prey cDNA needs to be transformed and amplified in bacteria for further characterization.

1. Transform competent bacteria with 1–5  $\mu$ L of isolated prey cDNA plasmids by either electroporation or the chemical method.
2. Grow overnight and isolate plasmid DNA by miniprep from bacterial transformants.

3. Restriction enzyme digest to verify the cDNA insert.
4. Sequence the insert cDNA using the GAL4 AD primer.

#### 3.4.4.6. OTHER METHODS TO VERIFY POSITIVE INTERACTIONS

Once an interaction has been identified by the yeast two-hybrid system, several other methods are available to confirm further the interaction in vitro and in vivo (*see Note 7*), such as these:

1. Pulldown assays to verify the interaction in vitro.
2. Coimmunoprecipitation to verify the interaction in mammalian cells.
3. Functional studies, such as transfection, to verify the biologic relevance of the interaction.

### 3.5. Mapping Interaction Domains of Two Known Proteins

Another main use of the yeast two-hybrid system is to identify interacting domains of two known proteins. We also examined the interaction domains of Smad1 and Hoxc-8 by the yeast two-hybrid technology described next (*see Note 8*).

1. Analyze sequence characteristics of two proteins to locate important structural or functional domains.
2. Make deleted forms (cDNA fragments) of each by polymerase chain reaction.
3. Clone each cDNA fragment into the respective bait or prey vector.
4. Transform Y190/pGBT9/Smad1 with deletions of pACT2/Hoxc-8 or Y190/pACT2/Hoxc-8 with deleted forms of pGBT9/Smad1.
5. Plate on SD/–Trp–Leu–His+3-AT (25 mM) plates and incubate at 30°C for 3–5 d.
6. Assay by filter lift and liquid  $\beta$ -gal to access the strength of each pair of interacting domains.

## 4. Notes

1. Strain Y190 is very leaky for the His3 expression, so 3-AT must be included when growing Y190 transformants on plates with appropriate SD medium. We found that 45 mM 3-AT is required to eliminate background growth. However, 3-AT is omitted when we grow liquid culture and is lowered to 25 mM when we grow clones on plates for the  $\beta$ -gal assays. If desired, optimize the 3-AT concentration by growing Y190/pGBT9 bait transformants on SD/–Trp–His plates containing a range of 25–60 mM. Use the lowest concentration that allows only small (<1 mm in diameter) colonies to form after 1 wk.
2. Y190 carries the *ade2-101* mutation that confers a pink color to colonies and its colonies can grow to >2 mm in diameter. Spontaneous white mutants and smaller colonies may form at a rate of 1 to 2% (we found that the rate is higher). Select pink colonies when inoculating cultures.

3. Smads are signaling mediators and transcription factors, located in both cytoplasm and nucleus, which is convenient for the use of the two-hybrid assay in the yeast. However, some Smads might activate reporter gene transcription when fused to GAL4 DBD; thus, the transactivation activity of Smads must be tested before using them as baits in the interactor hunt. If they activate reporter gene transcription, modification is required to remove the ADs.
4. After two rounds of screening for Smad1 interactor, we have obtained 25 positive clones. DNA sequence analysis found one clone as Hoxc-8 and two clones as Smad4. Smad4 is known to interact with Smad1, which provided a positive control for the system. The interaction between Smad1 and Hoxc-8 was confirmed by a pulldown assay in vitro and a coimmunoprecipitation in COS-1 cells (19). The interaction domains of the two proteins were mapped at the N-terminal domains of Smad1 and the homeodomain of Hoxc-8 (23).
5. Transformation efficiency is critical to ensure that all the possible target interactors are trapped. Generally, an efficiency of  $10^4$  transformants/ $\mu$ g of DNA or greater is acceptable. In our practice, we used a two-step transformation by generating first a bait-carrying strain, and then transformed it with the cDNA library plasmids. We usually obtain an efficiency of  $10^5$  or greater when performing a large-scale transformation. Two-step transformation sometimes can be a problem if the bait protein is toxic or introduces some growth advantage to the yeast host cells. If this is a concern, one can break the large-scale transformation into 5–10 small-scale ones to transform the bait and prey plasmids simultaneously. Small-scale transformation can sometimes give a drastically higher efficiency.
6. Theoretically, 10–20% of the yeast cells will lose their plasmid spontaneously when the selective pressure is absent. Patching 30–50 colonies should give at least a few Trp–Leu+ clones. However, we sometimes have to patch more than 100 colonies to isolate one Trp–Leu+ clone. An alternative is to isolate prey plasmids from yeast and transform into bacteria to isolate Leu+ clones. In this case, the HB101 bacterial strain can be used as the host because the yeast *leu2* gene in pACT2 can complement the *leuB* mutation of HB101.
7. Because the yeast two-hybrid uses the transcriptional activity of reporter genes as a readout, the interaction must take place in the nucleus. For membrane-bound proteins and proteins that normally do not enter the nucleus easily, the yeast two-hybrid system, as presented, may not be suitable. In some cases, a nuclear localization signal can be fused in front of the bait gene or the transmembrane domain removed to assist the target proteins in entering the nucleus.
8. The production of  $\beta$ -gal can be used to assess the binding affinity of the bait and the prey proteins. However, the  $\beta$ -gal activity may not necessarily correlate with the in vivo strength of interaction. We have experienced difficulties when trying to test the interaction of Hoxc-8 with Smad1 deletions. Positive interactions (confirmed by gel-shift assays) only activated *His3* gene transcription but  $\beta$ -gal activity was only barely detectable by filter lift assays. The fusion proteins appear to be expressed at levels ranging from 50 nM to 1  $\mu$ M (24). At this concentration,



it should be possible to detect very low-affinity interactions, but when compared with other methods, the two-hybrid sometimes seems to be more sensitive and sometimes less. It is speculated that the folding of fusion bait or prey proteins can be somewhat distorted, causing steric hindrance to interfere with the interaction (24).

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## Protein Interactions Important in Eukaryotic Translation Initiation

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### 1. Introduction

The process of translation initiation involves the formation of a ribosomal initiation complex in which the anticodon of methionyl initiator tRNA (Met-tRNA<sub>i</sub>) is base paired with the start codon of the mRNA. In eukaryotes, AUG is selected with high stringency as the start codon, and in most cases, the AUG triplet closest to the 5' end is used regardless of the structure of the mRNA. Numerous protein factors called eukaryotic initiation factors (eIFs) interact with the 40S ribosomal subunit to stimulate assembly of this initiation complex. A large multisubunit factor called eIF3 binds to the free 40S subunit and impedes association with the 60S ribosomal subunit. The Met-tRNA<sub>i</sub> is delivered to the 40S subunit in a ternary complex consisting of eIF2 (a heterotrimeric factor) bound to a molecule of guanosine 5'-triphosphate (GTP). The binding of ternary complex to the 40S subunit is promoted by eIF3 and a single-subunit factor known as eIF1A. The resulting 40S preinitiation complex interacts with an mRNA molecule containing eIF4F bound to the 5' cap structure, in a reaction that also is stimulated by eIF3. It is thought that interaction between eIF3 and eIF4F directs the 40S preinitiation complex to the 5' end of the mRNA, after which it migrates downstream to the start site in a process known as scanning. The RNA helicase activity of eIF4F may remove secondary structure from the mRNA and thereby facilitate the recognition of AUG triplets by the anticodon of Met-tRNA<sub>i</sub>. The correct codon-anticodon pairing stimulates hydrolysis of the GTP bound to eIF2 in a reaction that depends on the GTPase-activating protein (GAP) eIF5 and is also modulated by eIF1. The GTP hydrolysis triggers the release of eIF2 and eIF3 from the ribosome, producing

a 40S initiation complex that can join with the 60S subunit. The resulting 80S initiation complex begins synthesizing a polypeptide chain with the methionine linked to initiator tRNA. Because only the GTP-bound form of eIF2 can bind Met-tRNA<sub>i</sub>, the guanosine 5'-diphosphate (GDP) bound to eIF2 at the end of this process must be replaced by GTP. This nucleotide exchange reaction is dependent on the heteropentameric factor eIF2B (for a review *see* **ref. 1**).

eIFs are highly conserved in sequence from yeast to mammals (**1**). They were initially characterized biochemically in mammalian cell extracts, and many of the corresponding structural genes have been cloned using the results of peptide sequencing or specific antibodies to screen cDNA libraries. On the other hand, many of the eIFs from the yeast *Saccharomyces cerevisiae* were first identified and cloned by genetic approaches and found to be similar in sequence to the corresponding mammalian factors. The recent completion of the *S. cerevisiae* genome sequence revealed additional homologs of mammalian eIFs that had not been identified genetically. *S. cerevisiae* appears to contain homologs of 25 of the 29 known polypeptides that comprise mammalian eIFs, lacking only 4 of the 10 subunits of mammalian eIF3 (**2,3**). Thus, in-depth analysis of the yeast eIFs, which is greatly facilitated by the powerful genetics developed for this organism, should be highly relevant to the mammalian system.

In this chapter, we summarize the use of two-hybrid analysis and in vitro protein-binding assays to detect protein interactions among the yeast eIFs involved in Met-tRNA<sub>i</sub> binding and stringent AUG selection. The results of these studies suggest that eIF1, -2, -3, and -5 are physically linked by numerous interactions, which raises the possibility that these eIFs interact with the 40S ribosome as a preformed multifactor complex.

## 2. Materials

### 2.1. Yeast eIFs Analyzed for Two-Hybrid Interactions

The yeast eIF subunits that were analyzed for two-hybrid interactions are given in **Table 1**. Their known or predicted functions and the corresponding mammalian subunit designations are also described. Yeast eIF1 and eIF5 and all three subunits of eIF2 were previously implicated in AUG recognition by the isolation of mutations with Sui<sup>-</sup> phenotypes (for *suppression of initiation codon mutations*). Sui<sup>-</sup> mutations allow translation to initiate from a UUG codon on *HIS4* mRNA and, thus, impair the stringent selection of AUG as a start codon (**4–6**). Hence, it was interesting to determine whether any of the individual subunits of eIF2 could physically interact with eIF1 or eIF5. Yeast eIF3 was less well characterized than these other factors (**7,8**); however, sequence comparisons of cloned mammalian eIF3 subunits with the yeast

**Table 1**  
**Two-Hybrid Assay for Pairwise Interaction Between Yeast eIFs**

Factor	Function	DBD fusion		AD fusion									
		Mammalian homolog	Yeast gene	<i>SUI2</i>	<i>GCD11</i>	<i>SUI3</i>	<i>TIF5</i>	<i>SUI1</i>	<i>TIF32</i>	<i>NIP1</i>	<i>PRT1</i>	<i>TIF34</i>	<i>TIF35</i>
eIF2	Binds Met-tRNA <sub>i</sub> to ribosomes; AUG selection; binds GTP	eIF2 $\alpha$	<i>SUI2</i>	—	Blue	—	—	—	—	—	—	—	—
		eIF2 $\gamma$	<i>GCD11</i>	>30	—	>30	—	—	—	—	—	—	—
		eIF2 $\beta$	<i>SUI3</i>	10	>30	—	—	—	—	—	—	—	—
eIF5	GAP for eIF2	eIF5	<i>TIF5</i>	—	—	20	—	—	—	>30	—	—	—
eIF1	GAP modulator?	eIF1	<i>SUI1</i>	—	—	—	—	—	—	>30	—	—	—
eIF3	Promotes Met-tRNA <sub>i</sub> and mRNA binding to ribosomes; scaffold for other eIFs?	eIF3-p170	<i>TIF32</i>	—	—	—	—	—	—	—	—	—	—
		eIF3-p110	<i>NIP1</i>	—	—	—	>30	15	—	—	—	—	—
		eIF3-p116	<i>PRT1</i>	—	—	—	—	—	—	—	—	—	—
		eIF3-p36	<i>TIF34</i>	—	—	—	—	—	—	—	5	—	Blue
		eIF3-p44	<i>TIF35</i>	—	—	—	—	—	—	—	10	Blue	—

"Strain Y187 transformants bearing pGAD424 derivatives were mated with transformants of strain Y190 bearing pGBT9 derivatives, and the resulting diploid clones were replica plated to SC–His–Trp–Leu medium with different concentrations of 3-AT. The first two columns describe eukaryotic translation initiation factors analyzed in this chapter. The third and fourth columns list the mammalian subunits and their yeast counterparts. The remaining columns show the extent of interaction between the indicated AD fusions listed across the top and the DBD fusions listed in column 4, indicated by the maximum 3-AT concentration (millimolar) that allowed diploids bearing the relevant combination of two-hybrid constructs to form a confluent patch within 6 d of incubation at 30°C.—, no detectable growth at the lowest 3-AT concentration after 6 d; Blue, strongest interaction, turning blue on X-gal and conferring growth at 30 mM 3-AT. Brackets indicate classification of positive interactions into three groups.

genome sequence revealed five probable yeast homologs of mammalian eIF3 subunits (2,3,9–11) (Table 1). Among these five, only PRT1 and TIF34 were known to be components of yeast eIF3 (8,12). Accordingly, we wished to determine whether PRT1 or TIF34 could interact with any of the other putative yeast eIF3 subunits. Because yeast eIF3 copurified with eIF1/SUI1 (13) and eIF5 (14), we suspected that eIF3 might be involved in recruiting these factors to the 40S ribosome. If so, we might detect interactions between a particular subunit of eIF3 and eIF1 or eIF5. Finally, considering that mammalian eIF3 promotes ternary complex binding to 40S subunits, we might observe interactions between subunits of eIF2 and eIF3.

## 2.2. Two-Hybrid Constructs and Yeast Strains

### 2.2.1. Plasmids

To analyze interactions between subunits of eIF1, -2, -3, and -5, we subcloned DNA fragments encoding each of the known or predicted subunits into the two-hybrid expression vectors pGBT9 (*TRP1*) and pGAD424 (*LEU2*) (15), fusing the yeast proteins to the GAL4 DNA-binding domain (DBD) and activation domains (AD), respectively (see Subheading 3.1.).

### 2.2.2. Yeast Strains

Y190 ( $\alpha$  *leu2 trp1GAL-lacZ GAL-HIS3*) and Y187 (*a leu2 trp1GAL-lacZ*) (16) were transformed with the resulting pGBT9- and pGAD424-derived plasmids, respectively. We mated the transformants of Y190 ( $\text{Trp}^+$ ) and Y187 ( $\text{Leu}^+$ ) as described in Fig. 1 to produce  $\text{Trp}^+ \text{Leu}^+$  diploid strains bearing all pairwise combinations of the pGBT9 and pGAD424 constructs (method modified from ref. 17). The Y190/Y187 diploids contain two reporter genes, *GAL-HIS3* and *GAL-lacZ*, which allowed us to judge the strength of the two-hybrid interactions by two independent assays: the growth of cells on medium containing 3-amino-1,2,4-triazole (3-AT), a sensitive indicator of *HIS3* expression (18), and the amount of  $\beta$ -galactosidase activity expressed from the *GAL-lacZ* fusion (19). Thus, each diploid strain was replica plated to medium containing different concentrations of 3-AT (see Subheading 3.2.) and to a nitrocellulose membrane that was subsequently permeated with 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal), a chromogenic substrate for  $\beta$ -galactosidase (see Subheading 3.3.). It was important to test expression from both reporters by these assays to confirm the authenticity of the interactions we observed (see Note 1).

As summarized in Table 1, we detected three groups of interactions, one involving the three subunits of eIF2, the second involving eIF1, eIF2 $\beta$ , eIF5 and eIF3-NIP1, and the third involving the eIF3 subunits PRT1, TIF34, and TIF35 (see Note 2).

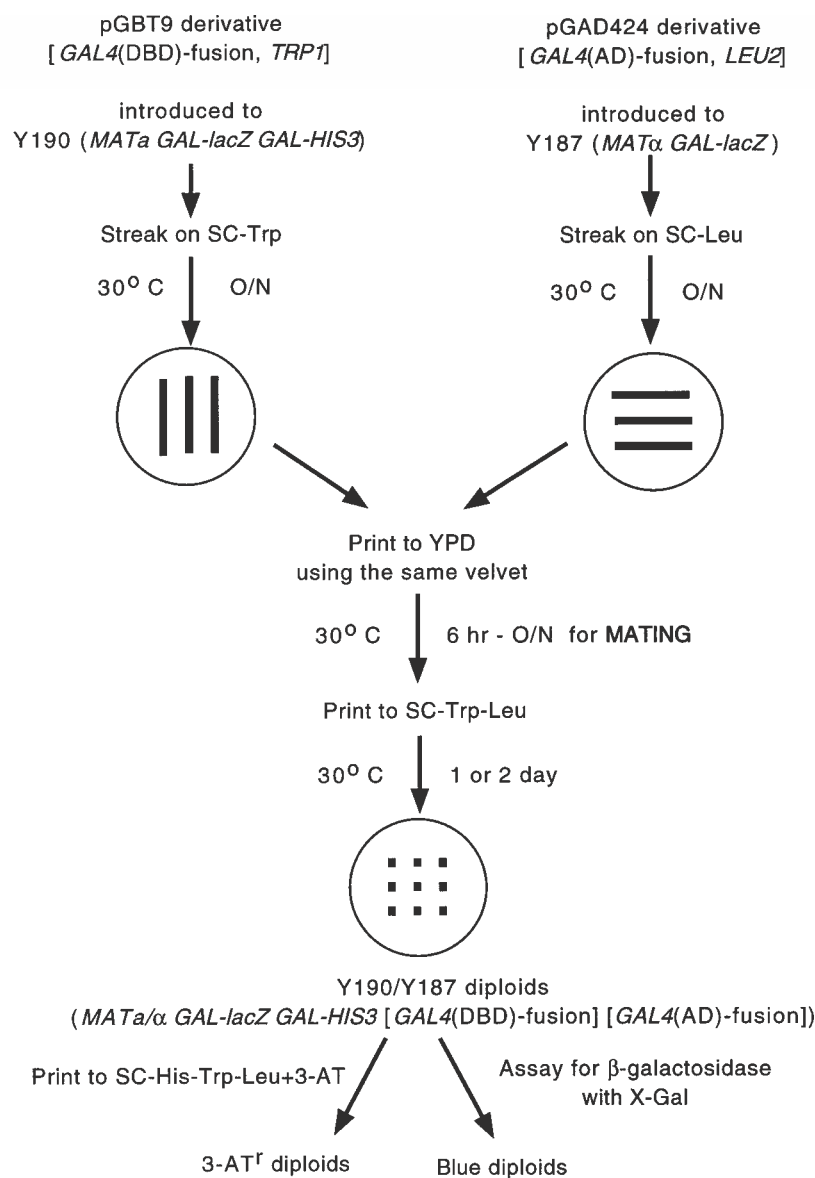


Fig. 1. Formation of diploid yeast strains containing all pairwise combinations of GAL4 DBD and AD fusions. Y190 (**left**) or Y187 (**right**) transformants bearing pGBT9 or pGAD424 derivatives were inoculated in parallel lines on SC-Trp and SC-Leu plates, respectively. After incubation overnight at 30°C, both plates were replica plated at 90° angles to a single velvet and then plated to rich medium (YPD). After incubation overnight at 30°C, during which mating occurs at the cross-sections between the transformants of opposite mating type, the mating plate was replica plated to SC-Trp-Leu. Only the diploids grew on this medium and were subsequently tested for growth on SC-Trp-Leu-His plates containing 3-AT and for β-galactosidase activity with X-gal as substrate (see text for further details).

### 2.3. Other Reagents

1. Z buffer: 16.1 g/L of  $\text{Na}_2\text{HPO}_4$ , 5.5 g/L of  $\text{NaH}_2\text{PO}_4$ , 0.75 g/L of KCl, and 0.246 g/L of  $\text{MgSO}_4$ . Adjust the pH to 7.0. At the time of use add  $\beta$ -mercaptoethanol at 2.7 ml/L.
2. Medium: Synthetic defined medium and plates lacking leucine and tryptophan ( $\text{LT}^-$ ) as well as lacking leucine, tryptophan, and histidine ( $\text{LTH}^-$ ): 6.7 g of Difco yeast nitrogen base without amino acids,  $\text{H}_2\text{O}$  to 850 mL (for plates add 20 g of agar). Autoclave and cool to  $<65^\circ\text{C}$ , and then add 100 mL of appropriate 10X dropout solution ( $\text{LT}^-$  or  $\text{LTH}^-$ ) and 50 mL of 40% D-glucose.
3. Nitrocellulose filters: 45  $\mu\text{m}$  (BA85; Schleicher & Schuell, Keene, NH).
4. 3-AT: Prepare a 2.5 M stock solution by dissolving 21 g of 3-AT in 100 mL of water, and sterilize by filtration.
5. X-gal: Prepare a concentrated stock by dissolving X-gal in *N,N*-dimethyl-formamide at a concentration of 20 mg/mL. Store at  $-20^\circ\text{C}$ .

## 3. Methods

### 3.1. Construction of Two-Hybrid Plasmids Encoding eIFs or eIF Subunits

DNA fragments encoding the proteins listed in **Table 1** were synthesized by polymerase chain reaction (PCR) using oligonucleotide primers that introduced restriction enzyme sites at both ends of the fragments (5'-*Bam*HI and 3'-*Pst*I sites for *GCD11* and *SUI2*; refer to **refs. 14, 20, and 21** for the other genes), and the resulting fragments were subcloned into pGAD424 or pGBT9 (**15**).

### 3.2. Simple Assay for Expression Levels from GAL-HIS3

The collection of Y190/Y187 yeast hybrids containing all combinations of pGAD424 and pGBT9 constructs were grown in patches on synthetic complete medium (**22**) lacking leucine and tryptophan (SC-Leu-Trp), incubated overnight at  $30^\circ\text{C}$ , and replica-plated to the same medium also lacking histidine (SC-Leu-Trp-His) and supplemented with 3-AT at 5, 10, 15, 20, 25, and 30 mM. The strength of each interaction was scored qualitatively by the density of cell growth observed at  $30^\circ\text{C}$  for up to 6 d after plating. Stronger two-hybrid interactions allowed cells to produce more *HIS3* product from the *GAL* promoter and, hence, to grow on medium containing higher concentrations of 3-AT (*see Note 3*).

### 3.3. Plate Assay for Measuring GAL-lacZ Expression

We assayed  $\beta$ -galactosidase activity in cells transferred to a filter as described previously (**19**) with a slight modification (**23**).

1. Permeabilize yeast colonies or patches printed to a 45- $\mu\text{m}$  nitrocellulose filter (BA85; Schleicher & Schuell) by placing the filter on a sheet of aluminum foil floating on the surface of liquid nitrogen for 5–10 s.

2. Placed the filter cell side up on a Whatman filter paper presoaked with 3 mL of Z buffer (24) containing 1 mg/mL of X-gal.
3. Incubate the filters at 30°C for 3 to 4 h for appearance of the blue product of X-gal cleavage by  $\beta$ -galactosidase.

### **3.4. Identification of a WD40 Protein, TIF34, as a Core Subunit of eIF3**

The three proteins involved in group 3 interactions, including the two known eIF3 subunits TIF34 and PRT1, plus TIF35, all contain interesting sequence motifs. TIF35 and PRT1 contain the RNA recognition motifs (RRMs) (25), as shown schematically in **Fig. 2B,C**, and TIF34 contains seven repeats of the WD40 motif (9,20,26). Since WD40 proteins are expected to fold into a circular  $\beta$ -propeller structure and interact with multiple proteins (27), we hypothesized that TIF34 is a core subunit of eIF3 subunit. To test this possibility, and to identify the TIF34-binding domains present in other eIF3 subunit homologs, we constructed libraries of random fragments encoding about 100 amino acids of the possible eIF3 proteins and conducted two-hybrid screenings with full-length TIF34 as the bait (see **Fig. 2A** and **Subheadings 3.5 and 3.6**).

As shown in **Fig. 2B,C**, we identified fragments containing ~100 C-terminal amino acids of PRT1 and ~150 N-terminal amino acids of TIF35 as binding partners of TIF34. We confirmed that these interactions are direct by in vitro binding studies between TIF34 synthesized in vitro and glutathione-S-transferase (GST) fusions to selected PRT1 and TIF35 segments (20). These findings were consistent with the idea that the WD40 subunit TIF34 provides a scaffold for two RRM-containing subunits, as illustrated in **Fig. 2D**. Interestingly, we identified identical C-terminal fragments of PRT1 when the same random fragment libraries described in **Subheading 3.5** were screened with full-length TIF35 as the bait (**Fig. 2B**). However, GST-fusions to the C-terminal segments of PRT1 failed to bind recombinant TIF35 in vitro (20). Thus, the two-hybrid interaction between TIF35 and the C-terminus of PRT1 was probably bridged by the native TIF34 present in yeast cells.

We observed no two-hybrid interactions among TIF34, TIF35, and PRT1 with full-length TIF32 or NIP1, or with random fragments of the latter two proteins. However, GST-TIF32 bound to full-length NIP1 and PRT1, both synthesized in vitro, and to a truncated PRT1 polypeptide lacking the C-terminal TIF34-binding domain (20). Thus, we proposed that TIF32 bridges the interaction between NIP1 and the N-terminal region of PRT1.

Independent evidence that TIF34 and TIF35 interact in vivo was provided by the finding that point mutations in *TIF34* with temperature-sensitive phenotypes weaken the interaction between TIF34 and a GST-TIF35 fusion in vitro; moreover, the Ts<sup>-</sup> phenotypes of *tif34* mutations could be suppressed by



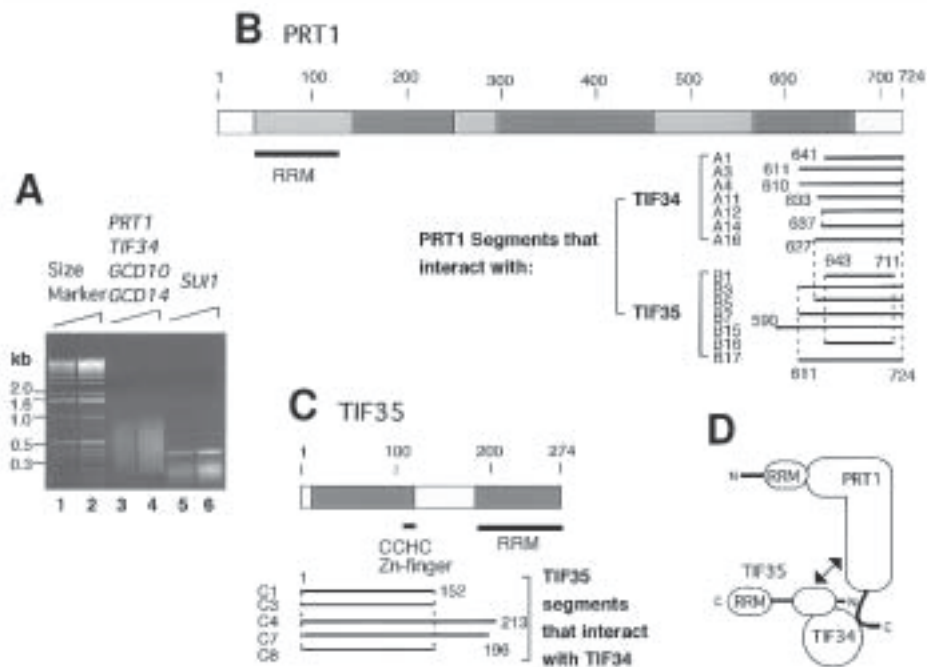


Fig. 2. Segments of PRT1 and TIF35 that interact with TIF34 or TIF35, the WD40 and RRM-containing subunits of yeast eIF3. (A) Agarose gel electrophoretic pattern of DNA fragments used to construct library 1. Two and one-half (lanes 3 and 5) or 5  $\mu$ L (lanes 4 and 6) of DNA fragments from the genes listed across the top, prepared as described under **Subheading 3.5.**, were resolved by 1.2% agarose gel electrophoresis, along with 2.5 or 5  $\mu$ L of size standards (1-kb ladder [Gibco-BRL]; lane 1 or 2, respectively). The sizes of DNA fragments in kilobases are shown on the left. (B) Two-hybrid interactions detected between random segments of PRT1 from library 1 and full-length TIF34 or TIF35. At the top is shown a schematic of the 724 amino acid PRT1 polypeptide, shaded to indicate its sequence similarities to human eIF3-p116 and the putative *Schizosaccharomyces pombe* homologs (dark gray boxes, regions of 21–34% identity; light gray boxes, regions of 9–11% identity; unshaded boxes, regions of 0–4% identity). Segments of PRT1 that interacted with full-length TIF34 or TIF35 are listed below the schematic along with clone designations and locations in the sequence. Thick bar indicates the location of the RNA recognition motif (RRM). (C) Interactions detected between random segments of TIF35 in library 2 and full-length TIF34. The 274 amino acid TIF35 polypeptide is shown schematically, shaded as in (B), to indicate sequence similarities to mouse eIF3-p44 and the putative *Caenorhabditis elegans* homolog. The locations of a zinc-finger motif (CCHC zinc finger) and RRM are shown below, and beneath them are depicted TIF35 segments that interacted with TIF34 as in (B). (D) Model of complex formation by TIF34, PRT1, and TIF35. TIF34 is depicted as a circle representing a  $\beta$ -propeller structure, by analogy with the crystal structure of  $\beta$ -transducin (27). The evolutionarily conserved domains of TIF35 and PRT1 are shown as ovals and the nonconserved regions as thick lines. The arrow indicates the direct interaction between the N-terminal half of TIF35 and the C-terminal region of PRT1 (longer than the TIF34-binding domain) that was detected by in vitro binding studies (20).

overexpressing TIF35 (20,28). In addition, PRT1, NIP1, and TIF35 specifically coimmunoprecipitated with TIF34 tagged with the hemagglutinin epitope in cell extracts and a ribosomal salt wash fraction (14,20). Likewise, myc-tagged TIF34 coimmunoprecipitated with MRSG-tagged TIF35 (28). Finally, a complex containing polyhistidine-tagged PRT1 was purified with nickel-affinity resin and gel filtration chromatography and shown to contain TIF32, TIF34, TIF35, and NIP1. This purified complex could rescue mRNA translation and Met-tRNA<sub>i</sub> binding to 40S ribosomes in a heat-treated extract from the Ts<sup>-</sup> *prt1-1* mutant and, thus, harbored an expected biochemical activity of eIF3 (14). Together, these experiments provided firm evidence that the five yeast homologs of human eIF3 subunits comprise a conserved eIF3 complex.

### **3.5. Construction of Libraries of Plasmids Encoding Segments of Possible eIF3 Subunits**

The libraries of DNA fragments encoding random segments of ~100 to 150 amino acids of the putative eIF3 subunits TIF32, NIP1, PRT1, TIF34, TIF35, GCD10, GCD14, and SUI1 were constructed according to Bartel et al. (29), except that we used PCR-amplified fragments containing the full-length genes as starting materials. The last three proteins were included because SUI1 (13) and GCD10 (30) copurified with eIF3 activity, and GCD14 showed genetic links to GCD10 (31) (see Note 4). We subdivided these genes into the following three groups to increase the likelihood that the libraries would contain DNA segments representative of all the genes: (1) *PRT1*, *TIF34*, *GCD10*, and *GCD14*; (2) *TIF32*, *NIP1*, and *TIF35*; and (3) *SUI1*. *SUI1* was separated from the others because, at ~300 bp, it is smaller than the expected size (~0.5 kb) of the sheared DNA fragments used for constructing the other libraries. Libraries containing fragments from each group were constructed according to the following procedure (see Note 5). Library 1 consists of a 20:1 mixture of group 1 and 3 libraries. Library 2 derived from group 2 genes.

1. PCR amplify the DNA fragments containing the complete open reading frames (ORFs) of the relevant genes using oligonucleotide primers corresponding to the 5' and 3' ends of the ORFs. We recommend the use of high-fidelity DNA polymerases, minimal amplification (e.g., 25 cycles), and plasmid DNAs bearing cloned genes rather than genomic DNA as the templates.
2. Combine the amplified DNA fragments in equimolar amounts to a final DNA concentration of 20 µg/mL in sterilized water.
3. Transfer 1 mL of DNA mixture to a Falcon 2059 tube and sonicate with a Type 550 Sonic Dismembrator (Fisher), or an equivalent instrument, holding the tube on ice throughout. Sonicate for 2-min pulses with 30 s of cooling between pulses. Analyze 10 µL after every 10 min of sonication by agarose gel electrophoresis. About 30–40 min of sonication was required to shear DNA to fragments with a

mass average length of ~0.5 kb, and 150 min was required for an average length of 0.15 kb.

4. Phenol extract the sheared DNA mixture, ethanol precipitate, and resuspend in 100  $\mu$ L of sterilized water. Remove small DNA fragments or nucleotides carried over from the PCR using a spin-column separation (e.g., Centri-Sep, Princeton Separations, and MicroSpin<sup>TM</sup> G-50; Pharmacia).
5. End fill the DNAs from **step 4** with T4 DNA polymerase at 37°C for 1 h. Purify the DNA with a spin-column separation as in **step 4**.
6. Phosphorylate the ends of the sheared, end-filled DNA fragments with T4 polynucleotide kinase at 37°C for 30 min.
7. Resolve the DNA fragments on a preparative low-melting-temperature 1.2% agarose gel (e.g., SeaPlaque<sup>®</sup> agarose; FMC BioProducts). Excise the gel slice containing DNA fragments between 0.3 and 0.8 kb in length (or between 0.1 and 0.3 kb for fragments of ~an average length of ~0.15 kb). Dissolve the gel pieces in 2 to 3 gel vol of TE (**32**) at 65°C for 10 min, followed by two phenol extractions and ethanol precipitation. Resuspend the purified DNA in 100  $\mu$ L of sterilized water. **Figure 2A** shows the gel electrophoretic pattern of the final purified DNAs.
8. Linearize pGAD424 DNA with *Sma*I and treat with alkaline phosphatase. Optimize the ligation condition by ligating a fixed amount of the purified DNA fragments to different amounts of linearized pGAD424 DNA with T4 DNA ligase, transforming an *Escherichia coli* strain with the ligation mixtures by electroporation, and checking for the presence of inserts in 10–20 independent Ap<sup>r</sup> colonies from each transformation.
9. Repeat the ligation reaction that yielded more than 30% of the transformants containing inserts with the expected sizes, and obtain >10<sup>4</sup> colonies of independent transformants. Collect these colonies with a sterile spatula, and prepare plasmid DNA from the transformants en masse.

### **3.6. Screening of Libraries of Plasmids Encoding Segments of Possible eIF3 Subunits**

1. Transform Y190 with the DBD fusion of interest. Check that the resulting transformants do not grow on SC–Leu–Trp–His medium containing 30 mM 3-AT to ensure that the fusion lacks transcriptional activation function on its own.
2. Transform the Y190 transformant with the library DNA. Plate one-tenth of the competent cell-DNA mixture on SC–Leu–Trp, to quantify the efficiency of transformation, and the remainder on SC–Leu–Trp–His medium containing 30 mM 3-AT. Incubate the plates at 30°C.
3. On d 2, count the number of colonies on the SC–Leu–Trp plate. This number multiplied by 10 should be several times larger than the number of independent clones in the library (the number of *E. coli* transformants used for preparing the library DNA at **step 9, subheading 3.5**). Repeat **step 2** if necessary to obtain the recommended number of yeast transformants.

4. On d 5, pick the transformants that grew on the SC–Leu–Trp–His plates containing 3-AT and streak to isolate single colonies from each. Check the expression from *GAL-lacZ* and *GAL-HIS3* as described under **Subheadings 3.2** and **3.3**. Eliminate any transformants that appeared on the 3-AT plates within 2 d but did not turn blue in the X-gal  $\beta$ -galactosidase assay, because these are most likely false positives.
5. Rescue plasmid DNA from the purified transformants of interest as described (**33**). We use a simplified buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulfate) for the resuspension of yeast cells.
6. Transform *E. coli* strain MC1065 ( $\Delta lacX74 \Delta [ara, leu] galU galK strAr hsdR-trpC9830 leuB6$ ) (**34**) with the DNA prepared in **step 5**. Plate the competent cells on M9 medium (**24**) supplemented with 400  $\mu$ M tryptophan, selecting for the Leu<sup>+</sup> transformants containing pGAD424 (*LEU2*) derivatives.
7. Prepare pGAD424-derivative DNAs from the MC1065 transformants.
8. Transform the Y190 transformants containing the DBD fusion plasmid (as in **step 1**) with the plasmid DNAs prepared in **step 7**. Verify that the resulting transformants show the same 3-AT resistance and blue color in the X-gal  $\beta$ -galactosidase assay as the original candidate strains isolated in **step 4**. Discard plasmids that do not recapitulate these phenotypes.
9. Sequence the insert in the purified pGAD424 derivative, prepared in **step 7**.

### **3.7. C-Terminal Third of eIF5, Containing Conserved Bipartite Motif, Is Responsible for Its Interaction with eIF3 and eIF2**

eIF5, the GAP for the Met-tRNA<sub>i</sub> binding factor eIF2, interacted in the two-hybrid assay with both the  $\beta$ -subunit of eIF2 and the NIP1 subunit of eIF3 (**Table 1**, group 2). This finding led us to analyze the binding domain in eIF5 responsible for these interactions (**21**). We made systematic deletions in the eIF5-coding region of the GAL4 DBD fusion plasmid, as shown schematically in **Fig. 3A** and described in **Subheading 3.8**. The resulting plasmids were tested for two hybrid interaction with full-length eIF2 $\beta$  or eIF3-NIP1 fused to the GAL4 AD. As summarized in **Fig. 3A**, we found that the same C-terminal third of eIF5 was both necessary and sufficient for its binding to eIF3-NIP1 and eIF2 $\beta$  in the two-hybrid assay. These results were confirmed by in vitro binding assays showing direct interaction of eIF2 $\beta$  and eIF3-NIP1 synthesized in vitro to a GST fusion bearing the C-terminal third of eIF5 (**21**).

The sequence of this C-terminal segment of eIF5 is conserved among yeast, humans, *C. elegans* and *Zea mays* and contains a bipartite sequence motif also present in the C-terminus of the catalytic ( $\epsilon$ ) subunit of eIF2B (**35**). This motif is composed of two segments (AA-boxes 1 and 2), both rich in acidic and aromatic residues (**Fig. 3B**). Because the eIF5-A9 construct lacked AA-box 2 and had reduced binding to eIF2 $\beta$  and eIF3-NIP1 (**Fig. 3A**), we hypothesized that the bipartite motif in eIF5 is critical for these interactions. Consistent with this idea, multiple alanine-substitutions altering all the conserved residues in AA-

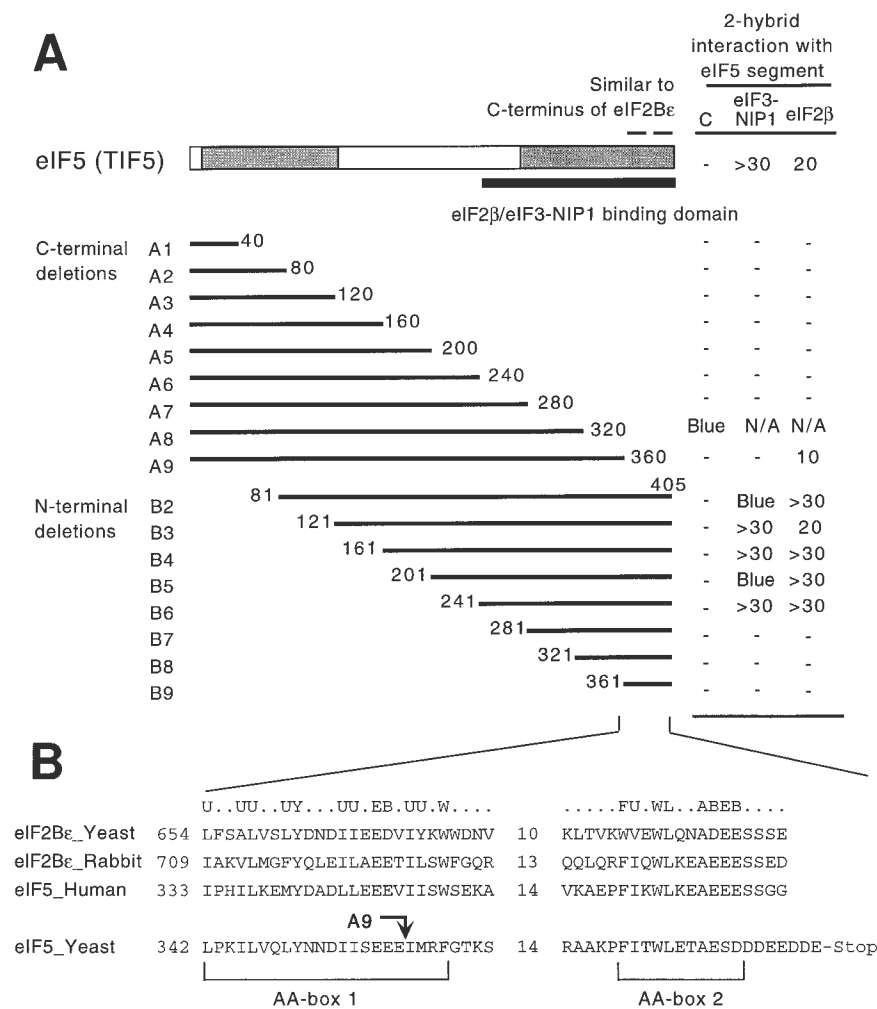


Fig. 3. Mapping the binding domain for eIF2β and eIF3-NIP1 in yeast eIF5. (A) The schematic at the top represents the amino acid sequence of eIF5 from *S. cerevisiae*. Shaded regions are conserved with eIF5 from higher eukaryotes (see text). Lines below the schematic depict the segments of eIF5 present in the deletion constructs, with amino acid positions at the termini indicated above the lines and clone names indicated on the left. The three columns on the right summarize the two-hybrid interactions of each eIF5 segment with eIF2β or eIF3-NIP1, scored as in Table 1; column C gives the results with the empty pGAD424 vector. N/A, not applicable because the eIF5-A8 fusion alone activated *GAL-HIS3* transcription. (B) The alignment of amino acid sequences of eIF5 and eIF2Bε from mammals and yeast in the conserved C-termini (35). The number of nonconserved residues between the conserved AA boxes are indicated. The symbols for conserved amino acids are shown at the top; besides the one-letter code for amino acids, U indicates hydrophobic amino acid, and B indicates glutamate or aspartate. The bent arrow above the yeast eIF5 sequence indicates the end point of the deletion in eIF5-A9 shown in (A).

box 1 or 2 of GST-eIF5 reduced its binding to eIF2 $\beta$  and eIF3-NIP1 in vitro to the background levels seen with GST alone (21).

We showed that the AA-box 1 mutation in eIF5 is lethal in yeast, whereas the AA-box 2 mutation in eIF5 conferred temperature-sensitive growth. This temperature-sensitive phenotype was partially suppressed by overexpressing all three subunits of eIF2. Furthermore, the native eIF2 and eIF3 complexes coimmunoprecipitated with FLAG-epitope-tagged eIF5 from whole-cell extracts in a manner strongly dependent on AA-box 2. We proposed that the bipartite motif in eIF5 mediates physical interactions with eIF2 and eIF3 in vivo, via eIF2 $\beta$  and NIP1, respectively. At least in the case of eIF2, this interaction with eIF5 appears to be important for translation initiation in vivo (21).

### **3.8. Construction of Deletions in eIF5 and Analysis of Their Interactions with eIF3-NIP1 and eIF2 $\beta$**

Plasmids encoding the truncated eIF5 fragments fused to the GAL4 DBD shown in Fig. 3A were constructed by synthesizing DNA fragments containing the corresponding coding regions by PCR using oligonucleotides that introduced restriction enzyme sites at both ends (5' *Eco*RI and 3' *Bam*HI for C-terminal deletions and 5' *Bam*HI and 3' *Sal*I for N-terminal deletions) and inserting them into pGBT9 (15). The Y190 transformants carrying the resulting plasmids were mated with the Y187 transformants carrying pGAD424, pGAD-NIP1, or pGAD-SUI3 as described in Fig. 1 and were tested for expression from *GAL-HIS3* or *GAL-lacZ* as described under Subheadings 3.2 and 3.3.

### **3.9. Conclusion**

Figure 4 summarizes the protein interactions we detected between yeast eIFs. Two-hybrid and in vitro binding assays, as well as genetic suppression data, support the protein linkage map for the yeast eIF3 complex depicted there (see Subheading 3.4.) (14,20). eIF3 binds through its NIP1 subunit to eIF1/SUI1 and eIF5 (Table 1), both implicated in stringent AUG selection (5,6). Thus, eIF3 may recruit eIF1 and eIF5 to the 40S ribosome and orchestrate their functions during the scanning process to establish pairing of an AUG triplet with the anticodon of Met-tRNA<sub>i</sub>. In addition to eIF3, eIF5 binds to eIF2 $\beta$  (Table 1), and both interactions were localized to its C-terminal one-third containing the bipartite motif, or AA-boxes (Fig. 3) (see Subheading 3.7.). These AA-boxes were shown to be important for interactions of eIF5 with both eIF2 and eIF3 in vivo (21).

The importance of the protein interactions summarized here is supported by analyses of the corresponding mammalian factors. Protein linkage of the mammalian eIF3 subunits has yet to be reported, but it was shown that eIF3-p116 (the PRT1 homolog) interacts directly with eIF3-p170 (the TIF32 homolog) by

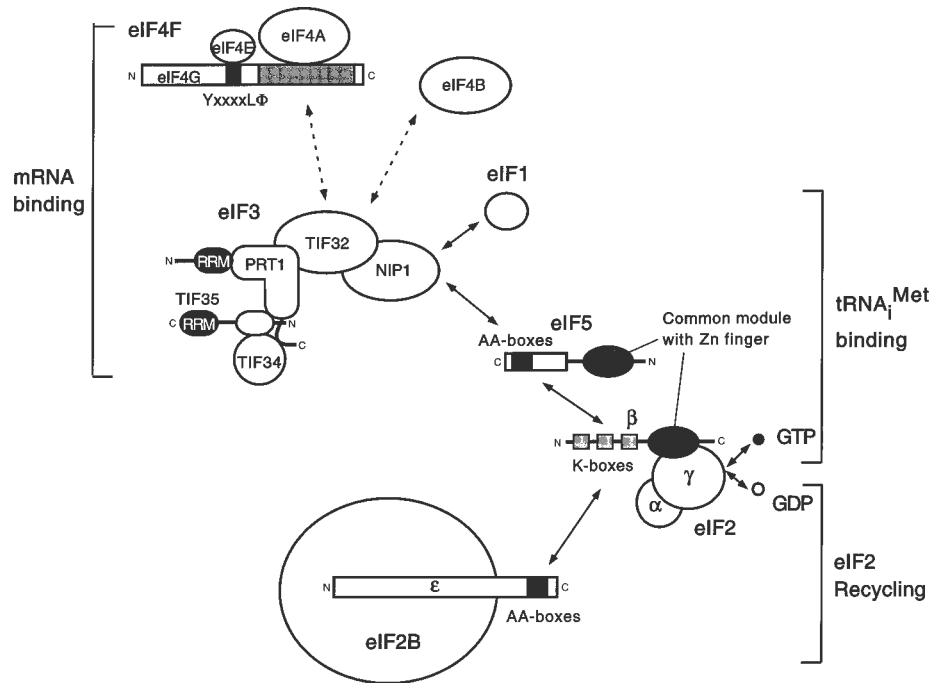


Fig. 4. Protein interactions between yeast eIFs. Circles and boxes indicate the subunits of *S. cerevisiae* eIFs, except that the largest circle labeled eIF2B depicts the four other subunits of this complex besides the  $\epsilon$  subunit, shown as a rectangle. Small solid and empty circles denote GTP and GDP, respectively. Solid arrows indicate direct interactions. The dotted arrows indicate an interaction suggested by results on the corresponding mammalian factors. The modular structures of PRT1, TIF35, eIF5, eIF2 $\beta$ , eIF2B $\epsilon$ , and eIF4G are described with their NH<sub>2</sub> or COOH-termini indicated as N or C, respectively; solid circles represent conserved modules or segments; solid boxes indicate conserved motifs; gray boxes indicate the K-boxes of eIF2 $\beta$  or the eIF3- and eIF4A-binding domain in eIF4G (see text for further details). The YxxxxL $\Phi$  motif, conserved in eIF4G from yeast to humans, is responsible for its binding to the cognate eIF4E cap-binding subunit (46).

far-Western analysis (10). Also conserved in mammalian systems are the interactions between eIF1 and eIF3-p110 (the NIP1 homolog) (36) and between eIF5 and the K-boxes of eIF2 $\beta$  (37). Because mammalian eIF5 appears to bind to the cognate eIF3 complex, this interaction may also be mediated through the mammalian NIP1 homolog (38).

Because eIF5 is believed to function at the 60S subunit joining step, the eIF3-eIF5 interaction that presumably facilitates recruitment of eIF5 to the initiation complex may be superseded by the eIF2-eIF5 interaction necessary for



stimulation of GTP hydrolysis by eIF2 on AUG recognition. Alternatively, eIF5 may be capable of binding simultaneously to eIF2 $\beta$  and eIF3-NIP1 and play an important role in anchoring the ternary complex and eIF3 to the 40S ribosome (**Fig. 4**). eIF3 is crucial for binding mRNA as well as the ternary complex to the 40S subunit, through interactions between eIF3 and the mRNA binding factors eIF4G (**39**) and eIF4B (**40**) (**Fig. 4**). Although these latter interactions have been observed in the mammalian system, they have not been demonstrated in yeast. In any case, eIF5 might assist in juxtaposing the ternary complex and an eIF3-eIF4F-mRNA complex on the 40S subunit in a manner critical for accurate scanning.

AA-boxes are also present in the catalytic ( $\epsilon$ ) subunit of the exchange factor eIF2B and were shown to be important for in vivo interaction between eIF2 and eIF2B and, most likely, recycling of eIF2-GDP to eIF2-GTP (**21**). eIF5 and eIF2B $\epsilon$  bind to the same segment of eIF2 $\beta$  containing three stretches of seven lysines (K-boxes). In this case, the eIF2-eIF5 and eIF2-eIF2B inter-action appears to be mutually exclusive (**21**), as expected from the fact that eIF5 and eIF2B have opposing effects on the guanine nucleotide status of eIF2. Presumably the interaction between eIF2 and eIF5 is restricted to the active GTP-bound form of eIF2 in the ternary complex that binds to the 40S subunit and scans the mRNA, whereas eIF2B replaces eIF5 when eIF2 is released from the ribosome in the inactive GDP-bound state and must be recycled to eIF2-GTP (**Fig. 4**).

Interestingly, the C- and N-terminal halves of eIF2 $\beta$  and eIF5, outside of the K-box- and AA-box-containing domains, respectively, are similar to one another and contain a typical zinc finger (solid circles in **Fig. 4**). The observed interaction between eIF5 and eIF2 $\beta$  (**Table 1** and **Fig. 3**) raises the interesting possibility that these proteins can form a heterodimer. Because mutations in eIF2 $\beta$  can deregulate the GTPase activity of eIF2 $\gamma$  (**6**), heterodimer formation between eIF5 and eIF2 $\beta$  may allosterically stimulate GTP hydrolysis in response to correct codon-anticodon pairing. Alternatively, heterodimerization might enable the C-terminal domain of eIF5 to directly contact eIF2 $\gamma$  to carry out its GAP function. This latter possibility is consistent with the fact that the N-terminal domain of eIF5 contains one of the conserved motifs in G-proteins (**41**) corresponding to the phosphate-binding site (**42**) and that an Sui<sup>-</sup> mutation in eIF5 mapping in this motif deregulates GTP hydrolysis by eIF2 (**6**).

#### 4. Notes

1. Interactions that can be detected by the  $\beta$ -galactosidase assay with X-gal are generally strong and correspond to the extent of *GAL-HIS3* expression conferring growth on medium containing >30 mM 3-AT.
2. The group 1 interactions confirmed complex formation by the subunits of eIF2, one of the best characterized eIFs in both yeast and mammals (**1**). The strong interactions of eIF2 $\gamma$  (GCD11) with eIF2 $\alpha$  (SUI2) and eIF2 $\beta$  (SUI3) are in accor-



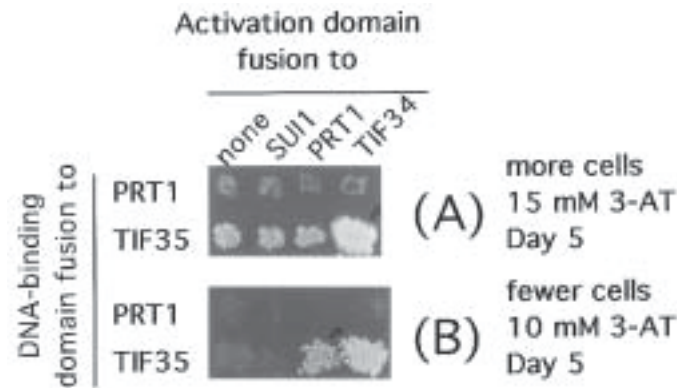


Fig. 5. An example of two-hybrid assays between possible eIF3 subunits. Patches of Y187/Y190 diploid strains carrying the indicated combinations of two-hybrid plasmids were grown on an SC-Trp-Leu plate and replica plated to SC-Trp-Leu-His medium containing 15 or 10 mM 3-AT and incubated for 5 d. The experiment shown in (A) was repeated in (B) with a reduced amount of cells in each patch prior to replica plating.

dance with previous proposals that the  $\gamma$ -subunit, which appears to bind both GTP and Met-tRNA<sub>i</sub>, is the core subunit of eIF2 (6,43), and that the  $\alpha$ - and  $\beta$ -subunits serve to regulate the GTP-binding and GTPase activities of eIF2 $\gamma$  (6,44). Interactions observed among groups 2 and 3 were novel. The group 2 interactions suggested that eIF1 and eIF5 both interact with eIF3 via the *NIP1*-encoded subunit, and that eIF5 additionally interacts with the  $\beta$ -subunit of eIF2. The group 3 interactions suggested that TIF35 interacted with the known eIF3 subunits PRT1 and TIF34. These interactions were characterized further as described in **Subheadings 3.4.–3.8.**

- It is possible that the DBD fusion to the protein of interest activates transcription from *GAL-HIS3* to some extent. As shown in **Fig. 5A**, the DBD fusion to TIF35 activated *GAL-HIS3* and conferred a weak 3-AT<sup>r</sup> phenotype. However, we could overcome this problem by reducing the amount of cells on each print, e.g., by reducing the time of preincubation on the SC-Trp-Leu medium. Thus, in the optimal condition, the TIF35 fusion strongly activated *GAL-HIS3* only when combined with AD fusions to PRT1 or TIF34, as shown in **Fig. 5B**. Strong and weak 3-AT<sup>r</sup> phenotypes also can be distinguished by measuring the sizes of colonies formed from individual cells on 3-AT plates.
- GCD10 and GCD14 were shown recently to be components of a nuclear complex required for the methylation, maturation, and stability of tRNA<sub>i</sub><sup>Met</sup> (45). Since the eIF3 complexes purified by Danaie et al. (7) and Phan et al. (14) do not contain GCD10, more work is needed to elucidate the cytoplasmic role of GCD10 in eIF3 function.

5. Standard DNA manipulations were conducted according to Maniatis et al. (32). We used enzymes, reagents, and kits, as recommended by the manufacturers.

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## Steroid Receptor and Ligand-Dependent Interaction with Coactivator Proteins

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### 1. Introduction

Steroid receptors belong to a large family of transcription factors that regulate hormone-responsive genes and thereby cellular growth and differentiation. In the absence of hormones, the receptor is maintained in an inactive or repressive state by association with heat-shock proteins (hsps) and corepressors. Activation of the aporeceptor by ligand binding involves structural and functional changes in the receptor, which promote release from the inactive or repressive state to bind specific DNA hormone response elements. In addition, the ligand-bound receptor promotes the recruitment of coactivators to the receptor-DNA complex to activate the transcription of hormonally regulated target genes (for a review *see* refs. 1 and 2).

Steroid receptors are modular proteins composed of functional domains that can operate as separate independent entities outside of their natural context and position. This includes separate functional domains for ligand binding located in the carboxy terminus, for DNA binding located centrally, and the amino-terminal domain that is required for maximal transcription function. At least two activation functional (AF) domains have been identified for the steroid/nuclear hormone receptors. These include a ligand-independent AF1 located at the amino-terminus (A/B) domain and the AF2 located at the carboxy terminus (D/E) domain, which is responsible for the ligand-inducible transcription activity of the steroid receptors (1).

Activation of gene expression by steroid receptors is a multistep process that involves functional interactions with several accessory proteins required for proper receptor function. As shown in **Fig. 1**, in the absence of the hor-

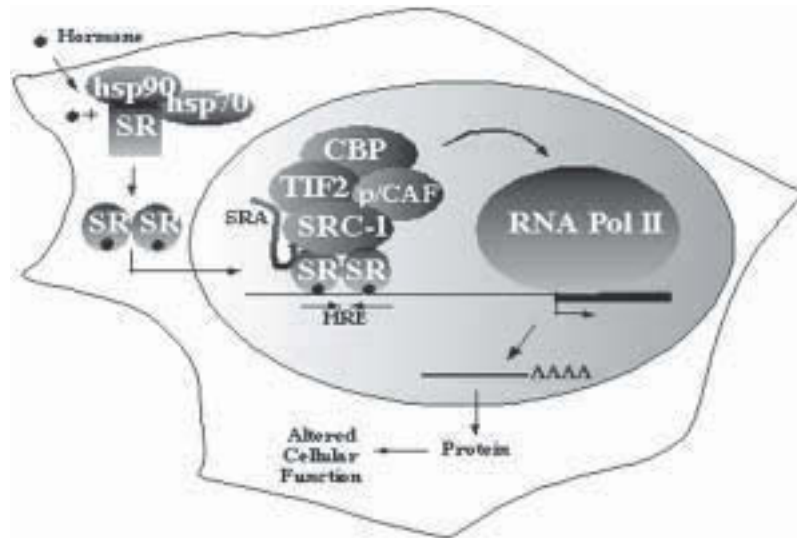


Fig. 1. Model for steroid receptor target gene activation. In the absence of ligand, the steroid receptor is inactive and associated with hsp90 and hsp70. Hormone binding induces an allosteric conformational change in the aporeceptor resulting in the release from the hsp complex, dimerization, and translocation to the nuclear compartment, where the receptor interacts as a dimer with specific DNA sequences called hormone response elements (HREs). In addition, the receptor promotes the recruitment of a multisubunit coactivator complex that may include SRC-1, TIF2, CBP, p/CAF, and the RNA coactivator SRA. The net result of these interactions is the formation of receptor and coactivators complex that activates transcription of target genes and thereby alters cellular function.

mon, the receptor is maintained in an inactive state by association with hsps, including hsp90 and hsp70. Steroid receptors can also repress the expression of target genes by steric interference or by competing with the binding of other transcription factors to specific DNA sequences at the promoter elements of a target gene (3). In addition, proteins interacting with nuclear receptors in the absence of a hormone, namely corepressors, are also actively involved in the repression of target genes (2,4,5). Activation of the receptor by ligand binding involves structural/functional changes in the receptor molecule. The ligand-activated receptor is capable of forming dimers and binding to specific DNA hormone response elements. Furthermore, the receptor promotes the recruitment of a number of ligand-dependent receptor-interacting proteins, namely coactivators that are required for receptors to achieve their full transcriptional



capacity (for a review *see* refs. 2, 5, and 6). Thus, different mechanisms for gene activation and repression by steroid receptors can be visualized depending on the ability of the steroid receptor to recruit different cellular cofactors, either corepressors or coactivators. Therefore, on binding of hormone, many nuclear receptors can be mobilized from a transcription inactive hetero-oligomeric complex containing corepressors, to a receptor complex actively involved in transcription containing the ligand-bound receptor and coactivators.

The yeast two-hybrid system has provided a method for the identification of gene-encoding proteins that interact with steroid receptors under several physiologic conditions, including the identification and characterization of proteins that interact with the nuclear receptors in a hormone-dependent manner. The steroid receptor coactivator-1 (SRC-1) was the first bonafide coactivator for nuclear receptors isolated in a two-hybrid screening using the ligand-binding domain (LBD) of the progesterone receptor (PR) as a bait (7). The physiologic role of SRC-1 in steroid hormone action *in vivo* has been demonstrated by the targeted ablation of SRC-1 gene, which causes partial hormone insensitivity (8,9). Subsequently, several coactivators have been isolated and characterized. Sequence comparisons indicate that SRC-1 belongs to a family of proteins that include the SRC-1 (7,10), the SRC-2/TIF2/GRIP1 (11,12), and the SRC-3/ACTR/RAC3/AIB1/p/CIP (13–16). Several other protein factors that interact with receptors in a ligand-dependent manner have been described, including the general coregulator CREB-binding protein and its homologous p300 (for a review *see* refs. 2, 5, and 17). By fulfilling a number of criteria, these protein factors have been defined as coactivators for nuclear receptors. First, they can enhance the transcriptional activity mediated by the receptor without altering the basal activity of the promoter. Second, they can specifically reverse the squelching observed between different receptors. Third, they contain independent and transferable receptor-interacting and activation domains (ADs) (7).

This chapter describes the two-hybrid technique used to isolate and characterize proteins that interact *in vivo* with steroid receptors in a ligand-dependent manner with properties of a coactivator. The example presented is the cloning and characterization of the SRC-1 isolated by its ability to interact with the PR in a hormone-dependent manner (7). The two-hybrid system is based on the modular properties of the gal4 activator consisting of two distinct domains: the DNA-binding domain (DBD) and the AD. In the steroid receptor two-hybrid system, a hybrid between the yeast GAL4 DBD and the PR LBD was used as a bait protein and the GAL4 AD fused to a cDNA expression library was used as the target. Neither of the hybrid proteins is capable of initiating specific transcription of reporter genes in yeast in the absence of specific interaction with the other hybrid protein. Positive interaction between the library-encoded protein and the bait protein results in the activation of two of the reporter genes, the *leu2* and the *lacZ* encoding genes,



which allows the yeast cells to grow in medium lacking leucine and to develop a blue color in the presence of 5-bromo-4-chloro-3-indoyl- $\beta$ -D-lactopyranoside (X-gal). Thus, it was possible to identify proteins that interact with PR in a ligand-dependent manner with the properties of a coactivator.

## 2. Materials

### 2.1. Reagents and Medium

1. Progesterone and other steroid hormones are obtained from New England Nuclear (Boston, MA) (*see Note 1*).
2. Nitrocellulose filters (0.45- $\mu$ m pore size) are obtained from Schleicher & Schuell (Keene, NH).
3. Medium components: All reagents and materials for the preparation of medium to support growth and maintenance of yeast, including Bacto brand agar, peptone, yeast extract, dextrose, and yeast-nitrogen base without amino acids can be obtained from Difco (Detroit, MI). Amino acid supplement mixtures (complete and dropout mixtures) are obtained from Bio-101 (Vista, CA). Adenine hemisulfate salt is obtained from Sigma (St. Louis, MO).
4. YEPD is a nutrient-rich medium for the routine propagation of yeast strains. This medium is prepared by combining 10 g of yeast extract, 20 g of peptone, 20 g of dextrose, and 40 mg of adenine sulfate in 1 L of distilled water and autoclaving for 15 min at 15 lb/in.<sup>2</sup>. Adenine is included in culturing medium to inhibit reversion of *ade1* and *ade2* mutations.
5. Synthetic defined (SD) minimal dropout medium is used for selection of yeast strains transformed with specific plasmids. It contains 6.7 g of yeast nitrogen base with ammonium sulfate, 2% (w/v) glucose, and the appropriate amino acid dropout mixture (Bio-101). The dropout mixture is composed of specific amino acids required for the growth of the yeast. The omission of Trp from SD medium selects for the pAS1-CyH bait vector. The omission of Leu selects for the AD plasmid. The omission of Leu, Trp, and His from SD medium selects for both the DBD-bait plasmid and the expression library vector as well as for the hybrid bait-protein interaction inside the yeast cell.
6. 3-Amino-1,2,4-triazole (3-AT): Most yeast strains exhibit low-level expression of the *HIS3* marker in the absence of gal4-activated transcription, which leads to background growth under histidine-selection conditions, mainly because a few molecules of the enzyme are sufficient to permit histidine synthesis. Therefore, 3-AT, a chemical inhibitor of imidazole glycerol phosphate dehydratase (the product of the *HIS3* gene) is used to inhibit yeast growth owing to “leaky” *HIS3* expression (*see Note 2*). The final concentration of 3-AT to be used during the screening is dependent on the yeast strain and the particular Gal4 DBD bait fusion protein. We successfully used 25 mM 3-AT when screening for proteins that interact with the PR in a ligand-dependent manner (*see Note 3*).
7. Lithium acetate/TE buffer: 0.1 M LiAc, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. LiAc buffer is used to prepare competent yeast strains for transformation.

8. 40% Polyethylene glycol (PEG): PEG 4000 (P-3640; Sigma) is prepared by dissolving 40 g of PEG in 100 mL of 1X LiAc/TE. This solution is filtered overnight or autoclaved.
9. Sorbitol solution: 10 mM Tris-HCl, pH 8.0, 100 mM LiAc, 1 mM EDTA, and 1 M sorbitol.
10. X-Gal solution: 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol, and 1 mg/mL of X-gal.
11. Denatured salmon sperm DNA: 10 mg/mL in distilled water. Dissolve overnight at 4°C. Denature by boiling for 5 min and snap-freeze in a dry-ice ethanol bath. Store in small aliquots at -20°C. Prior to use, boil for 5 min and store on ice.

## 2.2. Plasmid DNA and Yeast Strains

The yeast host strain Y190 (Mato *ura3-52 his3 ade2-101 trp1-901 leu2-3,-112 gal4 gal80 LYS2::UAS<sub>GAL417mers</sub>-HIS3 URA3::UAS<sub>GAL417mers</sub>-lacZ*) contains two reporter genes for the detection of in vivo protein-protein interactions—*lacZ* and *HIS3*, which are integrated into the yeast genome. A mutation in the *Gal4* gene ensures that the endogenous *GAL4* gene is not expressed. In addition, *GAL80*, whose product inhibits function of the *GAL4* gene product, is mutated. This strain also carries the auxotrophic markers leucine (*leu2*) and tryptophan (*trp*) for selection of yeast strains that have been transformed with the pAS1 Gal4 DBD and pACT Gal4 AD plasmids, respectively. The auxotrophic marker histidine (*his3*) and LacZ are for selection of yeast transformed with interacting proteins.

The vector plasmid pAS1-CYH, harboring the Gal4 DBD, and pACT, harboring the Gal4 AD, contain the 2 $\mu$  origin for replication and an *fl* origin for production of single-stranded DNA. Both vectors contain the  $\beta$ -lactamase (*la*) for selection with ampicillin in *Escherichia coli*. The pAS1-CYH vector also contains the chloramphenicol-resistance (*Cam<sup>r</sup>*) genes for selection with chloramphenicol in *E. coli* and plasmid lost to generate a Y190 strain containing only the library plasmid to facilitate plasmid recovery. For selection in yeast, the pAS1-CYH vector contains the *LEU2* gene and the pACT vector contains the *TRP1* gene. The hybrid proteins are expressed by the alcohol dehydrogenase 1 (ADH1) promoter (p<sub>ADH1</sub>) and are terminated by the ADH1 terminator (t<sub>ADH1</sub>). The DNA fragment encoding the bait protein must be inserted so that the bait protein is expressed in the same reading frame as the GAL4 DBD. The UAG amber suppressor is present in the same translational reading frame as the GAL4 DBD.

## 3. Methods

### 3.1. Characterization of Bait Receptor Protein: General Considerations

The first step in the cloning of steroid receptor-interacting proteins is to construct a plasmid that expresses the Gal4 DBD fused to the steroid receptor

protein. The major requirement for this bait protein is that it be inactive on transcription when expressed in the yeast strain (*see Note 4*). The intrinsic transcriptional activity of the LBD fails to activate transcription of the *His3* and *LacZ* reporter genes in yeast. Therefore, the LBD of the steroid receptor is the preferred domain to be used in the two-hybrid system (*see Note 5*).

Using standard cloning techniques, insert the DNA fragment encoding the LBD of the receptor of interest into the polylinker of the pAS1 vector (Trp selection marker). In this instance, a polymerase chain reaction cloning strategy was used to generate a DNA fragment coding for the PR LBD (amino acid 632-933) with flanking sequences containing the *SmaI* and the *SalI* restriction sites at the 5' and 3' end, respectively.

Perform an LiAc transformation of the yeast strain Y190 with the bait protein. The resulting transforming yeast strain should be tested for its growth properties on SC-His-Trp plates containing different concentrations of 3-AT, as well as for its ability to activate the transcription of the *LacZ* reporter. Note that the growth properties of the transformed Y190 yeast strain and the transcriptional activity of the bait fusion protein to activate the *LacZ* reporter should be tested in the presence of progesterone at a final concentration of 1  $\mu$ M. High concentrations of hormone in the assay are necessary to facilitate uptake of steroid hormone into yeast (*see Note 6*).

Since the transcriptional activity of the PR bait protein is undetectable in the presence of the hormone, it is necessary to confirm that the Gal4 fusion protein is being synthesized in the Y190 yeast strain. Therefore, a Western immunoblot analysis using antibodies directed against the LBD of the PR (C262 monoclonal antibody) is required to determine whether the fusion protein is expressed. Western blotting using the Gal4 DBD or the hemagglutinin antibodies, which are commercially available, can be used to test for protein synthesis if antisera are not available for the protein of interest.

If the bait fusion protein activates transcription alone, it cannot be used to detect protein-protein interactions in this assay. If this is the case, it is possible to generate truncated versions of the bait protein to generate Gal4 DBD fusion constructs that do not activate transcription of the *His3* and *LacZ* reporter genes.

If the antibody used fails to detect expression of the bait protein, it may not necessarily indicate that the bait protein is not expressed. This may be owing to a low expression of the bait fusion protein. Low expression of the bait fusion protein may be advantageous, mainly because overexpression of exogenous proteins may be toxic and inhibit cell growth or induce lethality in yeast. In addition, overexpression can also result in an excess of unbound bait proteins to the promoter of the reporter gene. This may directly interfere with the ability of the target protein to interact with the bait fusion protein when bound to the

promoter and, consequently, with the activation of the reporter activity. If the bait fusion protein fails to activate transcription like most of the fusion proteins, then you may proceed with the library transformation step.

### **3.2. Library Transformation and Screening for Interacting Proteins**

Yeast are generally transformed with the bait and the target plasmids in a cotransformation protocol. However, when using a cDNA expression library for screening and cloning purposes, we prefer to use a sequential transformation protocol with the bait fusion protein followed by transformation with the cDNA expression library. Note that to determine hormone-dependent interactions of the steroid receptor with the target proteins, the SC–His, Trp, Leu plates should also include the steroid hormone (progesterone at a final concentration of 1  $\mu$ M).

1. Use a colony of the Y190 yeast strain transformed with the bait fusion protein expression vector to inoculate 10 mL of SC–Trp medium and grow overnight at 30°C. Use this culture to inoculate 200 mL of SC–Trp medium. This will ensure that the 200-mL culture will grow to saturation by the next day.
2. Use the culture in **step 1** to grow 500 mL of YEPD medium to log phase. The absorbance at 600 nm should be approx 0.6.
3. Harvest the cells by centrifuging at 1000g for 10 min and wash once with approx 100 mL of sterile water. Resuspend the cell pellet in 50 mL of sorbitol solution (10 mM Tris, pH 8.0), containing 100 mM LiAc, 1 M EDTA, and 1 M sorbitol). Incubate the cell suspension at 30°C for 30 min. Spin down as above and resuspend the cell pellet in approx 1 mL of sorbitol solution. This yeast cell suspension can be kept on ice while preparing the DNA mixture containing the cDNA expression library DNA to be used in the screening. Although several protocols recommend that the transformation procedure be performed in small aliquots (0.1 mL of the above yeast suspension), we have successfully used the entire cell culture of yeast to efficiently transform with the cDNA expression library in bulk.
4. Prepare a carrier DNA mixture containing 4 mg of denatured single-stranded salmon sperm DNA and 40  $\mu$ g of cDNA library in a final volume of 1 mL of sorbitol solution. Mix and add the DNA mixture to the competent yeast cells prepared in **step 3** above and incubate for 30 min at 30°C with slow shaking (*see Notes 7 and 8*).
5. Add 10 mL of 40% PEG prepared in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 100 mM LiAc, and continue the incubation for an additional 30 min. Then, heat-shock the yeast cell suspension containing the DNA mixture by incubating at 42°C for 15 min in a water bath.
6. After heat-shocking, add 100 mL of SC(–His–Trp–Leu) liquid medium. Allow the yeast cells to recover by incubating at 30°C for at least 3 h. Then, harvest the

cells and resuspend the pellet in 10 mL of SC(–His–Trp–Leu) medium. Plate 0.5 mL of the transformed cell suspensions onto 150-mm SC(–His–Trp–Leu) agar plates containing 25 mM 3-AT to select for histidine prototrophy (*see Note 9*).

7. To determine the transformation efficiency of the Y190 carrying the bait-fusion protein with the cDNA expression library, plate an aliquot of the transformed yeast suspension on SC(–Trp–Leu) medium and incubate at 30°C for 2 d. Transformation efficiencies of 10<sup>5</sup> colonies/μg of cDNA library can be obtained by this procedure.

### 3.3. $\beta$ -Galactosidase Filter Assay

Colonies that grow after 3–5 d are qualitatively tested for  $\beta$ -galactosidase activity by colony filter assay. This assay will allow the identification of potential positive clones containing cDNA-encoding proteins that interact with the bait fusion protein.

1. Place a circular nitrocellulose filter onto the plate of yeast colonies that grew in SC(–His–Trp–Leu) agar plates and place orientation markers as needed.
2. Lift the filters and immerse in liquid nitrogen for 5 s to permeabilize the cells.
3. Carefully place the filters onto 3MM filter paper saturated with X-gal solution (*see Subheading 2.1., item 10*) and incubate at 30°C. Strong positive interactions can be observed after 30 min of incubation by the appearance of a blue color. Weak interactions may require prolonged incubation time and can be detected in a few hours or overnight.

### 3.4. Final Considerations

Putative positive clones can be rescued directly from the nitrocellulose filter or from the master plate, if needed (*see Note 10*). All putative positive clones should be tested again to confirm interactions. Once the cDNA expressing the target protein is isolated, it is then used to retransform the Y190 yeast strain. At this point, a quantitative X-gal liquid assay is needed to determine the relative strength of the interactions of the isolated clones with the bait protein. In addition, it is recommended that the effect of the hormone on the strength of the interactions between the bait fusion protein and the isolated cDNA-encoding potential target proteins be determined. Because the rate of occurrence of false positives is a common problem of the two-hybrid system, a secondary criterion for distinguishing a true from a false positive is required. This may be accomplished, at least in part, by performing in vitro coimmunoprecipitation assays of epitope-tagged bait protein with in vitro transcribed and translated cDNA clones encoding the target protein (*see Chapters 8–10*).

## 4. Notes

1. In general, steroid hormone agonists exhibit higher affinity for their cognate receptor, as compared to their natural hormones. Because less is known about the

properties of steroid agonists in yeast, we recommend the use of cognate hormones for steroid receptors to perform the screening to identify receptor-interacting proteins—in this case, progesterone instead of the progesterone against R5020.

2. The level of growth owing to *HIS3* expression, as well as the amount of 3-AT required to suppress growth, is dependent on the strain in use. The Y190 strain requires 25–50 mM 3-AT to reduce this background. However, one should empirically establish the minimal level of 3-AT that is required to inhibit the activity of residual Y190 *HIS3* gene product. This is accomplished by introducing the pAS1 bait and the pACT expression vectors into the strain. The transformation is then plated onto SC-leu-trp-his containing increasing concentrations of 3-AT. Background colony growth is examined after 4 d at 30°C.
3. It is also convenient to add the steroid hormone into the medium when the Y190 yeast strain is transformed with the bait protein. Thus, the growth property of the Y190 strain harboring the bait protein is tested in the presence of 3-AT inhibitor and the steroid hormone. Again, a rather high concentration of steroid hormones ( $10^{-6}$  M final concentration) should be used to accomplish saturation of receptor inside the yeast cell.
4. It is important to rule out in the initial experiments the possibility that the receptor-bait fusion protein fails to activate reporter gene expression on its own. Members of the nuclear receptor superfamily of transcription factors harbor two main activation domains: the AF1 located at the amino-terminus LBD, and the ligand-inducible AF2 located at the carboxy-terminus LBD. These activation functions have been shown to work as transactivators when over-expressed in yeast. If the bait construct is a weak activator by itself, it can lead to a number of false positive interactions and should not be used as two-hybrid bait.
5. Interestingly, we found that the intrinsic transcriptional activity of the full-length human PR A-isoform, as well as the transcriptional activity of the AF1- and AF2-containing region of the receptor, is undetectable when fused to the Gal4 DBD of the pAS1-CyH expression vector. The transcriptional activity of the receptor, as assessed by the colony filter and liquid X-gal assay, remains undetectable when tested in the presence of  $10^{-6}$  M final concentration of progesterone. The inability of the PR bait proteins to activate reporter expression in the Y190 yeast may be owing, at least in part, to the relatively low expression level of the bait-receptor protein.
6. The Y190 strain harbors a single copy of the *His3* and the *LacZ* reporters that are integrated into the yeast genome. The use of two reporters as a single copy integrated into the genome seems to contribute to the low-background activity when proteins harboring intrinsic transcriptional activity are used as a bait protein. To determine further the effect of hormone on the transcriptional activity of the bait protein, we recommend performing a quantitative *LacZ* liquid assay, in both the presence and absence of the steroid hormone.
7. In general, the volume of DNA mix employed to transform the yeast strain should not exceed 10% of the volume of competent cells. For routine experiments, stan-

dard miniprep of DNA by standard alkaline lysis works well in the transformation procedure. The presence of RNA in the DNA sample seems to increase the transformation efficiency. However, when highest efficiencies are required for library screening, it is recommended that more purified plasmid DNA preparation, such as CsCl-banded DNA preparation, be used.

8. Replacing the single stranded DNA carrier by total RNA isolated from yeast can further increase the transformation efficiency of the yeast strain. Total RNA from yeast can be isolated by standard phenol-chlorophorm extraction procedures or it can be obtained from Boehringer Mannheim (cat. no. 109-223).
9. During the screening for steroid receptor-interacting proteins, we often place an aliquot of the transformation reaction (about 5% of the total volume of the transformation mixture) directly from the heat-shocked cells that are in the 40% PEG solution onto SD-Leu, Trp, His plates. The remaining transformation mixture was routinely rescued from the transformation procedure by incubating in SC-Leu-Trp-His liquid medium for 3 h at 30°C. Usually, we add 5 vol of prewarmed SD-Trp, Leu, His medium (without the 3-AT inhibitor) to the transformation mixture. After recovery, transformed cells can be concentrated by centrifugation and plated at your own leisure.
10. After screening for receptor-interacting protein, each individual positive yeast colony is grown to recover the plasmid cDNA encoding for the target protein. The cDNA is then used to retransform the Y190 yeast strain together with the bait protein to confirm further the ligand effect on the protein-protein interactions. Recovering the plasmid from the yeast can be laborious, mainly because the presence of polysaccharides in the plasmid DNA preparation from the yeast wall can strongly decrease the efficiency of bacterial transformation. Therefore, the use of highly competent strains of bacteria and electroporation may be the transformation method of choice to rescue plasmids from yeast.

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## **Interaction of Cellular Apoptosis Regulating Proteins with Adenovirus Anti-apoptosis Protein E1B-19K**

**Thirugnana Subramanian and G. Chinnadurai**

### **1. Introduction**

Human adenoviruses have been excellent models for gene expression in mammalian cells. They have also been very important tools in dissecting complex cellular processes such as control of cell proliferation, oncogenesis, and apoptosis. The adenovirus genome contains 36 kb of linear double-stranded DNA that is organized as early and late genes. The left 14% of the viral genome contains two early gene regions, E1A and E1B, that encode proteins that mediate oncogenic transformation of cultured cells and regulate apoptosis. The E1A region codes for two major proteins of 289 amino acids (289R) and 243 amino acids (243R). The E1A proteins are sufficient to immortalize and partially transform primary rodent cells in vitro (*1*). The E1B region codes for two major proteins of 55K (496R) and 19K (175R), which cooperate with E1A in transformation individually (*2,3*). A major function of E1A is induction of proliferation of quiescent host cells to facilitate viral replication. The unscheduled synthesis of cellular DNA initiated by E1A expression also leads to apoptosis. Both E1B proteins suppress apoptosis induced by E1A. One of the consequences of E1A expression is elevation of expression of the tumor suppressor protein p53 (*4*). The 55K protein appears to mediate its anti-apoptosis activity primarily by binding to p53 and antagonizing the activity of p53. On the other hand, the E1B-19K protein exhibits a more general anti-apoptosis activity and efficiently suppresses apoptosis induced by a multitude of stimuli and is a functional homolog of the cellular anti-apoptosis protein BCL-2 (*5-7*).

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To understand the mechanism by which E1B-19K suppresses apoptosis, we undertook a search for cellular proteins that complex with the 19K protein. Several conventional biochemical approaches were unsuccessful in identifying any 19K-interacting proteins. We then used the yeast two-hybrid screening approach to identify and clone 19K-interacting proteins. Using this approach we have identified four different pro-apoptotic proteins, BNIP1-3, and BIK (8,9) that specifically interact with E1B-19K and the cellular anti-apoptosis protein BCL-2. The availability of a number of well-characterized functionally defective mutants of 19K has facilitated the identification of functionally relevant interacting proteins. Furthermore, we were also able to introduce another layer of specificity in our selection using the cellular (BCL-2) and viral functional homologs (EBV-BHRF1) of 19K (10,11). The detailed protocols for the screening of cDNA libraries to identify interacting proteins are described in other chapters. In addition to identifying and cloning interacting proteins, the yeast two-hybrid analysis is a valuable tool for genetic analysis of protein-protein interaction and for mapping the interacting domains. In this chapter, we describe a qualitative as well as quantitative method for the determination of interaction of a panel of E1B-19K mutants with pro-apoptotic proteins BNIP1, -2, and -3 and BIK. This protocol employs the “first generation” of two-hybrid vectors and yeast strains. In our experience, this system appears to be useful for mutational analysis of protein-protein interaction because weak interactions of mutants could be quantitatively analyzed.

## 2. Materials

1. Plasmids: The following plasmids (8,9) can be obtained from the authors by request:
  - a. pMA424-E1B 19K, which expresses adenovirus 2 E1B 19K as a fusion protein with the GAL4 DNA-binding domain (DBD) (aa 1-147) and derivatives of various 19K mutants.
  - b. pACT-BNIP1.
  - c. pACT-BNIP2.
  - d. pACT-BNIP3.
  - e. pACT-BIK.

Plasmids b–e express cellular apoptotic regulators BNIP1, BNIP2, BNIP3, and BIK as fusion proteins with the GAL4 activation domain (Ad) (amino acids 768–881). Empty vectors pMA424 (12) and pACT (13), which express the GAL4 DBD and the AD, respectively as well heterologous bait proteins are used as negative controls.

2. Yeast strain: GGY1 : 171 (14) can be obtained from the authors (14).
3. 50% Glucose solution: Dissolve 50 g of glucose (Sigma, St. Louis, MO) in water and make up to 100 mL, filter sterilize, and store at 4°C.
4. Yeast extract, peptone, dextrose (YPD) plates: Autoclave 1 g of yeast extract (Difco, Detroit, MI), 2 g of peptone (Difco), and 1.75 g of bacto-agar (Difco) in

- 96 mL of water for 20 min. Cool to 56°C, add 4 mL of 50% glucose, and pour 20–25 mL/Petri dish (100 mm). After solidification, store the plates at 4°C.
5. 5X YP medium: Autoclave 50 g of yeast extract and 100 g of peptone in 900 mL of water and store at 4°C.
  6. 100X Adenine sulfate solution: Dissolve 30 mg of adenine sulfate (Sigma) in 10 mL of water, filter sterilize, and store at –20°C.
  7. 10X TE: Prepare 1 M Tris-HCl (Sigma), pH 7.5, and 200 mM EDTA (Sigma). Autoclave and store at room temperature. To make 10X TE, mix 10 mL of 1 M Tris, 5 mL of 200 mM EDTA, and 85 mL of water. Filter sterilize and store at room temperature.
  8. 10X Lithium acetate solution: Dissolve 10.2 g of lithium acetate (Sigma) in 90 mL of water, adjust the pH to 7.5 with dilute acetic acid, and make up the volume to 100 mL with water. Filter sterilize the solution and store at room temperature.
  9. Salmon sperm DNA solution: Dissolve 200 mg of salmon sperm DNA (Sigma) in 10 mL of 1X TE and store at 4°C.
  10. 50% Polyethylene glycol (PEG) solution: Dissolve 50 g of PEG 4000 (Sigma) in 100 mL of water, autoclave, and store at room temperature.
  11. Z buffer: Dissolve the following chemicals (Sigma) in 900 mL of water: 8.53 g of Na<sub>2</sub>HPO<sub>4</sub>, 5.5 g of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.75 g of KCl, 0.25 g of MgSO<sub>4</sub>·7 H<sub>2</sub>O. Adjust the pH to 7.0 and make up the volume to 1 L. Autoclave and store at room temperature.
  12. 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) solution: Prepare 10% X-gal (Gibco-BRL, Gaithersburg, MD) in dimethylformamide and store at –20°C.
  13. 100X Leu<sup>–</sup> His<sup>–</sup> amino acid mix: Dissolve the following chemicals (Sigma) in 1 L of water by stirring it with a magnetic stirrer at 4°C: 300 mg of L-isoleucine, 200 mg of L-arginine, 200 mg of L-methionine, 2000 mg of L-threonine, 300 mg of L-tyrosine, 1500 mg of L-valine, 300 mg of L-lysine, 500 mg of L-phenylalanine, 500 mg of L-tryptophan, 200 mg of adenine, 200 mg of uracil. Filter sterilize the solution and store at 4°C.
  14. Synthetic defined (SD) Leu<sup>–</sup> His<sup>–</sup> plates: Autoclave 6.7 g of yeast nitrogen base (Difco) without amino acids, 15 g of bacto-agar, and 860 mL of water in a 2-L flask for 20 min. Cool to 56°C and add 100 mL of 100X Leu<sup>–</sup> His<sup>–</sup> amino acid mix and 40 mL of 50% glucose solution. Pour 40–50 mL/Petri dish (150 mm) and store at 4°C after solidification.
  15. Whatman no. 3 filter circles (12.5 cm).
  16. Nitrocellulose membranes: Purchase 12.5-cm circles (0.45 μm) of nitrocellulose membrane from Schleicher & Schuell (Keene, NH).
  17. (o-Nitrophenyl-β-D-galactopyranoside (ONPG) solution: Dissolve 40 mg of ONPG (Sigma) in 10 mL of water and store at –20°C.
  18. 20% Na<sub>2</sub>CO<sub>3</sub> solution: Dissolve 20 g of Na<sub>2</sub>CO<sub>3</sub> (Sigma) in 100 mL of water and store at room temperature.
  19. Breaking buffer: Prepare 100 mM Tris-HCl, pH 8.0, with 1 mM dithiothreitol (Sigma) and 20% glycerol (Gibco-BRL).

20. 0.45- to 0.5-mm glass beads (Sigma).
21. Phenylmethylsulfonyl fluoride (PMSF): Prepare a 40 mM stock solution in isopropanol and store at  $-20^{\circ}\text{C}$ .

### 3. Methods

#### 3.1. Maintenance and Storage of Yeast Strain

1. For short-term storage, streak yeast stocks on YPD plates, grow for 2 to 3 d at  $30^{\circ}\text{C}$ , wrap the plates with parafilm, and store at  $4^{\circ}\text{C}$  (*see Note 1*).
2. For long-term storage, scrape the yeast from the YPD plate with a sterile toothpick and suspend in 1 mL of 15% sterile glycerol. Transfer the yeast-glycerol suspension to 2-mL sterile vials and store in a  $-60^{\circ}\text{C}$  freezer.

#### 3.2. Preparation of Yeast Competent Cells

1. Prepare 330 mL of yeast extract, peptone, adenine, dextrose (YPAD) medium by mixing 66 mL of 5X YP, 3.3 mL of 100X adenine sulfate, 13.2 mL of 50% glucose solution, and 247.5 mL of sterile water in a 1-L flask.
2. Remove 30 mL of YPAD medium into a 500-mL flask and inoculate with a small portion of yeast strain (GGY1 : : 171) using a sterile toothpick. Grow the culture overnight at  $30^{\circ}\text{C}$  in a shaker.
3. The next day, transfer the 30-mL yeast culture to 300 mL YPAD medium and continue to grow for 3 h.
4. Collect the yeast pellet by centrifuging at 1000g for 5 min at room temperature.
5. Suspend the pellet in 10 mL of sterile water and transfer to a 40-mL sterile tube.
6. Centrifuge again at 1000g for 5 min and suspend the yeast cells in 2.06 mL of 1X TE/lithium acetate solution. Prepare 1X TE/lithium acetate every time by mixing 10X TE, 10X lithium acetate, and sterile water.
7. Transfer the yeast-TE/lithium acetate mixture to a 5-mL snap-cap tube and rotate in a rotator for 1 h at  $30^{\circ}\text{C}$ . Use these competent cells for transformation. Store the cells at  $4^{\circ}\text{C}$  for further use and discard after 1 wk (*see Note 2*).

#### 3.3. Interaction of Adenovirus E1B-19K Protein with Apoptotic Regulators

The specific protocol to determine the patterns of interaction of various apoptosis regulating cellular proteins with adenovirus E1B-19K and other viral (EBV-BHRF1) and cellular (BCL-2) homologs is given next.

##### 3.3.1. Yeast Transformation

Introduction of DNA into *Saccharomyces cerevisiae* can be accomplished in several ways. In earlier methods, spheroplasts were incubated with DNA, PEG, and calcium chloride (15). Later, Ito et al. (16) treated the yeast cells with alkaline salts such as lithium acetate and incubated with DNA and PEG. Agitation of yeast cells with glass beads and incubation with DNA is one of the

simplest methods to introduce DNA into yeast cells through physical damage (17). Electroporation has also been used successfully to transform yeast (18). In our protocol, we use a modification of the lithium acetate method described by Gietz and Schiestl (19).

1. Boil 50  $\mu$ L of salmon sperm DNA in an Eppendorf tube.
2. Add 450  $\mu$ L of 1X TE/lithium acetate (freshly prepare by mixing 10X solutions with water) and aliquot 15  $\mu$ L into a fresh Eppendorf tube.
3. Add 1  $\mu$ g of each pMA424 (negative control) or pMA424-E1B 19K (test plasmid) and pACT (negative control), pACT-BNIP1, pACT-BNIP2, pACT-BNIP3, or pACT-BIK (test plasmids) along with 30  $\mu$ L of yeast competent cells.
4. Rotate these mixtures for 30 min in a rotator inside a 30°C incubator.
5. Add 180  $\mu$ L of 50% PEG solution and rotate for another 30 min.
6. Heat-shock for 15 min at 42°C in a water bath.
7. Plate the whole mixture in a 150-mm SD Leu<sup>-</sup> His<sup>-</sup> plate and incubate at 30°C for colony formation (3 to 4 d) (see **Note 3**).

### 3.3.2. X-Gal Colony Filter Assay

1. Add 270  $\mu$ L of  $\beta$ -mercaptoethanol (Sigma) and 1 mL of 10% X-gal to 100 mL of Z buffer.
2. Transfer 8 mL of X-gal containing Z buffer to each 150-mm Petri dish, place in it a Whatman filter circle paper, and soak the filter well.
3. Lay a nitrocellulose filter onto the yeast colonies and allow it to wet completely.
4. Using forceps, lift the filter off the yeast plate carefully without smearing the colonies, and place it over an aluminum foil float over liquid nitrogen facing the colony side up to permeabilize the cells.
5. After 10 s, carefully remove the filter (see **Note 4**) and place it over the wet Whatman filter facing the colony side up and incubate at 30°C (up to a maximum of 16 h). During this period, monitor the filters for color development (blue) at regular intervals (see **Note 5**).
6. For each interaction, pick up three to five colonies at random, and patch onto SD Leu<sup>-</sup> His<sup>-</sup> plates at 30°C for quantitative determination of the interaction.

### 3.3.3. Quantitative Determination of Protein-Protein Interaction

Using the following protocol, we performed an *in vivo* interaction of adenovirus E1B-19K, EBV-BHRF1, and human BCL-2 with apoptotic regulators and other proteins. In quantitative assay (**Table 1**) the wt 19K and its functionally positive mutants showed interaction (stronger) whereas the defective mutants showed very little or no interaction with BNIP1, -2, and -3. In qualitative assay (**Table 2**) only the apoptotic regulators BNIP 1, -2, -3, and BIK showed interaction with E1B-19K, EBV-BHRF1, and human BCL-2, whereas they did not interact with human CtBP, CtIP, HIV Tat, and HIV Rev, showing that the assay employing the yeast two-hybrid system is extremely sensitive and useful for studying protein-protein interaction.

**Table 1**  
**Interaction of Cellular Apoptosis Regulating Proteins**  
**with E1B-19K, EBV-BHRF1, and Human BCL-2<sup>a</sup>**

AD fusion proteins	DBD fusion with			
	E1B-19K	EBV-BHRF1	Human BCL-2	None
1. None	W	W	W	W
2. BNIP 1	B	B	B	W
3. BNIP 2	B	B	B	W
4. BNIP 3	B	B	B	W
5. BIK	B	B	B	W
6. CtBP	W	W	W	W
7. CtIP	W	W	W	W
8. HIV Tat	W	W	W	W
9. HIV Rev	W	W	W	W

<sup>a</sup>The protein interaction studies were carried out in yeast GGY1 : : 171, and the interactions are based on the  $\beta$ -galactosidase activity. B, blue; W, white.

**Table 2**  
**Interaction of Cellular Proteins with E1B-19K Mutants<sup>a</sup>**

Mutant	Cell death Suppression	Relative level of <i>LacZ</i> expression					
		BNIP1		BNIP2		BNIP3	
		X-Gal	ONPG	X-Gal	ONPG	X-Gal	ONPG
Wild type	+	B	1	B	1	B	1
14-5	(+)	B	0.61	B	0.55	LB	0.16
29-0	+	B	0.33	B	0.32	B	0.19
50-1	–	W	<0.01	W	0.09	W	0.01
75-6	+	B	1.72	B	1.6	B	0.22
90-6	–	W	<0.01	W	0.04	W	0.01
123-4	–	W	0.05	LB	0.24	LB	0.1
146-E	+	B	0.87	B	0.98	B	0.28

<sup>a</sup>The protein interaction studies were carried out in yeast strain GGY1 : : 171. Results of X-gal staining are indicated as B for blue, W for white or LB for light blue. The various 19K mutants and their effects on cell death are described (7). (+) indicates positive at reduced levels.  $\beta$ -Galactosidase activities were determined from three to four independent colonies selected at random.

1. Inoculate 3 mL of YPAD (Leu<sup>–</sup> His<sup>–</sup>) medium with the transformed yeast culture from the SD Leu<sup>–</sup> His<sup>–</sup> plates using sterile toothpicks and grow them at 30°C overnight.
2. Chill the cells on ice and harvest by centrifuging at 1000g for 5 min in a tabletop centrifuge.

3. Discard the supernatant and resuspend in 250  $\mu$ L of breaking buffer. The cells can be stored at  $-20^{\circ}\text{C}$  and assayed at a later date.
4. Add glass beads until the beads reach a level just below the meniscus of the liquid. Add 12.5  $\mu$ L of PMSF stock solution.
5. Vortex six times at top speed in 15-s bursts and add 250  $\mu$ L of breaking buffer.
6. Mix well and transfer the liquid extract to another tube using a pipetter.
7. Clarify the extract by centrifuging for 15 min in a microfuge. Measure the optical density (OD) of an aliquot at 280 nm to determine the protein concentration of the extract.
8. Add the extract to 400  $\mu$ L of Z buffer and adjust the volume to 500  $\mu$ L with breaking buffer (use the extract with an equal amount of protein).
9. Incubate the mixture in a water bath for 5 min at  $30^{\circ}\text{C}$  and initiate the reaction by adding 100  $\mu$ L of ONPG solution. Continue to incubate until the mixture becomes pale yellow.
10. Terminate the reaction by adding 250  $\mu$ L of  $\text{Na}_2\text{CO}_3$  solution and measure the OD at 420 nm.

#### 4. Notes

1. The yeast plates have to be incubated in an incubator humidified with water to prevent evaporation and dryness of the plates.
2. The yeast competent cells (kept in an ice bath) can be used for several days within a week period.
3. Do not grow the transformed yeast colonies more than 5 d for colony filter assay. Colonies grown for longer periods tend to give a false positive reaction in X-gal colony filter assay.
4. Extra care should be taken when removing the frozen nitrocellulose filters from the liquid nitrogen to avoid breaking.
5. In this assay stronger interacting proteins develop color faster (within an hour) than the weaker interacting proteins that may develop color even with overnight incubation.

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## **IV**\_\_\_\_\_

### **ALTERNATIVE S**TRATEGIES

## Mammalian Two-Hybrid Assays

### *Analyzing Protein-Protein Interactions in Transforming Growth Factor- $\beta$ Signaling Pathway*

Xin-Hua Feng and Rik Derynck

#### 1. Introduction

Mammalian two-hybrid assays are used to analyze protein-protein interactions in transiently transfected mammalian cells. The concepts and principles that form the basis for mammalian two-hybrid assays are essentially the same as for the yeast two-hybrid assays (**1**), discussed in other chapters, except that the analyses are carried out in transfected mammalian cells (**2**). Thus, similar to yeast two-hybrid assays, mammalian two-hybrid assays score interactions between proteins that are localized in the nucleus (**Fig. 1**). Many transcription factors contain two functional modular domains: a DNA-binding domain (DBD) and a transcription activation domain (TAD). Whereas these domains are normally present in a single protein, their indirect interaction through protein-protein interactions can activate transcription as well (**3**). Thus, like an intact transcription factor, tethering of these two domains in a protein complex can promote transcription by interaction of the TAD with the basal transcription machinery. Typically, in two-hybrid assays, one protein is fused to the DBD and another protein of interest is fused to the TAD, and the direct or indirect interaction through another protein (or proteins) then activates transcription of a reporter gene under the control of a DBD binding promoter. Quantitation of the reporter expression then allows measurement of the protein interaction. Several combinations of DBDs and TADs have been successfully used in mammalian two-hybrid assays. The GAL4- and LexA-DBDs are commonly used DBD modules and are derived from the yeast GAL4 and *Escheri-*

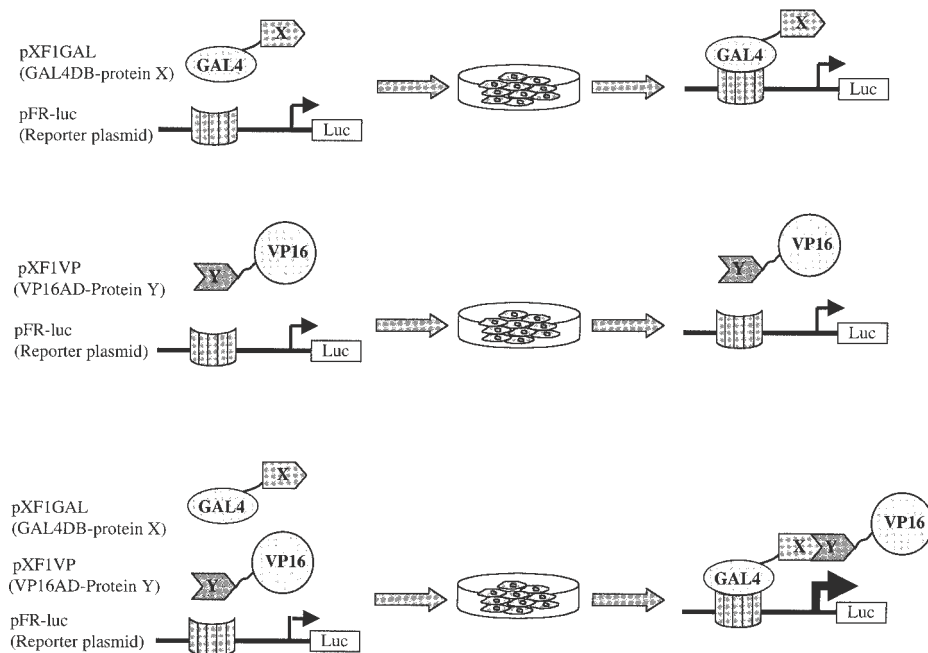


Fig. 1. Schematic representation of the mammalian two-hybrid system. The Gal4 DBD, fused to protein X, binds to the Gal4 DNA sequence but does not activate transcription of the luciferase reporter, because it does not have a transcriptional activation domain or interact with one. The VP16 TAD, fused to protein Y, also does not activate transcription, because it lacks DNA binding. It is only when proteins X and Y interact with each other that the DBD is tethered to the transactivation domain, thus activating transcription of the luciferase gene.

*chia coli* LexA genes, respectively. These are essentially the same DBD fusion modules that are used in yeast two-hybrid assays (4–7). As TADs, the GAL4-, B42-, and VP16-TADs are commonly used and are derived from the yeast GAL4, the *E. coli* B42 polypeptide, and Herpes simplex VP16 genes, respectively. The same GAL4-TAD and B42-TAD are commonly used in yeast two-hybrid assays as partners for the GAL4-DBD and LexA-DBD, respectively (4–7). Finally, the genes for the enzymes  $\beta$ -galactosidase, luciferase, and chloramphenicol acetyltransferase (CAT) are commonly used as reporter genes and allow convenient quantitation of multiple samples (4–7). As will be apparent from the examples presented, we routinely combine in our studies the GAL4-DBD with the VP16-TAD and quantitate the transcription level by measuring the level of luciferase expression.

The evaluation of protein-protein interactions in mammalian cells provides a characterization that is distinct from the results obtained in yeast two-hybrid assays. Yeast two-hybrid assays are often aimed at characterizing interactions between mammalian proteins and are thus carried out in a nonphysiologic intracellular environment. By contrast, mammalian two-hybrid assays quantitate these protein interactions in an environment that is physiologic to the proteins of interest. This issue is particularly relevant for proteins that are normally present and active in the nucleus and participate in the formation of multiprotein complexes, such as transcription factors. Transcription factors act as components of multiprotein complexes and in this way often interact indirectly with each other. Whereas such interactions can be reliably assessed in mammalian two-hybrid assays, yeast two-hybrid assays only may score direct interactions or nonphysiologic indirect interactions, because of the differences in the presence and assembly of components of such multiprotein complexes between yeast and mammalian cells. Another distinction from yeast two-hybrid assays is that mammalian two-hybrid assays can be easily combined with transient transfections, in which the DBD- and TAD-fused proteins of choice are coexpressed with the reporter plasmid and possibly other proteins. On the other hand, the use of mammalian cells does not easily allow one to adapt the mammalian two-hybrid system to screen cDNA libraries for interacting proteins, as is done in yeast two-hybrid screening.

Finally, mammalian two-hybrid assays provide distinct advantages in assessing protein interactions that may depend on extracellular factors and their resulting signaling pathways, or on changes in cell differentiation. Changes in intracellular signaling and environment may result in protein modifications, (e.g., phosphorylation) or in the expression of additional proteins, which are required for the interactions of the proteins of interest. For example, protein interactions that are induced by cytokines or growth factors or depend on activation of defined signaling pathways can be reliably scored in mammalian two-hybrid assays, and the extent to which these interactions depend on such stimuli can be assessed as well. In our studies, we used the mammalian two-hybrid system to evaluate transforming growth factor- $\beta$  (TGF- $\beta$ )-inducible interactions of Smads with transcription factors.

Our studies using mammalian two-hybrid assays were aimed at characterizing the role of Smads in TGF- $\beta$  signaling. Smads are a family of structurally related, intracellular proteins, which serve as signaling effectors for TGF- $\beta$  and TGF- $\beta$ -related proteins (8,9). Based on their structure and function, the Smads can be subdivided into three classes. The receptor-activated Smads (i.e., Smad1, -2, -3, -5, and -8) interact transiently with the ligand-activated receptors, and their C-terminal phosphorylation by the receptors confers activation

and dissociation from the receptor. The common Smad, Smad4 in vertebrates, does not interact with the receptor but is a common partner for receptor-activated Smads following their dissociation from the receptor. This heteromeric complex with Smad4 (presumably a heterotrimer) then translocates into the nucleus where it exerts its transcriptional activities. Finally, the inhibitory Smads, Smad6 and Smad 7, inhibit Smad signaling presumably by competing with the receptor-activated Smads for receptor binding, thereby effectively preventing activation of receptor-activated Smads. In the case of TGF- $\beta$ , activation of TGF- $\beta$  receptors results in C-terminal phosphorylation and activation of Smad2 and Smad3, and the heteromeric complexes of Smad2 or Smad3 with Smad4 then translocate into the nucleus, where they activate or repress transcription of defined TGF- $\beta$ -responsive genes (**9,10**).

Recent progress has led to the characterization of how Smad2 or Smad3 in complex with Smad4 activate TGF- $\beta$ -induced transcription of some genes. These studies have led to a general model for how a heteromeric Smad complex, following ligand-induced translocation into the nucleus, activates transcription through physical interactions and functional cooperativity with a defined set of transcription factors (**9,10**). For example, in response to TGF- $\beta$ , Smad3 can interact with c-Jun, TFE3, the vitamin D receptor, or several other transcription factors, whereas in response to activin, Smad2 can interact with the winged-helix proteins FAST-1 and FAST-2, or the homeodomain transcription factors Mixer or Milk (for reviews *see* refs. **9** and **10**). At the same time, the heteromeric Smad complex also interacts with a favorable promoter DNA sequence adjacent to the binding sequence for the interacting transcription factor (**9,10**). Finally, the C-terminal sequences of the receptor-activated Smads interact with the coactivator CBP/p300, and this interaction is required for transcriptional activation (**11**). Smad4, by contrast, does not have a role similar to that of the receptor-activated Smads, but rather serves as a coactivator, which stabilizes the interaction of the receptor-activated Smad with CBP/p300 (**11**).

At the time of our studies, all this information was not yet known and, in fact, our mammalian two-hybrid assays allowed us to characterize the protein-protein interactions of the TGF- $\beta$ -activated Smad3 with c-Jun and CBP/p300, and the stabilizing role of Smad4 in the Smad3-CBP interaction (**11**). Some of these experiments are presented to illustrate the use of mammalian two-hybrid assays in assessing TGF- $\beta$ -induced protein-protein interactions.

## **2. Materials**

### **2.1. Oligonucleotide Primers**

The underlined sequences indicate restriction sites and the bold letters indicate start or stop codons.

1. For the VP16 activation domain:
  - a. VF: TCAGGCGCCACC **ATG** GCA CCC AAG AAG AAG.
  - b. VR: GGGGAATTC ATC GAT CCC ACC GTA CTC GTC AAT.
2. For the Smad3 coding region (amino acids 2–524):
  - a. S3F: AAGGAATTC TCG TCC ATC CTG CCT TTC AC.
  - b. S3R: CTTGTCGAC **CTA** AGA CAC ACT GGA ACA GC.
3. For the Smad4 coding region (amino acids 2–552):
  - a. S4F: GAAGAATTC GAC AAT ATG TCT ATT ACG AAT AC.
  - b. S4R: GTAGTCGAC **TCA** GTC TAA AGG TTG TGG GTC.
4. For the c-Jun coding region (amino acids 2–331):
  - a. JF: CCGGAATTC ACT GCA AAG ATG GAA ACG AC.
  - b. JR: GAGGTCGAC **TCA** AAA TGT TTG CAA CTG CTG CGT.

## 2.2. Expression and Reporter Plasmids

Plasmid construction, *E. coli* transformation, and plasmid DNA preparation were carried out using standard molecular biology methods (**12**). The nucleotide sequences of plasmid segments generated by polymerase chain reaction (PCR) amplification were sequenced to confirm their sequence integrity.

### 2.2.1. Construction of pXF1GAL

The GAL4-DBD plasmid was made by transferring the GAL4DB coding region of pGBT9 (Clontech) into pRK5 (**13**). The GAL4-DBD in pGBT9 was excised by digestion with *Hind*III and *Eco*RI, filled in with Klenow DNA polymerase I, and subsequently subcloned into the filled-in *Cla*I site of pRK5. This insertion inactivated the *Hind*III and *Cla*I sites but maintained the downstream *Eco*RI site. The new plasmid was named pXF1GAL.

### 2.2.2. Construction of pXF1VP

The VP16 activation domain (VP16-AD) plasmid was constructed by inserting into pRK5 the PCR-amplified coding sequence for the VP16-AD from pVP16 (**7**). The amplified DNA fragment containing the VP16-AD was then digested with *Nar*I and *Eco*RI and inserted into the *Cla*I-*Eco*RI sites of the mammalian expression plasmid pRK5, creating plasmid pXF1VP.

### 2.2.3. Other Mammalian Two-Hybrid Expression Vectors

To generate GAL4-DBD- or VP16-AD-fused Smad, c-Jun, or c-Fos sequences, the PCR-amplified coding sequences were cloned into the *Eco*RI-*Sal*I sites of either pXF1GAL or pXF1VP. This resulted in the generation of Gal-Smad3, Gal-Smad4, VP-Smad3, VP-Smad4, VP-c-Jun, and VP-c-Fos. Expression plasmids encoding the GAL4-DBD-fused CBP segments (Gal-CBP) (**14**) were a gift from L. Xu and M. G. Rosenfeld (University of California, San Diego).

#### 2.2.4. Reporter Gene Vectors

Plasmid pFR-Luc (Stratagene) was used as a GAL4-driven luciferase reporter vector. pFR-luc contains five copies of the GAL4-binding sites upstream of the minimal TATA box to drive expression of luciferase reporter. Other similarly constructed reporter genes such as CAT and secreted alkaline phosphatase (SEAP) reporter can also be used instead of the luciferase reporter but will require different methods to quantitate the CAT and SEAP activities. pSV $\beta$ gal, which expresses  $\beta$ -galactosidase under the control of the SV40 promoter (Promega), was cotransfected to allow normalization of transfection efficiency.

#### 2.3. Mammalian Cell Culture Reagents and Materials

1. Minimal essential medium (MEM), Eagle's with Earle's salts and L-glutamine (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (HyClone), 100  $\mu$ M nonessential amino acids (Gibco-BRL), and 1X Pen/Strep (100  $\mu$ g/mL of streptomycin sulfate plus 100 U/mL of penicillin).
2. Serum-free Opti-MEM medium (Gibco-BRL).
3. LipofectAMINE (Gibco-BRL).
4. DEAE-dextran (Pharmacia): Sterilize the stock solution of 2 mg/mL of DEAE-dextran using sterile 0.2- $\mu$ m filters, and store aliquots at  $-20^{\circ}\text{C}$ .
5. Dimethyl sulfoxide (DMSO, Sigma).
6. Luciferase Assay kit (Analytic Luminescence Laboratory).
7. Galacto Light Plus kit (Tropix), for the determination of  $\beta$ -galactosidase activity.
8. Luminometer Monolight 2010 (Analytic Luminescence Laboratory).

### 3. Methods

#### 3.1. PCR-Mediated Amplification

We carried out PCR reactions using *Pfu* DNA polymerase (Stratagene). A 50- $\mu$ L reaction contained the following components: 1X *Pfu* buffer (included with the enzyme), 100  $\mu$ M dNTP, 0.5  $\mu$ M of each primer, 100 ng of template DNA, and 5 U of *Pfu* enzyme. Reactions were first incubated at  $95^{\circ}\text{C}$ , 4 min before being subjected to 15 cycles of amplification: denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 60 s. PCR products were then concentrated using Microcon-50 (Amicon), digested with restriction enzymes as indicated, and purified by glass-wool filtration after agarose electrophoresis.

#### 3.2. Cell Culture, Transfections, and Reporter Assays

Mv1Lu (ATCC no. CCL-64) and its derivative RI-14 cells (*11*), and F9 and SW480.7 cells, were maintained in MEM Eagle's with Earle's salts and

L-glutamine, supplemented with 10% FBS, 100  $\mu$ M nonessential amino acids, and 1X Pen/Strep. Mv1Lu or RI14 cells were transfected using the DEAE-dextran method (15). For transfection of F9 and SW480.7 cells, LipofectAMINE (Gibco-BRL) was used following the manufacturer's instructions.

### 3.2.1. Cell Transfections and Lysate Preparation

1. Grow cells in 6-well plates (35 mm in diameter) containing MEM medium with supplements as indicated. Seeding of a number of cells that will result in 30–40% confluency on the next day will provide good results. Take into account that different cell lines have different growth rates.
2. The next day, prepare the transfection media:
  - a. Mv1Lu and RI-14 cells: Add DEAE-dextran to a final concentration of 125  $\mu$ g/mL and chloroquine to 0.1 mM in 1 mL of antibiotic-free MEM medium.
  - b. F9 and SW480.7 cells: Add 4  $\mu$ L of LipofectAMINE reagent to 100  $\mu$ L of serum-free Opti-MEM per well (Gibco-BRL).
3. For each well of Mv1Lu or RI-14 cells, add 250 ng of each plasmid (i.e., the Gal fusion, VP fusion, pFR-luc, and pSV $\beta$ gal) to 1 mL of DEAE transfection medium. For each well of F9 or SW480.7 cells, add 250 ng of each plasmid (i.e., the Gal fusion, VP fusion, pFR-luc, and pSV $\beta$ gal) to 100  $\mu$ L of serum-free Opti-MEM. Mix the DNA solution with the LipofectAMINE solution and incubate at room temperature for 15–45 min. Then add 800  $\mu$ L of Opti-MEM to the mixture.
4. Rinse the cells with 37°C, serum-free medium prior to transfection. Add the transfection mixture to prewashed cells and incubate for 3 h (Mv1Lu/RI-14) or 6 h (F9 or SW480.7).
5. Remove the transfection medium and shock the cells for 2 min with 10% DMSO (see Note 1).
6. Wash the cells with warm medium and incubate with regular medium for another 24 h.
7. Remove the medium and treat the cells for 24 h with 400 pM TGF- $\beta$ 1, as required, in MEM medium containing 0.2% FBS.
8. Wash the cells twice in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline, and lyse the cells for 15 min at room temperature in 300  $\mu$ L of 1X Reporter Lysis Buffer (Promega).

### 3.2.2. Luciferase Assay

The luciferase expression levels can be measured using assay reagents from Analytic Luminescence Laboratory or any other sources (e.g., Promega). Luciferase activities are measured using a Luminometer Monolight 2010 (Analytic Luminescence Laboratory). Depending on the sources of reagents and luminometer, the materials and procedures for luciferase measurement may be adjusted according to the manufacturer's instructions.



1. Prepare luciferase substrates A and B according to the manufacturer's instructions.
2. Transfer 100  $\mu$ L of cell lysate into a 12  $\times$  75 mm tube.
3. Add 100  $\mu$ L of Substrate A (buffer/substrate mixture) to the same tube.
4. Add 100  $\mu$ L of Substrate B (D-luciferin solution) to the same tube. Immediately place the sample into the cuvet in the luminometer and measure the luminescence (*see Note 2*).

### 3.2.3. $\beta$ -Galactosidase Assay

$\beta$ -Galactosidase activity was assayed using the Galacto Light Plus kit (Tropix) following the manufacturer's instructions. This kit contains Reaction Substrate and Accelerating Solution. Briefly, transfer 10  $\mu$ L of cell lysate into a 12  $\times$  75 mm tube. Add 100  $\mu$ L of Reaction Substrate to the tube and incubate at 37°C for 15–60 min. Place the tube in the luminometer to measure the  $\beta$ -galactosidase activity after injecting Accelerating Solution (*see Note 3*).

### 3.3. Yeast Two-Hybrid Assays

LexA-based yeast two-hybrid assays (**5**) were used to detect interactions between full-length Smads in bait plasmid pEG202 (**16**) and CBP fragments in prey plasmid pJG4-5. CBP(1–450), CBP(314–1100), CBP(1069–1891), and CBP(1891–2441) in pJG4-5 (**14**) were provided by L. Xu and M. G. Rosenfeld. Plasmids were transformed into yeast EGY48 using Alkali Cation (Bio 101), and protein interactions were assessed by scoring  $\beta$ -galactosidase activity as reporter, as previously described (**16**).

### 3.4. General Discussion of Results

#### 3.4.1. TGF- $\beta$ -Inducible Interaction of Smad3 and CBP

In our search for proteins that regulate the transcriptional activity of Smad3, we focused on CBP, a close homolog of p300. CBP/p300 is a transcriptional coactivator that is able to physically interact with several transcription factors and thereby links them to the general transcription machinery (**17**). Yeast two-hybrid assays revealed that the segment of CBP from amino acids 1891–2441 had the ability to interact with the C-domain (i.e., the C-terminal one-third) of Smad3 (**11**). Deletion of the C-terminal 35 amino acids of Smad3, which contain the two receptor-mediated phosphorylation sites, abolishes this interaction (**11**). We then evaluated the interaction of Smad3 fused to the Gal4-DBD with defined segments of CBP fused to the VP16-TAD in a mammalian two-hybrid system (**11**).

Four CBP segments, which together span the entire length of the 2441 amino acid polypeptide, were tested in these interaction assays, in both the absence and presence of TGF- $\beta$ . Thus, expression plasmids for individual Gal4-fused CBP segments and the VP-Smad3 expression plasmid were cotransfected with

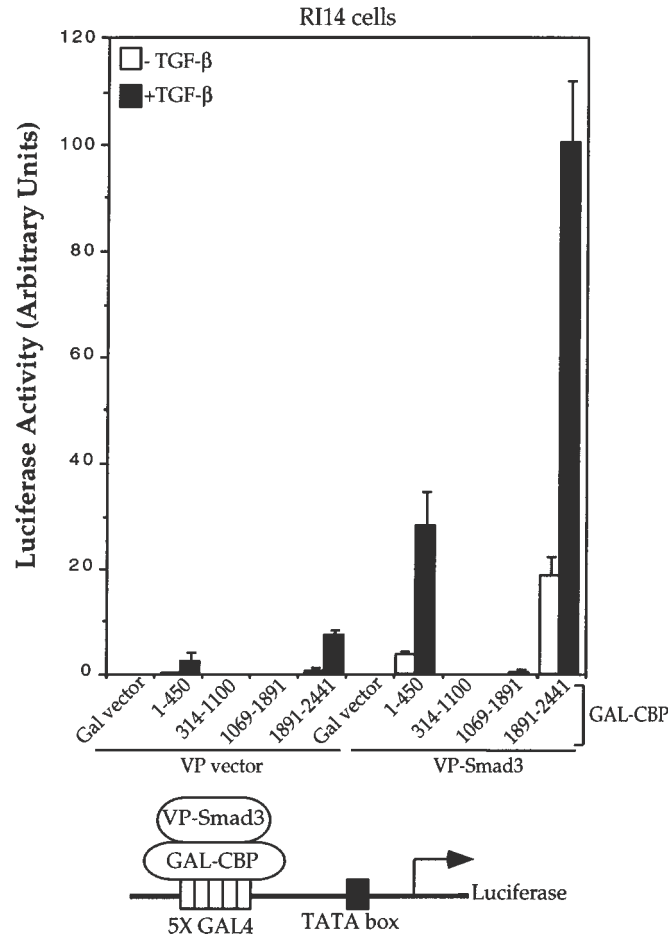


Fig. 2. TGF- $\beta$ -inducible interaction of Smad3 and CBP. RI-14 cells were transfected with an expression plasmid for individual CBP segments (amino acid numbers shown) fused to the Gal4 DBD, and an expression plasmid encoding Smad3 fused to the VP16 AD. The transcriptional activity from the Gal4 promoter is measured by the level of luciferase expression. Parallel measurements of TGF- $\beta$ -treated or untreated cells show the TGF- $\beta$ -inducible interaction between Smad3 and CBP segments 1–450 and 1891–2441 (**11**).

a 5xGal4-luciferase reporter plasmid (pFR-Luc) in the TGF- $\beta$ -responsive RI-14 cells. As shown in **Fig. 2**, two CBP segments were able to interact with Smad3. The segment from amino acids 1891–2441 showed the strongest interaction, and this finding was consistent with the yeast two-hybrid analyses (**11**). In addition, the CBP segment from amino acids 1–450 showed a weaker interac-

tion, which was not detected in yeast two-hybrid assays. Both interactions were strongly enhanced in the presence of TGF- $\beta$ . The TGF- $\beta$  inducibility of this interaction is consistent with the TGF- $\beta$ -induced activation and nuclear translocation of Smad3, whereas CBP is constitutively present in the nucleus. These results are consistent with the coimmunoprecipitation data, which also show a TGF- $\beta$ -inducible interaction of Smad3 and CBP (*II*).

These results demonstrate that the mammalian two-hybrid assay system allows a quantitative assessment of protein-protein interactions in transfected cells. These results also show that this system is convenient to characterize interactions that depend on activation of signaling pathways, e.g., by TGF- $\beta$ , or secondary protein modifications, such as C-terminal phosphorylation of Smad3 in response to activation of the type I TGF- $\beta$  receptor. In addition, the interaction of the CBP(1–450) with Smad3 in mammalian two-hybrid assays and our inability to detect this interaction in yeast two-hybrid assays (*II*) illustrate the high sensitivity of mammalian two-hybrid assays or its dependence on an unknown protein.

#### 3.4.2. Interaction of Smad4 with CBP

In another set of experiments, we used mammalian two-hybrid assays to characterize the interaction of Smad 4 with CBP and the role of Smad4 in TGF- $\beta$ -induced transcription. The results further highlight possible applications of mammalian two-hybrid assays in the characterization of protein-protein interactions in vivo.

On TGF- $\beta$  stimulation, Smad4 interacts with Smad3 (or Smad2) and, following nuclear translocation, cooperates with Smad3 to induce TGF- $\beta$ -induced transcription. Since Smad3 interacts through its C-terminus with CBP/p300, these observations raised the questions: What is the role of Smad4 in TGF- $\beta$ -induced transcriptional activation? We therefore used mammalian two-hybrid assays to evaluate whether Smad4 interacts with CBP. As shown in **Fig. 3**, the same Gal4-fused segments of CBP, which showed interaction with VP-Smad3 (**Fig. 2**), also interacted with VP-Smad4 in a TGF- $\beta$ -inducible manner. By contrast, we were unable to detect interaction of Smad4 with CBP segments in yeast two-hybrid assays (*II*). In addition, the CBP segment from amino acids 1678–2441 interacted directly with glutathione-S-transferase (GST)-fused Smad3 and not with GST-fused Smad4 (*II*). These observations strongly suggest that the mammalian two-hybrid interactions of Smad4 with CBP might be owing to an indirect interaction of Smad4 with CBP. This interaction could then occur through the ability of Smad4 to form a heteromeric complex with endogenous Smad3, which in turn interacts with CBP in a TGF- $\beta$ -dependent manner. This model is consistent with the heteromeric complex formation of Smad3 and Smad4, and the lack of interaction of Smad4 with CBP in GST-

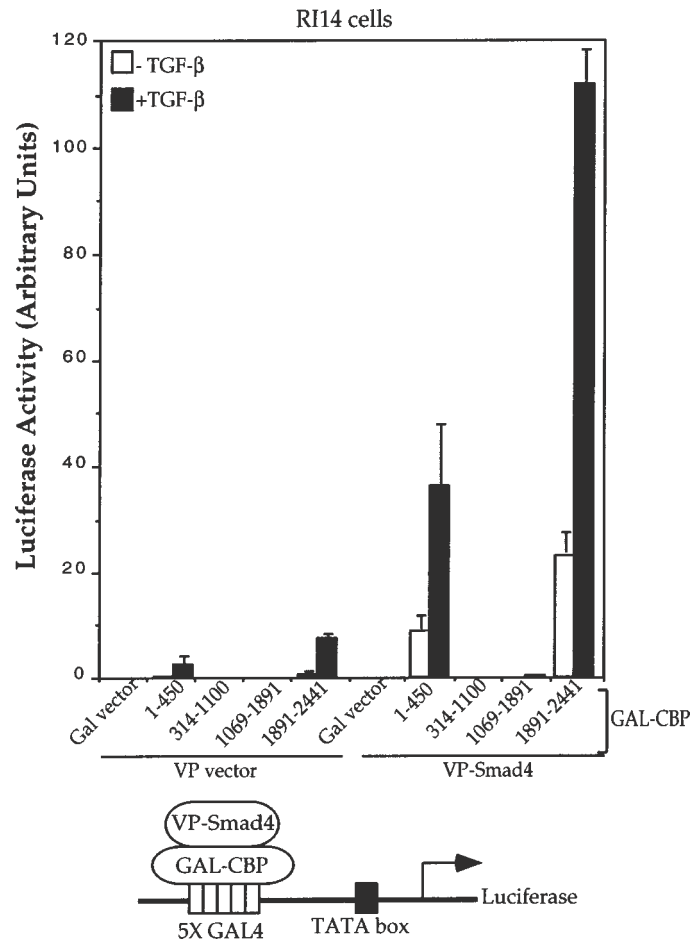


Fig. 3. TGF- $\beta$ -inducible interaction of Smad4 with CBP. The experimental design is quite similar to that of **Fig. 2**, except that the experiments measured the interaction of VP16-fused Smad4 with the CBP segments. Parallel measurements of TGF- $\beta$ -treated or untreated cells show the TGF- $\beta$ -inducible interaction of Smad4 with the same CBP segments 1–450 and 1891–2441, with which Smad3 interacts in **Fig. 2 (II)**.

adsorption and yeast two-hybrid assays (*II*). This model may also explain why in mammalian two-hybrid assays, like Smad3, Smad4 interacts with the same CBP segments in a TGF- $\beta$ -dependent manner.

Taking into consideration these results and the previous observations that Smad3 and Smad4 synergize to mediate TGF- $\beta$ -induced transcription, we assessed the effect of Smad4 on the TGF- $\beta$ -inducible interaction of Smad3

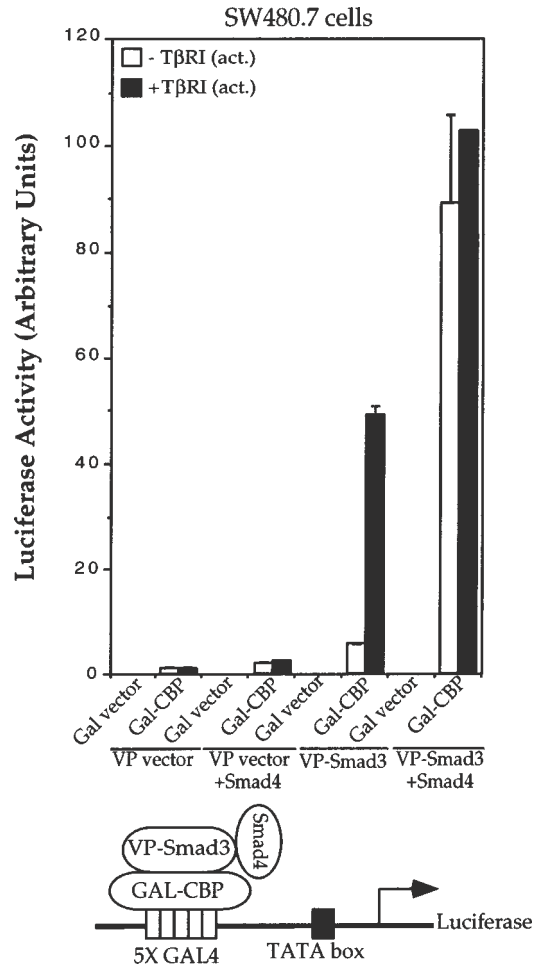


Fig. 4. Smad4 enhances the interaction of Smad3 with CBP. Smad4-defective SW480.7 cells were transfected with an expression plasmid for the CBP segment (1891–2441) fused to the Gal4 DBD, and an expression plasmid for the VP16-fused Smad3. While this resulted in a TGF- $\beta$ -inducible interaction, coexpression of Smad4 strongly enhanced this interaction (*II*).

with CBP. We took advantage of the availability of Smad4-deficient cells (i.e., SW480.7 cells) (*18*). As shown in **Fig. 4**, Gal-CBP(1–450) and Gal-CBP(1891–2441) interacted with VP-Smad3 in a TGF- $\beta$ -inducible manner, consistent with the results in RI-14 cells. However, coexpression of Smad4 enhanced this interaction, strongly suggesting that the primary role of Smad4 is to enhance the efficiency or to stabilize the interaction of Smad3 with CBP. This could

then explain the ability of Smad4 to function as a TGF- $\beta$ -inducible coactivator of Smad3, even though Smad4 does not directly interact with CBP and has no transcriptional activity by itself.

Collectively, these results illustrate the use of the mammalian two-hybrid assay system to detect indirect protein interactions and to assess the role of a protein in stabilizing or enhancing the interaction between two other proteins.

#### 3.4.3. TGF- $\beta$ -Inducible Interaction of Smad3 with c-Jun and c-Fos

As a third example to illustrate the use of mammalian two-hybrid assays, we present some experiments to characterize the TGF- $\beta$ -inducible interaction of Smad3 with c-Jun and c-Fos. As is now known, Smads cooperate with other transcription factors in a TGF- $\beta$ -inducible manner, and this cooperativity is responsible for TGF- $\beta$ -induced gene expression (*11*). Since at that time we had determined that TGF- $\beta$  and Smad3/4 were able to activate transcription from the AP-1 binding sequence in the collagenase I promoter, we used mammalian two-hybrid and other assays to examine the physical interaction of Smad3 with c-Jun and c-Fos (*19*). c-Jun and c-Fos form a dimer at the AP-1 binding site and mediate constitutive transcription from that sequence.

To evaluate the interaction of Smad3 with c-Jun and c-Fos, we assessed the ability of Gal-Smad3 to interact with VP-c-Jun or VP-c-Fos in the TGF- $\beta$ -responsive Mv1Lu cells. As shown in **Fig. 5**, Smad3 and c-Jun did not interact with each other in the absence of TGF- $\beta$ , but TGF- $\beta$  treatment resulted in a strong ligand-dependent interaction between both proteins. This TGF- $\beta$ -inducible interaction was also apparent in coimmunoprecipitation assays and was consistent with the ability of c-Jun to directly interact with Smad3 in GST-adsorption assays (*19*). By contrast, we were unable to detect an interaction between c-Jun and Smad3 in yeast two-hybrid assays. This may be owing to the fact that the sequence in Smad3, which is required for interaction with c-Jun, is not exposed in yeast and that this exposure requires Smad3 activation through C-terminal phosphorylation in response to TGF- $\beta$ . Accordingly, it is believed that the TGF- $\beta$ -induced phosphorylation of Smad3 in mammalian cells results in a conformational change, which exposes both the C-domain (the C-terminal third) and the N-domain (the N-terminal third). This N-domain would then be exposed and able to interact with c-Jun. This model is consistent with the increased direct interaction of c-Jun with a Smad3 mutant, which lacks the C-domain, when compared to full-size Smad3, in GST-adsorption assays (*19*).

Mammalian two-hybrid assays in Mv1Lu cells also revealed a TGF- $\beta$ -inducible interaction between Smad3 and c-Fos (**Fig. 5**). Although this interaction was less than with c-Jun, it nevertheless contrasted with the very low levels of Smad3 interaction with c-Fos in GST-adsorption assays (*19*). This raised the possibility that the Smad3 interaction with c-Fos, as scored in mam-

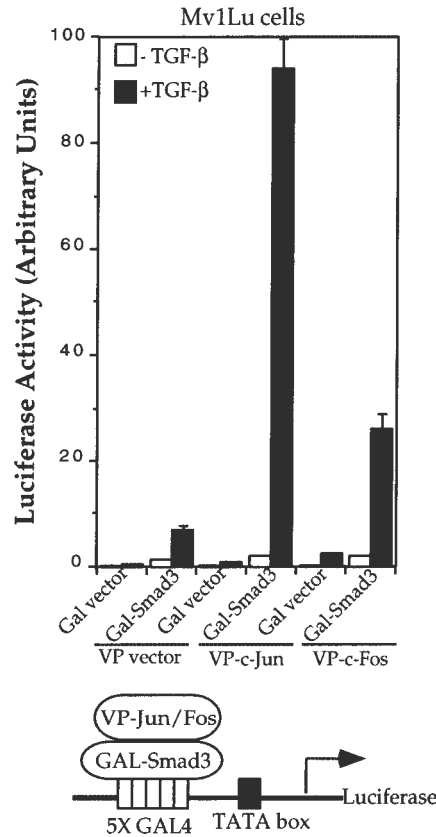


Fig. 5. TGF- $\beta$ -inducible interaction of Smad3 with c-Jun and c-Fos. Mv1Lu cells, which endogenously express c-Jun and c-Fos, were transfected with an expression plasmid for Smad3 fused to the Gal4 DBD, and an expression plasmid for VP16-fused c-Jun or c-Fos. Comparison of the luciferase levels in the absence or presence of TGF- $\beta$  shows TGF- $\beta$ -inducible interactions of Smad3 with c-Jun and c-Fos (19).

malian two-hybrid assays, reflected an indirect interaction owing to the interactions of Gal-Smad3 with endogenous c-Jun, and of endogenous c-Jun with VP-c-Fos. We therefore tested the interactions of Gal-Smad3 with VP-c-Jun or VP-c-Fos in F9 teratocarcinoma cells, which are known to have minimal endogenous levels, if any, of c-Jun and c-Fos. As shown in **Fig. 6**, Gal-Smad3 interacted in a TGF- $\beta$ -inducible manner with VP-c-Jun, consistent with the mammalian two-hybrid assays in Mv1Lu cells, as well as the coimmunoprecipitation and GST-adsorption data (19). By contrast, Gal-Smad3 showed a low level of constitutive interaction with VP-c-Fos. These data therefore suggest that the TGF- $\beta$ -inducible interaction of Gal-Smad3 with VP-c-Fos

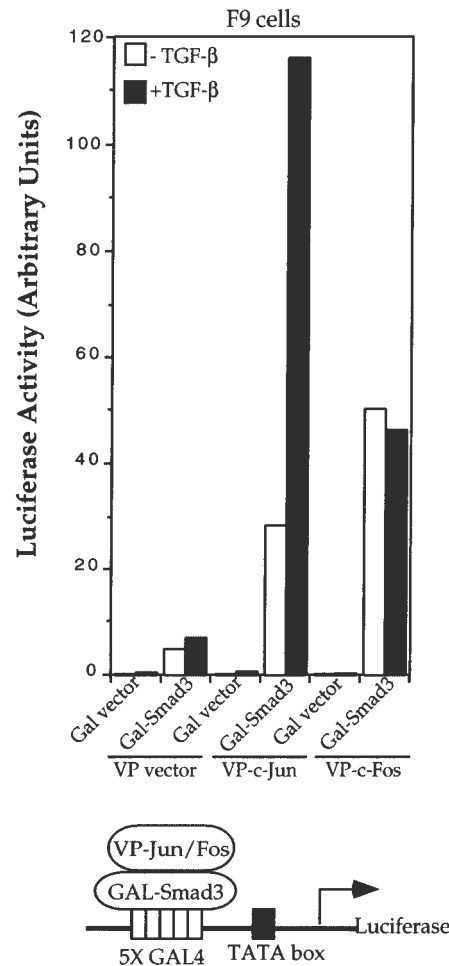


Fig. 6. Interaction of Smad3 with c-Jun and c-Fos in the absence of detectable c-Jun and c-Fos levels. F9 cells, which lack or have very low endogenous c-Jun or c-Fos, were transfected with an expression plasmid for Smad3 fused to the Gal4 DBD, and an expression plasmid for VP16-fused c-Jun or c-Fos. Measurement of the luciferase levels in the absence or presence of TGF- $\beta$  shows a TGF- $\beta$ -inducible interaction of Smad3 with c-Jun and a constitutive interaction of Smad3 with c-Fos (19).

in Mv1Lu cells may have been owing primarily to an indirect interaction through endogenous c-Jun.

As with the two previous examples, these results illustrate that mammalian two-hybrid assays can score both direct and indirect interactions, and that they can detect interactions that depend on activation of signaling pathways and



consequent modifications of one of the proteins. In this case, the direct interaction of c-Jun and the indirect interaction of c-Fos with Smad3 depend on the TGF- $\beta$ -induced phosphorylation and changes in conformation of Smad3, as well as on the nuclear translocation of the Smad3/4 complex. Finally, this example illustrates how mammalian two-hybrid assays can detect interactions, that, even though direct, were not detectable in yeast two-hybrid assays.

### **3.5. General Discussion of Mammalian Two-Hybrid System**

Most cell biologic processes depend on the ability of proteins to interact with each other. For example, intracellular signaling initiated by extracellular ligands results from cascades of transient or stable protein interactions, while transcriptional activation depends on the formation of multiprotein complexes at the promoter DNA. Protein-protein interactions can be assessed using several commonly used methods. Direct protein interactions are often assessed *in vitro* by the ability of a purified or *in vitro* translated,  $^{35}\text{S}$ -labeled protein to interact with a purified protein of interest. The latter protein is thereby often fused to a GST protein segment or a (His)6-sequence tag, thus allowing its purification and specific adsorption of the protein with its associated proteins (20). In addition, far-Western protein blotting allows assessment of the ability of a radiolabeled protein to directly interact with proteins, following gel electrophoretic separation and subsequent transfer onto a membrane (20). Although both methods are highly informative about the ability of proteins to interact, they do not provide information whether and under which conditions these interactions occur *in vivo*. Similarly, yeast two-hybrid assays only allow assessment of the ability of two proteins to interact with each other, and it still remains to be determined how far these interactions occur in the intact cell. *In vivo* protein-protein interactions are most frequently assessed using coimmunoprecipitation analyses. In this chapter, we have outlined the use of mammalian two-hybrid assays as an alternative method to assess the interaction of protein associations *in vivo*.

As illustrated with the few examples in this chapter, mammalian two-hybrid assays can be used to study interactions between proteins of vertebrate origin *in vivo* and provide several advantages over coimmunoprecipitation analyses or yeast two-hybrid assays. In contrast to yeast two-hybrid assays, mammalian two-hybrid assays allow assessment of protein interactions in the more physiologic context of an intact cell, which is subject to regulation by extracellular ligands. Thus, protein-protein interactions, which depend on induction by extracellular stimuli, are often detectable using mammalian two-hybrid assays, but are not necessarily apparent in yeast two-hybrid assays nor can their regulation be assessed in yeast two-hybrid assays. For example, the TGF- $\beta$ -inducible Smad3 interaction with c-Jun (Figs. 5 and 6) is not detected in yeast

two-hybrid assays, whereas the TGF- $\beta$ -induced Smad3 interaction with CBP (**Figs. 2–4**) is assessed as constitutive in yeast two-hybrid assays (**II**). Additionally, regulated protein-protein interactions often depend on secondary protein modifications, such as phosphorylation or fatty acylation, and such regulated interactions may not be apparent in yeast two-hybrid assays, yet are detected using mammalian two-hybrid assays. For example, the regulated phosphorylation of c-Jun and Smad3 may explain why both proteins do not detectably interact with each other in yeast two-hybrid assays, yet strongly interact in mammalian two-hybrid (**Figs. 5 and 6**) and coimmunoprecipitation assays (**19**). Finally, whereas yeast two-hybrid assays most frequently score direct protein-protein interactions, mammalian two-hybrid interactions can score both direct and indirect protein interactions, and protein associations that depend on accessory proteins. This allowed us to detect a TGF- $\beta$ -inducible interaction of Smad4 and CBP (**Fig. 3**), even though no direct interaction between both proteins could be detected (**II**).

Mammalian two-hybrid assays also complement coimmunoprecipitation assays in several ways. First, mammalian two-hybrid assays often provide a much higher sensitivity than coimmunoprecipitation analyses. For example, we found Smad3 interactions with an N-terminal segment of CBP (**Fig. 2**), which were not detectable in coimmunoprecipitation analyses. Second, mammalian two-hybrid analyses provide the ability to quantitate interactions, thus providing some assessment of the efficiency and affinity of interactions. This is not possible using coimmunoprecipitation analyses. Finally, the sensitivity of the method allows mammalian two-hybrid assays to detect indirect interactions as well as the role of interacting proteins in multiprotein complex formation. For example, comparison of Mv1Lu cells, which contain both c-Jun and c-Fos, and F9 cells, which lack detectable c-Jun and c-Fos, allowed us to assess the role of c-Jun in the c-Fos/Smad3 interaction (**Figs. 5 and 6**). Indeed, our results revealed that the TGF- $\beta$ -inducible interaction between c-Fos and Smad3 in Mv1Lu cells is largely, but not exclusively, owing to the interaction of c-Fos with c-Jun, which in turn displays a strong, TGF- $\beta$ -inducible interaction with Smad3 (**19**). As another example, we showed using mammalian two-hybrid assays that Smad4 stabilizes and enhances the TGF- $\beta$ -inducible interaction of Smad3 with CBP (**Fig. 4**), even though no direct interaction of Smad4 with CBP was detectable in yeast two-hybrid or GST-adsorption assays.

As shown by these examples, mammalian two-hybrid assays clearly present a highly sensitive method to detect both direct and indirect interactions *in vivo* in a cell physiologic context. This method greatly complements more established methods of coimmunoprecipitation and *in vitro* analyses of direct interactions.

#### 4. Notes

1. This step is only for Mv1Lu/RI-14 cells. Treating certain cell lines with 10% DMSO dramatically improves transfection efficiency. Preliminary experiments of individual cell lines should examine the effects of DMSO or glycerol shock on transfection efficiencies.
2. Substrate solutions can be added to the sample cuvet by injection if the luminometer has two injectors; alternatively, manual pipetting is required if no injectors are installed.
3. The luciferase activities were normalized to  $\beta$ -galactosidase expression for transfection efficiency. All assays are done in triplicate. Statistical and graphic analysis of luciferase and  $\beta$ -galactosidase activity may be performed using the Excel 98 program.

#### Acknowledgments

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## One-Hybrid Systems for Detecting Protein-DNA Interactions

Mary Kate Alexander, Brenda D. Bourns, and Virginia A. Zakian

### 1. Introduction

The yeast two-hybrid assay has proven useful for detecting protein-protein interactions. A variation on the theme can be used for finding proteins that interact with a particular DNA sequence. The one-hybrid assay, so far carried out only in *Saccharomyces cerevisiae*, in its simplest form (**Fig. 1**) consists of a DNA sequence of interest placed upstream of a reporter gene. The reporter gene can be either on a plasmid (**1**) or integrated into the chromosome (**2**). The protein or library being tested is cloned into a vector that expresses that protein fused to a transcription activation domain (TAD), the equivalent of the prey protein in a two-hybrid assay. This hybrid protein is expressed in the strain carrying the reporter gene. If the protein is able to interact with the sequence of interest, by either binding directly to the DNA or indirectly via interaction with a DNA-binding protein, transcription of the reporter gene is activated.

There are several variations on the basic one-hybrid assay. As already mentioned, the reporter gene can be placed on either a plasmid or a chromosome; most frequently, it is integrated into the chromosome. In addition, the binding site can either be an “artificial” site, such as several tandem repeats of a partial sequence (**1,2**), or a fully functional site (**3,4**). This chapter will concentrate primarily on the latter type of assay.

To use yeast to detect proteins interacting with nonyeast binding sites, it is sometimes also useful to express a protein already known to bind to the site, in order to detect proteins that interact with the sequence indirectly, via interaction with this protein (**5**). This modified one-hybrid assay has also been called the double interaction screen (**6**). Another variant, the one-two-hybrid assay,

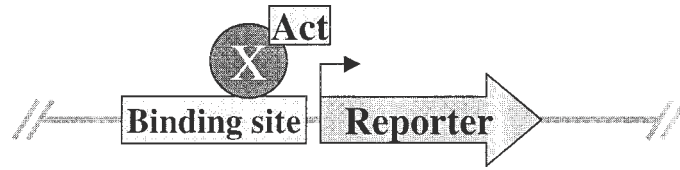


Fig. 1. A generic one-hybrid assay. The DNA sequence of interest is placed upstream of a reporter gene with a minimal promoter, either on a plasmid or integrated into a chromosome. A hybrid protein consisting of a TAD fused to a protein that interacts with the target site is able to activate transcription of the reporter gene.

combines reporters for one- and two-hybrid assays in the same strain (7). This setup may help differentiate between direct and indirect interactions of nonyeast proteins with the binding site, by determining whether the fusion protein is able to activate both reporter genes (direct), or only the two-hybrid reporter (indirect). Finally, the one-hybrid assay can be modified to screen a library of potential binding sites for the sequence that interacts with a known protein (8,9).

### 1.1. Artificial Sites

Most reported one-hybrid assays have used artificial or heterologous sites. One early use of a one-hybrid assay to screen for proteins binding to a site of interest utilized three tandem repeats of an 11-bp sequence that had been shown by gel-shift assays to be bound by an activity, Olf-1, present in olfactory nuclear extracts (1). This assay was able to identify a cDNA clone encoding the Olf-1 protein. Several months later, a different group used a one-hybrid assay in which the binding site was four direct repeats of the 11-bp ARS consensus sequence (ACS) from the yeast replication origin. The ACS is essential but not by itself sufficient for origin activity (10). This assay was used to isolate *ORC6*, a gene encoding a component of the origin recognition complex.

The most common use of the yeast one-hybrid assay has been to identify transcription factors and other regulatory proteins using DNA sequences that have been identified as *cis*-acting elements through other genetic or biochemical methods. One recent example of such a screen using an *S. cerevisiae* sequence is the identification of Rpn4p as a transcription factor that binds to a sequence found upstream of most known proteasomal genes (11). Screens have also been performed using *cis*-acting sequences involved in transcriptional regulation from other organisms, including *Caenorhabditis elegans* (12), sea urchins (13), *Arabidopsis* (14), *Drosophila* (6), and mammals (15).

The one-hybrid assay can also be used to find factors binding to other *cis*-acting sequences, such as the aforementioned yeast replication origin. A one-hybrid assay was used to identify the fission yeast telomere-binding protein

Taz1p; in this case, 32 bp of *Schizosaccharomyces pombe* telomeric DNA was placed upstream of a *LacZ* reporter gene in *S. cerevisiae*, and an *S. pombe* cDNA-GAL4 activation domain (AD) fusion library was screened for proteins that bound to the telomere sequence. The identification of Taz1p as a telomere-binding protein was corroborated by the increase in telomere length and decrease in telomeric silencing observed in an *S. pombe* strain disrupted for *taz1* (16). A one-hybrid assay has also been used to identify a protein that binds to *Drosophila* pericentromeric dodeca-satellite repeats; six copies of the 12-bp dodeca-satellite sequence were placed upstream of *HIS3* and *LacZ* reporters and used to screen a *Drosophila* cDNA library (17).

### 1.2. In Situ One-Hybrid Assays

The assays described in **Subheading 1.1.** take part of a *cis*-acting sequence out of its normal chromosomal context and place it next to a reporter gene. However, it is also possible in some cases to use a fully functional *cis*-acting site to identify binding in the natural chromosomal context. The use of authentic sites, as opposed to a partial site taken out of context, makes it more likely that all the proteins that normally associate with the site will be present. This type of assay has been used to detect *S. cerevisiae* telomere-binding proteins (3) and *S. cerevisiae* centromere-binding proteins (4).

In the telomere one-hybrid assay, a *HIS3* reporter gene with a minimal promoter is integrated at the left telomere of chromosome VII. In the plasmid used for construction of the strain, a 71-bp C<sub>1-3</sub>A sequence is located upstream of *HIS3* (**Fig. 2A**). The *URA3* selectable marker and homology to the *ADH4* gene, which is located about 20 kb from the native telomere, are downstream of the reporter. When this construct is integrated at the *ADH4* locus (**Fig. 2B,C**), the 71-bp sequence acts as a seed to form a functional telomere on the truncated chromosome. A chromosome modified in this way has been shown to have a loss rate indistinguishable from that of a wild-type chromosome (18) and, therefore, may be considered to be a fully functional telomere with all its associated proteins. In addition to this HIS-Tel strain, control strains are used that contain the same *HIS3* reporter gene integrated internally on the chromosome, within the *ADH4* locus, either with (HIS-Int-CA) or without (HIS-Int) 276 bp of telomeric sequence upstream of the promoter (**Fig. 2D**). The *HIS3* reporter has only a minimal promoter, so the strain is initially His<sup>-</sup>. To confirm specificity of binding, a telomere protein fused to a TAD should be able to activate *HIS3* expression in the HIS-Tel strain but not in the HIS-Int strain. The *HIS3* reporter is subject to silencing by the telomere position effect (TPE), but this silencing can be overcome by the presence of a TAD (discussed in more detail in **Subheading 1.4.**, p. 250).

To test the validity of such an approach, the known telomeric DNA-binding protein Rap1p (19) was shown to be strongly positive in the assay. The system

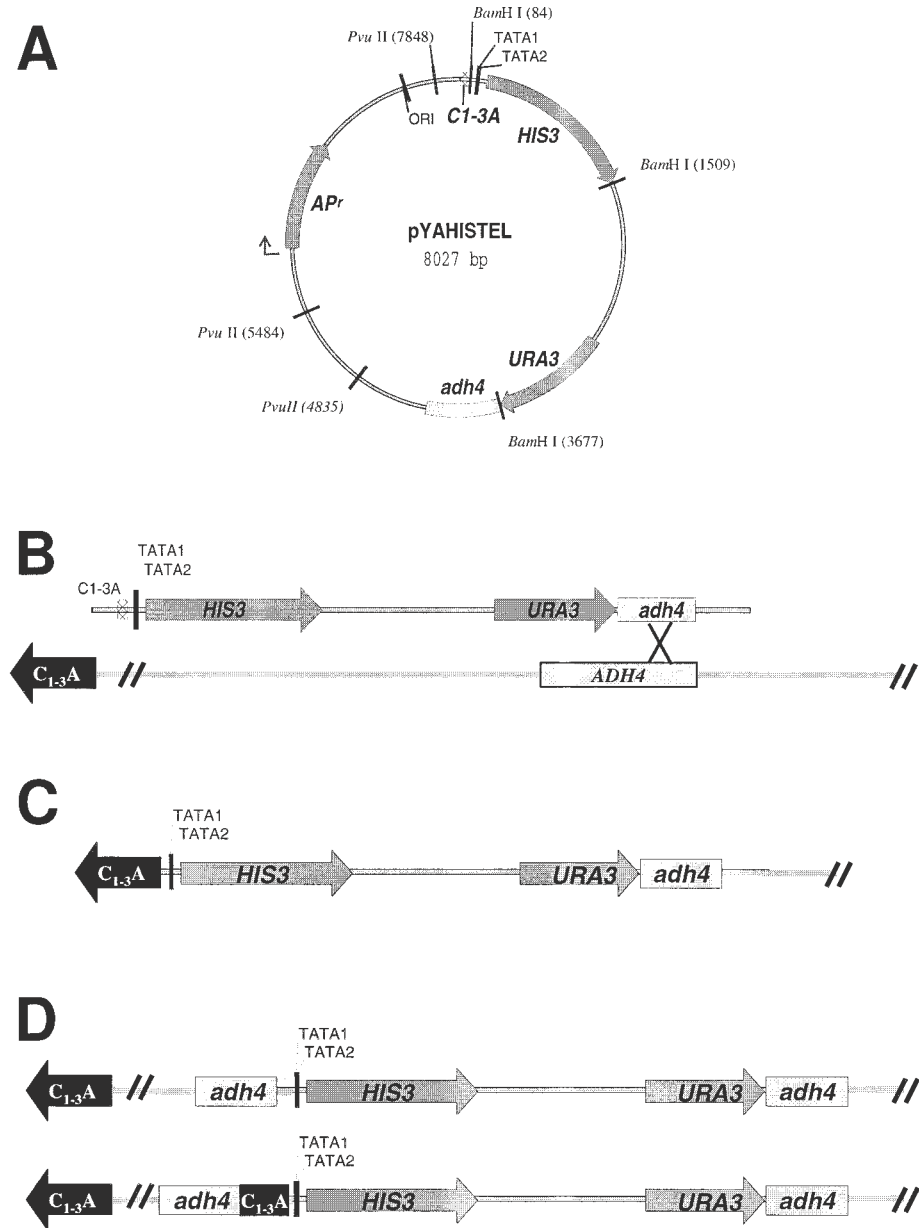


Fig. 2. Construction of HIS-Tel reporter strain. (A) Integrative plasmid pYAHISTEL. For integration, this plasmid is digested with *Pvu*II and transformed into a *his3 ura3* strain using the method of Gietz et al. (43) or a similar protocol. Integrants are selected on YC –Ura plates and checked by Southern blotting for the correct structure. (B) Integration occurs at the *ADH4* locus, about 20 kb from the left



was next used to demonstrate that the silencing proteins Sir2p, Sir3p, and Sir4p; the telomere length regulators Rif1p and Rif2p, and the single-stranded binding protein Cdc13p bind yeast telomeres in vivo. By comparing activation of the reporter in the HIS-Tel and HIS-Int-CA strains, those proteins whose binding requires the physical end of the chromosome (e.g., Cdc13p, which binds to the single-stranded overhang at the end of the chromosome) can be distinguished from the majority of telomere proteins, which can also bind to telomeric sequences at an internal site on the chromosome. The *LacZ* gene can also be used as a reporter, providing the ability to do either a quantitative assay (Fig. 3) or a color assay (Fig. 4) for enzyme activity (BDB and VAZ).

A second one-hybrid system that used a fully functional *cis*-acting site upstream of the reporter gene is a screen to identify *S. cerevisiae* kinetochore proteins (4). Since yeast centromeres are only 125 bp long, it is possible to use an entire centromere in the assay. In this experiment, the entire *CEN3* sequence from chromosome III was cloned upstream of a *HIS3* reporter gene, and this cassette was used to replace the endogenous *CEN3* DNA in the test strain, so that it provides the only centromere on chromosome III. As a control for the specificity of binding, another strain was used in which a mutant *CEN3* sequence, carrying a single base-pair change that renders the sequence non-functional in yeast, was placed in front of the reporter gene and integrated at the *LYS2* locus. The system was tested using AD fusions of the known kinetochore proteins Cbf3ap, Cbf3bp, Mif2p, and Cse4p, all of which were able to activate *HIS3* expression in the test strain with wild-type *CEN3* sequence, but not in the control strain carrying a mutant *CEN3* sequence. These strains were then used to screen a genomic library and a cDNA library for protein components of the yeast centromere. In addition to the known centromere protein Cbf3ap, three novel centromere proteins were identified by this screen: Ctf19p, Mcm21p, and Okp1p (4).

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Fig. 2. (*continued*) telomere of chromosome VII. (C) HIS-Tel. The 71-bp C<sub>1-3</sub>A tract acts as a seed for the addition of a new telomere adjacent to the *HIS3* reporter gene (29). This strain is used to test the telomere-binding ability of AD fusion proteins. (D) HIS-Int and HIS-Int-CA. Plasmids for construction of the HIS-Int and HIS-Int-CA strains have homology to the 5' end of *ADH4* either in place of or upstream of the C<sub>1-3</sub>A sequence, so that these constructs are integrated within the *ADH4* locus; the length of the C<sub>1-3</sub>A sequence in HIS-Int-CA is 276 bp (3). The HIS-Int strain is used as a negative control to eliminate proteins that might interact with DNA nonspecifically. The HIS-Int-CA strain can be used to test whether a given protein requires the physical end of the chromosome for its interaction with the telomere, or whether the presence of double-stranded telomeric DNA is sufficient for the interaction.

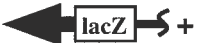

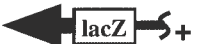



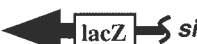
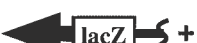

Row		$\beta$ -gal Activity ( $\times 10^{-3}$ nmol/min $\times 10^7$ cells)	
		RAP1	RIF1
1	 + 	<1 <1	9 +/- 0.00 8 +/- 0.00
2	 + 	99 +/- 4.50 56 +/- 2.05	48 +/- 0.47 34 +/- 0.94
3	 + 	2 +/- 0.94 <1	11 +/- 1.25 10 +/- 0.00
4	 <i>sir3::LYS2</i>	14 +/- 0.00 3 +/- 0.00	17 +/- 0.00 35 +/- 0.00
5	 +  <i>sir3::LYS2</i>	161 +/- 3.40 75 +/- 3.86	134 +/- 3.86 124 +/- 1.25

Fig. 3. Quantitative LacZ assays. AD fusions of Rap1p and Rif1p gave detectable results in the assay; Sir2-Actp, Sir3-Actp, Sir4-Actp, and Cdc13-Actp did not. Assays were done in wild-type *LacZ*-Tel and *LacZ*-Int strains as well as in a *LacZ*-Tel strain with a *sir3::LYS2* insertion mutation (44) (unpublished results). Rap1-Actp in *LacZ*-Tel showed a >50 to 100-fold increase in  $\beta$ -galactosidase levels compared to vector alone (RAP1 column, cf. rows 1 and 2) or when the *lacZ* gene was internal (cf. rows 2 and 3). Similarly, expression of Rif1-Actp in *LacZ*-Tel caused a three- to sixfold increase in *lacZ* expression relative to vector alone (RIF1 column, cf. rows 1 and 2), and a three- to fivefold increase relative to expression from the *lacZ* gene at an internal location (cf. rows 2 and 3). Because of loss of TPE, background levels of *lacZ* expression were increased in a strain carrying an insertion mutation in the *sir3* gene (*sir3::LYS2*); however, both Rap1-Actp and Rif1-Actp were still able to activate transcription above this background level (cf. rows 5 and 4). The strain and protein used in each case are shown at the right. The strain used is either the test strain *LacZ*-Tel or the control strain *LacZ*-Int. The protein expressed is either the AD alone (Act), an AD-telomere protein fusion (X-Act), or the telomere protein alone (X), as indicated.

### 1.3. Utility of the One-Hybrid Assay

Perhaps the most obvious use of the one-hybrid assay, as with the two-hybrid assay, is in screening AD fusion libraries for new binding proteins, as has been done in many cases to look for transcriptional regulators. The telom-

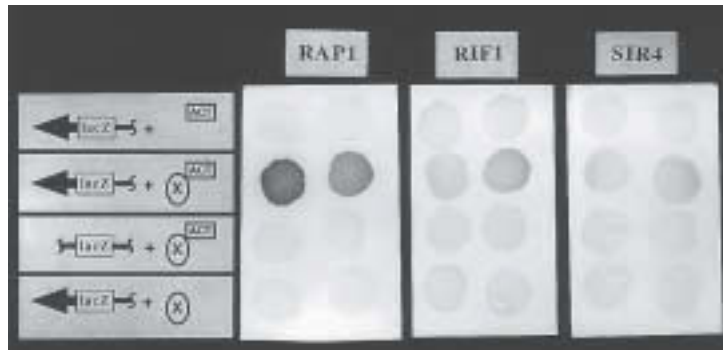


Fig. 4. LacZ color assays: *LacZ* filter assays with Rap1p, Rif1p, and Sir4p.  $\beta$ -Gal assays were performed according to the method of Breeden and Nasmyth (41). Rap1-Actp, Rif1-Actp, and Sir4-Actp activated reporter expression in the *LacZ*-Tel strain to a greater extent than the vector control (cf. rows 1 and 2); this activation was dependent on the presence of telomeric sequences upstream of the reporter (row 3) and on the presence of an AD (row 4). Sir3-Actp also gave a positive result, but the color was too faint to be reproduced photographically. Strains and proteins are indicated as in Fig. 3.

ere one-hybrid assay has been used to screen two yeast libraries, and a third screen is in progress (unpublished data). The first screen used a genomic library in the vector pJG4-5 (20); 1.38 million transformants were screened, and 26 positive clones were identified. Of these, 10 were the known telomere-binding proteins Rif1p (2 clones) or Sir4p (8 clones). One clone was also isolated that contained part of the *SIR4* gene in an inverted orientation. Overexpression of the carboxyl terminus of Sir4p is known to reduce TPE (21); thus, if this gene were somehow being expressed, it would be expected to give a false positive. The remaining eight clones contained either out-of-frame fusions or no open reading frame (unpublished data). The second screen utilized a yeast genomic library in the pGAD-1, -2, and -3 vectors (22); eight million transformants were screened, giving six positives, all of which contained the known telomere protein Rap1p (unpublished data). In the centromere one-hybrid assay, 80 million transformants were screened, and 120 positive clones were isolated, of which 30 plasmid-dependent clones were sequenced. The positive clones included Okp1p, Mcm21p, Ctf19p, Cbf3p, and the false positive Atr1p; the remaining 62 plasmid-dependent clones also carried one of these genes, as determined by polymerase chain reaction analysis.

As discussed, the inverse type of screen may also be done, in which a library of potential binding sites is placed upstream of the reporter gene and the sites are screened for their ability to interact with a known fusion protein (8,9). This method has been used to identify *Drosophila* genes regulated by *Ultrabithorax* (23).

Once a binding protein is identified, the one-hybrid system can be used to determine requirements for binding. For instance, deletion derivatives of the fusion protein can be constructed and tested in the one-hybrid assay in order to localize the domain necessary for binding. The mapped domain could either be a DNA-binding domain (DBD) or a protein-protein interaction domain. An example of the latter is the testing of deletion derivatives of the human high-mobility group factor SSRP1 for interaction with its *cis* site, a CArG box, through its binding to serum response factor (5). For a protein that binds DNA directly, the one-hybrid assay can be used to determine *in vivo* sequence requirements for binding by using different mutant binding sites. For a yeast protein that interacts with the target sequence through protein-protein interactions, binding requirements can be investigated through the use of strains mutant for other proteins that are involved in the same process or structure. For example, when the telomere one-hybrid assay is performed in a HIS-Tel *sir3* $\Delta$  strain, binding of Sir2p to the telomere is lost, whereas binding of Rap1p, Rif1p, Rif2p, Sir4p, and Cdc13p to the telomere is unaffected (3); thus, only Sir2p requires Sir3p to bind to the telomere. By using a quantitative LacZ assay to measure binding of Rap1p and Rif1p AD fusions to the telomere in a LacZ-Tel strain (Fig. 3), it can be seen that the binding of Rif1p to the telomere actually increases in the absence of Sir3p more than can be accounted for by the decrease in TPE, as might be expected if the Rif proteins compete with the Sir proteins for binding (24).

#### 1.4. Limitations

One of the most significant limitations of the one-hybrid assay is that, as with the two-hybrid assay, there are many possible sources of false negatives. For instance, the hybrid protein may be unable to fold correctly, or the accessibility of either the AD or the binding portion of the test protein may be blocked, especially if the protein is part of a multiprotein complex. This lack of accessibility may be a problem for the kinetochore protein Cbf3bp, which does not activate the *HIS3* reporter even though the fusion protein is functional and other proteins in the same complex give a positive signal in the assay (4). Misfolding may explain the inability of a Cbf3dp-AD fusion to complement a *cbf3d* mutation and its inability to activate the reporter gene (4). Even if the hybrid protein is able to interact with the sequence being tested, it may not compete well with the endogenous, full-length protein; in some cases, this problem can be circumvented by deleting the endogenous gene. Furthermore, testing a carboxyl-terminal AD fusion in addition to the more common amino-terminal fusions might circumvent any of the preceding problems, but this is impractical in the context of a large-scale screen.

Proteins that are expressed only at a certain point in the cell cycle or under certain conditions may require accessory proteins or modifications that are not present in mitotically dividing cells. For example, an AD fusion of the meiosis-specific protein Ndj1p does not activate a *HIS3* reporter gene in the telomere one-hybrid assay (unpublished data), even though the wild-type protein is localized to meiotic telomeres by immunofluorescence (25,26). This result may indicate that other meiotic proteins are needed to localize Ndj1p or that some meiosis-specific modification of the protein is required. In some cases, this problem may be solved by using the double interaction screen described in **Subheading 1.**, in which a protein required for the interaction is expressed along with the fusion protein being tested.

Another limitation is that transient interactions may be difficult to detect in the one-hybrid assay. For example, in the telomere one-hybrid assay, an Est2-Actp fusion protein does not increase *HIS3* transcription over background levels, even though, as the catalytic subunit of telomerase, this protein must be present at telomeres at least part of the time (3). Finally, some fusion proteins may be toxic to the cell, especially when overexpressed; the growth defects caused by overexpression of the hybrid protein may obscure any activation of the reporter gene. This last problem is likely to be at least a partial explanation for the weak activation of *HIS3* by a Sir2-Actp fusion in the telomere one-hybrid assay (3). Overexpression of Sir2p is known to be toxic (27); this effect was confirmed by the slow growth of strains expressing Sir2p or Sir2-Actp when grown on plates selecting only for the expression of the fusion protein (**Fig. 5**) (3).

False positives can also be a problem, but in a screen using *HIS3* as a reporter, most can be eliminated by increasing the stringency through an increase in the amount of 3-amino-1,2,4-triazole (3-AT) in the test plates. 3-AT is a competitive inhibitor of the *HIS3* gene product, so adding it to the plates means that a higher level of *HIS3* expression is required for growth (28). The drawback to this solution is that some weaker interactions may be missed when the stringency is increased. In the telomere one-hybrid assay, proteins that reduce telomere position effect when overexpressed, such as Sir4p, are potential false positives. These false positives can be eliminated by testing whether activation of *HIS3* is dependent on the presence of an AD; a true positive will activate the reporter to a greater extent when a fused AD is present. Note that some proteins, such as Sir4p, can be true binding proteins even though they promote some level of reporter gene expression in the absence of an AD. In the case of Sir4p in the telomere one-hybrid assay, this point was demonstrated by the fact that the amount of activation is greater when a Sir4p AD fusion protein is expressed than when the wild-type protein, with no AD, is overexpressed (**Fig. 6B**) (3). Another potential

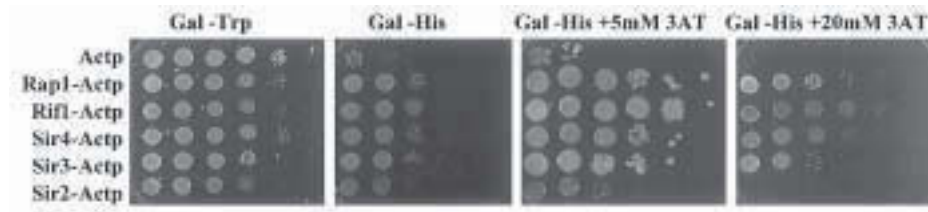


Fig. 5. Activation of His-Tel by telomere-binding proteins. Spot tests of HIS-Tel with vector control (Actp) or with Rap1p, Rif1p, Sir4p, Sir3p, or Sir2p AD fusions are shown. Tenfold serial dilutions were plated on YC -Trp + 3% galactose as a plating control, or on YC -His + 3% galactose + 0, 5 mM, or 20 mM 3-AT to select for activation of the reporter gene. All fusion proteins tested activated the *HIS3* reporter to a greater extent than vector alone on Gal -His + 0 mM or 5 mM 3-AT; Sir2-Actp was not able to grow on Gal -His + 20 mM 3-AT. Note that Sir2-Actp-expressing cells grow slowly even on Gal -Trp control plates.

false positive, isolated in the centromere one-hybrid assay, is Atr1p, which confers resistance to 3-AT when overexpressed (4).

Each particular target sequence may also introduce specific considerations. In the case of the telomere one-hybrid assay, one concern is that the reporter gene may be silenced by TPE (29). To minimize this potential problem, the promoter of the *HIS3* or *LacZ* reporter was positioned so that it would be in a region near the junction between the telomeric repeats and the subtelomeric DNA that is accessible to both HO endonuclease (18) and *Escherichia coli* dam methylase (30) in vivo. Nonetheless, the reporter gene was still subject to TPE, as can be seen by comparing background levels of expression in a wild-type HIS-Tel strain to a silencing-defective HIS-Tel *sir3* $\Delta$  strain. However, this silencing was overcome by the presence of a transcriptional activator, such as a Rap1-AD fusion (3). To correct for different levels of background *HIS3* expression, greater amounts of 3-AT were added to the test plates for strains that lack TPE, such as a *sir3* $\Delta$  strain.

Another problem specific to the telomere one-hybrid assay is that the  $C_{1-3}A$  sequences are able to activate gene expression by themselves (31), presumably because this sequence is bound by the Rap1p protein, which can act as a transcriptional activator (32). This is supported by the fact that overexpression of the carboxyl terminus of Rap1p is able to activate reporter gene expression independently of the presence of a fused AD (unpublished data). Activation of the one-hybrid reporter gene by Rap1p becomes dependent on the AD fusion when a construct is used that retains the DBD but lacks the endogenous AD (3). Because *RAP1* is an essential gene in *S. cerevisiae* (32), it is not possible to reduce the background level of expression by deleting this gene. The enhancer

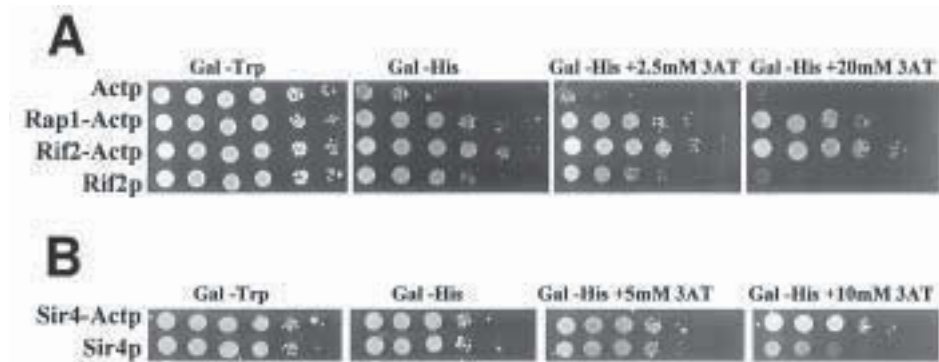


Fig. 6. 3-AT concentration can be used to modulate the sensitivity of the HIS-Tel reporter strain. Tenfold serial dilutions were plated on YC –Trp + 3% galactose as a plating control, or on YC –His + 3% galactose + 3-AT at the concentrations indicated to select for activation of the reporter gene. (A) Vector control, Rap1-Actp, Rif2-Actp, and Rif2p in HIS-Tel. Rif2-Actp grew ~10X better than Rap1-Actp on plates containing 3-AT. Overexpression of Rif2p increased reporter gene expression on Gal –His + 0 mM or 2.5 mM 3-AT, presumably through an effect on TPE; this background was eliminated by adding 20 mM 3-AT to the plates. (B) Sir4-Actp and Sir4p in HIS-Tel. Sir4p activated *HIS3* expression to about the same level with and without an AD fusion when assayed on plates containing 0 or 5 mM 3-AT; at concentrations of 3-AT  $\geq 10$  mM 3-AT, Sir4-Actp can be seen to grow more quickly than Sir4p.

effects of telomeric sequences likely explain why there is a higher level of background expression in the HIS-Tel and HIS-Int-CA test strains than in the HIS-Int control strain.

Although the *in situ* one-hybrid assay, as described in **Subheading 1.2.**, has advantages over a more artificial system, it may not be practical in all cases. *Saccharomyces* telomeres and centromeres are well suited for this type of assay because they are well-defined, relatively short elements (~300 bp for the telomere and 125 bp for the centromere). A larger *cis*-acting sequence might have to be broken down into smaller regions in order for a fusion protein bound to the *cis*-acting site to be able to access the promoter of the reporter gene. In addition, this type of assay is limited to sequences endogenous to the organism being used for the assay, in all cases to date, *S. cerevisiae*.

### 1.5. Future Directions

There are additional ways in which one-hybrid assays may be used. Other possible sites in *S. cerevisiae* that could be used in their entirety in a one-hybrid assay include boundary elements (33,34) and silencers. A telomere one-hybrid assay similar to that described here should be possible in any system in



which a reporter can be integrated next to a telomere seed sequence, including *S. pombe* (35) or mammalian tissue culture cells (36,37). Similarly, a functional *ARS* in *S. cerevisiae* or *S. pombe* could be used as the target site in a one-hybrid assay.

## 2. Materials

### 2.1. Reporter Genes

We have used two versions of the telomere one-hybrid assay, one with a *HIS3* reporter (described in **Subheading 1.2.** and in **ref. 3**) and one with a *LacZ* reporter (**Figs. 3** and **4**). The selectable *HIS3* marker, besides being more useful for a large-scale screen, proved to be a more sensitive reporter; fusion proteins that were weakly positive with a *HIS3* reporter appeared negative with the *LacZ* reporter (**Fig. 5** and data not shown). However, an advantage of the *LacZ* reporter gene is that it is easier to quantitate the results by doing enzyme assays (**Fig. 3**), although quantitation is only possible for fusions such as Rap1p and Rif1p that activate the reporter strongly. While the *HIS3* reporter can be weakly expressed in the absence of an activator protein, this background can be reduced by adding 3-AT, a competitive inhibitor of the *HIS3* gene product, to the selective plates. This also gives the system more flexibility; conditions that lead to an increased background level of reporter expression can be compensated for by adding 3-AT to the test plates. Chromosomal context can play a significant role in the amount of background expression; in the telomere one-hybrid assay, *HIS3* expression is greater in the HIS-Tel and HIS-Int-CA strains than in the HIS-Int strain, probably because the telomeric sequences act as a UAS in these strains. By contrast, the level of background expression in the centromere one-hybrid assay is lower, because the *CEN3* sequence on its own does not appear to increase transcription of the reporter gene (4). The amount of telomere position effect in the strain also affects background expression of the reporter; the amount of expression in a *sir3*Δ mutant of the HIS-Tel strain is much greater than in a wild-type HIS-Tel strain (3).

### 2.2. Yeast Strains, Libraries, and Plasmids

Any plasmids designed for expression of the prey protein of a two-hybrid assay (i.e., a fusion to a TAD) should be suitable for a one-hybrid assay as well. For the telomere one-hybrid assay as described here, the selectable marker on the fusion protein-expressing plasmid must be something other than *HIS3* or *URA3*. The *URA3* marker is no longer needed after integration of the *HIS3* reporter has been accomplished, but because it is subject to telomeric silencing, it cannot be easily recovered by selecting for spontaneous *ura3* mutants on



5-fluoro-orotic acid. We have used expression plasmids with either a *TRP1* or a *LEU2* marker. The promoter and AD of the expression plasmid will affect the choice of background strain for the assay. A plasmid or library with a galactose-inducible promoter requires the presence of wild-type *GAL4* and *GAL80* for its regulation; on the other hand, a plasmid or library that uses a *GAL4* AD requires a strain that is mutant for *gal80*, to allow activation in the absence of galactose (38). The telomere one-hybrid assay has been used in two backgrounds: YM701 (M. Johnston), a *GAL4 GAL80* strain, and B711 (unpublished data), a *leu2* derivative of YM710 (M. Johnston), a *gal4 gal80* strain. The former strain can be used with the pJG4-5 vector (20), and the latter may be used with the pGAD family of vectors (22). Convenient features of the pJG4-5 vector include galactose inducibility and a hemagglutinin tag fused to the protein, to allow confirmation that the hybrid protein is expressed and stable. The library itself can consist of either genomic DNA or cDNA; genomic DNA has the advantage of not being biased toward highly expressed proteins.

### 2.3. Media

Strains carrying AD fusion plasmids are grown on selective plates, YC –Trp or YC –Leu, with 2% glucose. To test for activation of the *HIS3* reporter, cells are grown overnight in the same selective medium (for constitutively expressed fusion proteins) or in selective medium with 3% raffinose (to remove glucose repression for galactose-inducible fusion proteins). Five microliters each of 10-fold serial dilutions of these cultures are plated on YC –His + 2% glucose or YC –His + 3% galactose, respectively (Figs. 5 and 6). These –His test plates may also be supplemented with 3-AT to reduce background *HIS3* expression. The same dilutions are plated on YC –Trp with glucose or galactose to control for viability and growth rate, both of which may be affected by overexpression of the fusion protein. When testing specific fusion proteins, duplicate plates of at least two independent transformants are tested on at least two different days.

The amount of 3-AT needed to bring growth of the vector-only control down to the first one or two dilutions is dependent on the particular strain being tested and also varies somewhat among different batches of 3-AT. In addition, the 3-AT seems to lose its potency over time, such that a strain will grow better on the same batch of plates when the plates are a couple of months old than when the plates are freshly made. One-hybrid assays reported in the literature use from 5 (4,39) to 50 mM 3-AT (17). For the telomere one-hybrid assay, 0–20 mM 3-AT is needed for a wild-type strain, and 35–50 mM 3-AT for a strain lacking TPE. The amount of 3-AT needed must be determined empirically, and it is usually best to use a range of concentrations when possible (e.g., see Fig. 5).

1. YC medium (for 1 L): 1.2 g of yeast nitrogen base (without amino acids), 6 g of NaOH, 5 g of  $(\text{NH}_4)_2\text{SO}_4$ , 10 g of succinic acid, 0.8 g of 5 dropout amino acid mix (see **item 2**). Add as needed 0.10 g of adenine, 0.05 g of histidine, 0.10 g of tryptophan, 0.10 g of uracil, and 0.10 g of leucine. For plates, add 20 g of bacto-agar. Bring to 900 mL with ddH<sub>2</sub>O; dissolve before autoclaving. In a separate flask, dissolve 20 g of glucose or 30 g of galactose or raffinose in 100 mL dH<sub>2</sub>O. Autoclave for 30 min for 1 L, 45 min for 2–3 L. Add autoclaved sugar solution to the medium (**40**).
2. Amino acid 5 dropout mix (for 50 L of plates): 5.0 g of lysine, 2.5 g of serine, 2.5 g of phenylalanine, 2.5 g of tryptophan, 2.5 g of valine, 5.0 g of cysteine, 2.5 g of methionine, 5.0 g of threonine, 2.5 g of aspartate, 5.0 g of arginine, 2.5 g of isoleucine, and 2.5 g of proline. Mix amino acids in a 100-mL bottle. Shake well.

### 3. Methods

#### 3.1. $\beta$ -Galactosidase Assays

$\beta$ -Galactosidase assays are performed using a filter-lift assay essentially as described by Breeden and Nasmyth (**41**), except that cells are picked from fresh growing colonies into 20  $\mu\text{L}$  of water and spotted onto nitrocellulose filters atop plates selecting for plasmid maintenance (**Fig. 4**). Galactose plates are incubated for 2 d and glucose plates 1 d at 30°C. The  $\beta$ -galactosidase reaction dishes contain 2.5 mL of Z buffer (**41**) with 0.75 mg of 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (Sigma, St. Louis, MO). Reactions are allowed to proceed for 2 d at 30°C. Liquid  $\beta$ -galactosidase assays (**Fig. 3**) are performed as described (**42**). Each experiment is performed at least twice.

#### 3.2. Controls

Several kinds of controls are used to ensure specificity and eliminate false positives. As mentioned, all strains are grown on control plates that select only for fusion protein expression to control for plating efficiency and to detect any effects of the overexpression of the fusion protein on growth rate. Two negative controls are used when testing a specific protein: the AD alone is expressed (vector control), or the protein is expressed with no AD. In the case of a telomere system, the latter is probably a better control for determining whether a given protein is binding to the target site, because it takes into account any reduction in TPE caused by overexpression of the protein. The AD alone appears to have some ability to activate the *HIS3* reporter in the telomere one-hybrid assay, because fusions that do not activate often grow more poorly than the vector control. When using vectors that express the fusion protein from a galactose-inducible promoter, it is also possible to determine whether activation depends on fusion protein expression by plating on both glucose- and galactose-containing media. However, in some cases, the very low level of

expression of proteins that occurs from the *GALI* promoter in glucose-grown cells may be sufficient for activation. Generally, both a negative and a positive control are included on every plate; for the telomere one-hybrid assay, these are usually vector alone and Rap1-Actp (**Figs. 5 and 6**). Finally, the target site should be changed to test the specificity of binding. Ideally, this would involve using a point mutation in the site that disrupts both the function of the site and binding of the proteins being tested, as was done in the centromere one-hybrid assay (**4**). In the telomere one-hybrid assay, a point mutation is not feasible owing to the repetitive nature of the sequence; instead, the control strain contained the *HIS3* reporter gene integrated internally on the chromosome (*HIS-Int*), with no telomeric sequence upstream of the promoter (**Fig. 2**).

### 3.3. Interpreting Results

Activation of the *HIS3* reporter is assayed by comparing growth of strains carrying empty vector with that of strains expressing fusion proteins (**Figs. 5 and 6**). For fusion proteins that are positive in the one hybrid assay, two kinds of growth difference are observed: a greater percentage of cells may form colonies, and individual colonies may grow more quickly than the control strain. For example, in the telomere one-hybrid assay, Rif2-Actp in the *HIS-Tel* strain consistently grows at a greater dilution than the vector control; close to 100% of the cells that grow on control plates are also able to grow on test plates (**Fig. 6A**, Gal –His). Sir4-Actp and Sir4p both grow to a greater dilution than the vector control (**Fig. 6B**, cf. Gal –His Actp to Sir4-Actp and Sir4p). The reduction in TPE caused by overexpression of Sir4p (**21**) probably accounts for the apparent activation of the reporter gene in the absence of an AD fusion. However, when the level of 3-AT in the test plates is high enough, cells expressing Sir4-Actp grow more quickly than those expressing Sir4p, although the former does not always grow to a greater dilution than the latter (**Fig. 6**, Gal –His + 10 mM 3-AT). These differences may be caused by the number of fusion proteins binding to the site, the stability or transience of the interactions, and the ability of the fusion protein to compete for binding with the endogenous protein.

In addition to reducing the background, 3-AT can be used to estimate the strength of an interaction. The fact that Sir2-Actp-expressing cells will stop growing on a lower amount of 3-AT than cells expressing Rif1-Actp (**Fig. 5**, 5 and 20 mM 3-AT) may indicate that Rif1p binds more strongly to the telomere. These comparisons must be done on the same batch of plates, on the same day, owing to the differences in 3-AT effectiveness described in **Subheading 2.3**. However, a difference in binding affinity is only one possible interpretation of observed growth differences; to rule out the possibility that the differences are owing to impaired functioning of one of the fusion proteins, this interpretation must be confirmed using biochemical methods.

### 3.4. Conclusion

One-hybrid assays provide a powerful method for identifying and analyzing proteins that interact with specific DNA sequences. In particular, one-hybrid assays that use a fully functional site have several advantages over those that use partial or artificial sites. Two published examples of this type of assay are the telomere and centromere one-hybrid assays. We have discussed here some of the practical considerations that apply to the telomere one-hybrid assay in particular, as well as to one-hybrid assays in general.

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## The Split-Hybrid System

*Uncoding Multiprotein Networks and Defining Mutations That Affect Protein Interactions*

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Richard H. Goodman, and Merl F. Hoekstra**

### 1. Introduction

Considering that intricate and regulated processes occur in the cell, it is not surprising that complex multiprotein networks mediate these processes. Characterization of protein-protein interactions has contributed to virtually all aspects of cellular biology. As research in these fields continues to advance and as genome and mass spectrometry sequencing efforts identify novel proteins, the study of protein-protein interactions will remain paramount to understanding cellular events.

Many techniques are useful in characterizing protein-protein interactions. Fields and Song (*1*) first introduced the yeast two-hybrid assay to allow investigators to study known protein-protein interactions and identify novel binding partners. This assay capitalizes on the observation that many eukaryotic transcription factors consist of separable functional domains: a DNA-binding domain (DBD) and a transcription activation domain (TAD). In the yeast two-hybrid assay (**Fig. 1**), one gene expressing a protein of interest is fused to a DBD while a second gene (or a cDNA library) is fused to a TAD such as *GAL4* or VP16. Interaction of the two proteins reconstitutes a functional transcription factor that can activate the expression of reporter genes. The reporter genes generally encode enzymes required for growth (*HIS3*, *LEU2*, or *URA3*), enzymes that can be detected colorimetrically (*lacZ*), or those detected visually or luminometrically (GFP). Identifying protein-protein interactions by

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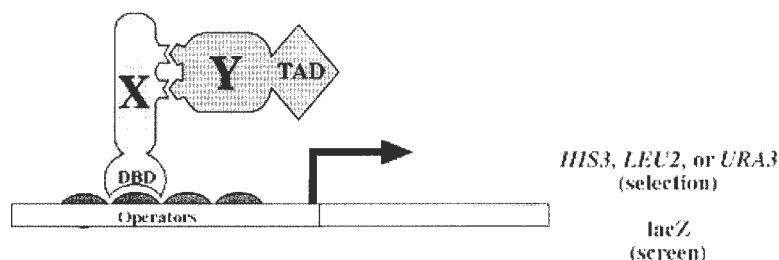


Fig. 1. Yeast two-hybrid system. Interaction of protein X fused to a DBD with protein Y fused to a TAD activates expression of nutritional (*HIS3*, *LEU2*, *URA3*) and colorimetric (*lacZ*) reporter genes.

growth selection, color, or light is fast, simple, inexpensive, and a likely reason for the yeast two-hybrid system to become a method of choice for studying protein-protein interactions. Since its inception, many modifications have been made to the yeast two-hybrid system. False positives from library screens have been decreased by incorporating a two-reporter gene system (2), and methods to screen libraries of proteins fused to the DBD have been developed (3).

Many variations of the basic two-hybrid have been devised. For instance, in one-hybrid systems, proteins that bind to specific DNA sequences can be identified by screening cDNA-TAD fusion libraries against a reporter gene driven by that particular DNA sequence (4,5). One three-hybrid assay screens for small ligands that interact with known receptors or for receptors that interact with known ligands (6), and another three-hybrid system detects RNA-protein interactions (7).

The yeast two-hybrid system can also be used to identify peptide aptamers that bind to a given protein (8,9). Large combinatorial peptide libraries can be screened rapidly in an *in vivo* setting, proving advantageous over other *in vitro* peptide-screening methods. Identified peptides may provide insight into protein-binding domains and, more important, may also be useful as inhibitors of protein-protein interactions. While most of the current hybrid technology concentrates on characterizing protein-protein interactions, the ability to study the disruption of protein-protein interactions should provide additional valuable information about protein networks.

Two recent technologies take a direct approach at identifying factors that disrupt protein-protein interactions. The split-hybrid (10) and reverse two-hybrid systems (11–14) convert the disruption of a protein-protein interaction into a positive selection. The split-hybrid (Fig. 2) employs many of the same components of the conventional two-hybrid system. However, the split-hybrid is a binary system that incorporates the *Escherichia coli* *tet*-repressor (TetR)

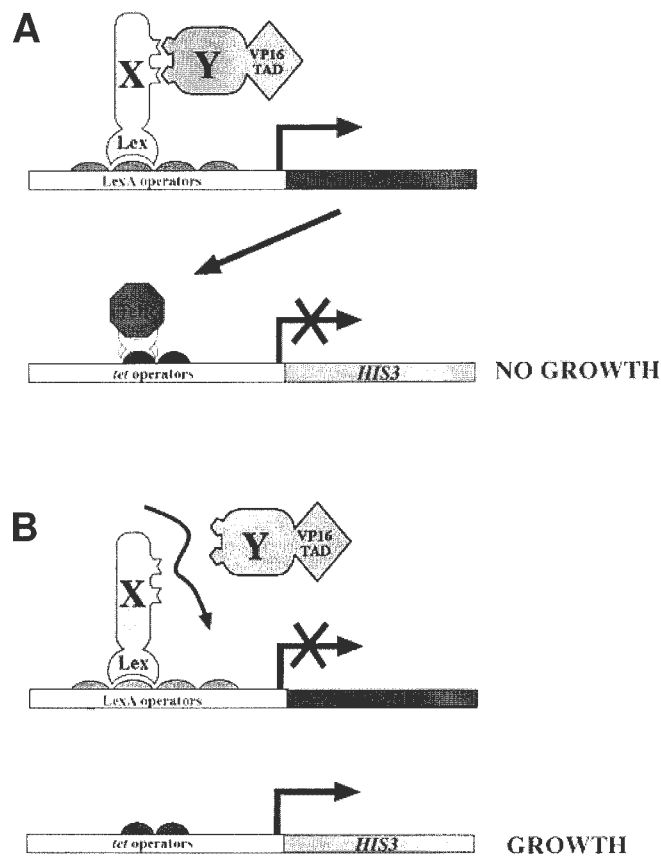


Fig. 2. Split-hybrid system. (A) Interaction of protein X fused to LexA with protein Y fused to the VP16 TAD activates the expression of TetR. By binding to *tet* operators located in the promoter of the *HIS3* gene, TetR prevents the expression of the *HIS3* gene product, blocking the growth of yeast in medium lacking histidine. (B) Disruption of the LexA-X and VP16-Y interaction prevents TetR expression, allowing *HIS3* expression and growth of yeast in medium lacking histidine.

operator system. Here, LexA operators are located in a yeast promoter that drives expression of the TetR gene. The interaction of one protein fused to the LexA DBD with a second protein fused to the VP16 TAD activates TetR, which subsequently binds to *tet* operators located upstream of *HIS3*. Binding of TetR to the *tet* operators represses *HIS3*, preventing yeast growth in the absence of histidine. If the protein-protein interaction is disrupted, no TetR protein is made, the *HIS3* gene is transcribed, histidine is produced, and yeast grow in the absence of histidine.

## 2. Materials

### 2.1. Yeast Strains and Media

Yeast strains are grown in yeast extract, peptone, dextrose (YPD) or selective medium using standard conditions (**15,16**). Selective medium is dropout or omission medium in which all amino acids and nucleosides are added except those required to select for specific auxotrophies/prototrophies. YPD and synthetic medium is purchased from Bio101 (Carlsbad, CA). Yeast strains YI584 and YI671 were derived from AMR69 and AMR70 (**17**). YI584 is MATa/MAT $\alpha$ , *his3 $\Delta$ 200/his3 $\Delta$ 200 trp1-901/trp1-901 leu2-3, 112/leu2-3, 112 ade2/ade2 URA3 : (LexA operator)8-TetR LYS2 : (Tet operator)2 : HIS3*. YI671 is a MAT $\alpha$  haploid derivative of YI154 that contains an *ADHI* construct driving expression of TetR.

### 2.2. Plasmids

The split-hybrid system is designed for use with lexA DBD constructs. Various plasmids, such as pBTM116 (**1**), are compatible with the system. Plasmid pVP16-lacZ is used for mutagenesis experiments. Both VP16 and GAL4 activation domains are amenable to use in the split hybrid (*see* **Note 1**).

## 3. Methods

### 3.1 Transformation and Analysis

Yeast strains are transformed as described previously (**10**) and plated on selective medium (*see* **Note 2**). After 3 d of growth, transformants are diluted in 5 mL of selective medium, vortexed, and mildly sonicated to disrupt clumps. Cells are seeded at 1000 cells/mL in selective medium. Tetracycline, 3-amino-1,2,4-triazole (3-AT), and histidine are supplemented as appropriate. Samples are incubated with shaking for 2 d at 30°C and are quantitated by measuring the OD<sub>600</sub>. The b-Galactosidase liquid assays are performed as described previously (**18**).

### 3.2 Use of the System

To date, the greatest utility of the split-hybrid system has been for mutagenesis studies (**10,19**). For this reason, we describe a general mutagenesis screen as an example of how to use the split hybrid.

#### 3.2.1. Plasmid Construction

The gene of interest should be cloned into a LexA vector. If a two-hybrid screen was previously performed, this already may have been constructed. If a new construct is being prepared, in particular for mutagenesis, we recommend plasmid pVP16-LacZ because the *lacZ* gene provides a screen for truncation or stop codon mutations.

**Table 1**  
**Split-Hybrid Plasmid Combinations<sup>a</sup>**

Transformant	Transformation medium
pLexA + pVP16-LacZ	SD–Leu–Lys–Trp–Ura
pLexA-YFG/T + pVP16-LacZ	SD–Leu–Lys–Trp–Ura
pLexA + pVP16-LacZ-YFG/B	SD–Leu–Lys–Trp–Ura
pLexA-YFG/T + pVP16-LacZ-YFG/B	SD–Leu–Lys–Trp–Ura

<sup>a</sup>YFG/T and YFG/B refer to your favorite gene target and bait. The medium given is recommended for identifying transformants because the LexA operator is integrated at URA3 and the Tet operator is integrated at LYS2. Medium lacking Ura and Lys results in minimal background growth. SD–Leu–Trp can be employed but background can be higher.

### 3.2.2. Identification of Optimal Growth Conditions for Yeast Transformants

YI584 and YI671 should be transformed with target and bait plasmids (*see Note 3*). YI671 has a strong promoter driving TetR and is used for weak interacting proteins. Controls are essential for the split hybrid and should include transformations with empty vectors. **Table 1** lists the desired plasmid combinations.

Transformants must be tested for intrinsic transactivation by the LexA fusion protein. Transformants (**Table 1**) should be tested on synthetic defined (SD) medium lacking histidine, leucine, lysine, tryptophan, and uracil (SD–His–Leu–Lys–Trp–Ura) diagnostic medium. Optimally, pLexA-YFG/T + pVP16-LacZ should show wild-type growth whereas pLexA-YFG/T + pVP16-LacZ-YFG/B should not grow. Liquid cultures are recommended and wild-type growth is measured by using complete medium containing all nutritional requirements. If growth is compromised for pLexA-YFG/T + pVP16-LacZ, this indicates that pLexA-YFG/T can affect the Tet gene and inhibit growth without disruption of pVP16-LacZ-YFG/B. To dampen this effect, YI584 should be compared to YI671 strains, or system modulation with tetracycline and 3-AT is required. If pLexA-YFG/T + pVP16-LacZ-YFG/B shows growth in diagnostic medium, background must be suppressed with 3-AT. For optimum use of the split hybrid, growth of this pair must be kept low.

### 3.2.3. System Modulation

If system modulation is required, transformants (**Table 1**) are picked, suspended in SD–His–Leu–Lys–Trp–Ura medium, and diluted to 1000 cells/mL and 100  $\mu$ L is seeded into 96-well microtiter plates. Increasing amounts of tetracycline (0–25  $\mu$ g/mL) and 3-AT (0–100 mM) are added (**Fig. 3**), and cul-

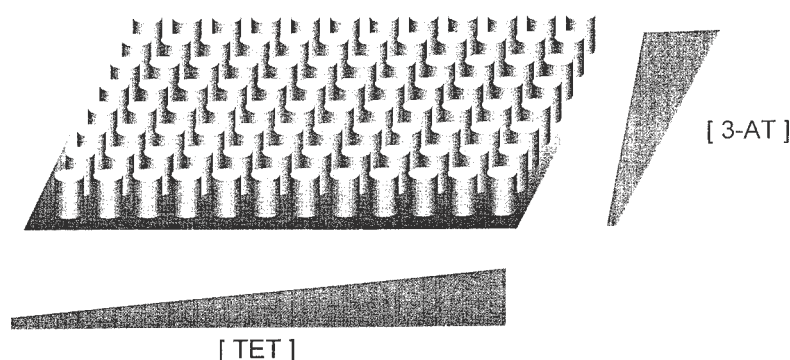


Fig. 3. Optimization grid. Yeast cells are plated into 96-well microtiter plates at a low cell concentration. Tetracycline and 3-AT are added at increasing concentrations on the X and Y grids, and optimum growth is monitored visually, microscopically with a hemocytometer, or spectrophotometrically.

tures with added histidine but without drug are included. Cells are grown with gentle rocking in a humidified incubator and are examined at 1 and 2 d for growth (*see Note 4*).

#### 3.2.4. Screening

Following mutagenesis, the pLexA-YFG/T-containing strain is transformed with a mutagenized pool of pVP16-LacZ-YFG/B. Transformants are selected on SD-His-Leu-Lys-Trp-Ura medium containing the appropriate concentration of tetracycline and 3-AT and grown until colonies form. Resulting colonies are screened for  $\beta$ -gal activity and LacZ colonies are excluded from further analysis. Plasmids are rescued into *E. coli* from positive colonies and are retransformed into both a traditional two-hybrid system (e.g., using strain L40) and the split-hybrid strain. The nonmutagenized parental plasmids should grow in the two-hybrid system but not in the split-hybrid system, whereas the mutagenized plasmid should not grow in the traditional two hybrid but grow in the split hybrid.

### 3.3. General Discussion of Split-Hybrid Assay

The TetR protein and *HIS3* components provide two points for regulation of the system. TetR binding to *tet* operators can be blocked with tetracycline, which is useful when a LexA fusion protein is able to activate the expression of TetR (and thus inhibit yeast growth) in the absence of the VP16 fusion protein. Here, the LexA fusion protein may be interacting directly with the transcrip-

tional machinery. However, the basal transactivating capabilities of these LexA fusion proteins are not stronger than VP16. Therefore, the LexA fusion protein alone will produce lower amounts of TetR protein than produced by the interaction of the LexA fusion with the VP16 fusion. Tetracycline can be titrated to suppress the activity of the TetR produced by the LexA fusion protein without suppressing the activity of the TetR produced by the actual LexA and VP16 interacting fusion proteins.

3-AT, an inhibitor of the *Saccharomyces cerevisiae* histidine pathway, is a second point for regulation. In cases in which protein interaction is weak, detection can be difficult. Because of the extreme sensitivity of yeast nutritional reporter genes (particularly *HIS3*), weak protein interactions often can be detected in the conventional yeast two-hybrid assay but not in other binding assays. In the split-hybrid system, weak interacting proteins may not produce sufficient TetR to fully shut down *HIS3*, resulting in background growth. 3-AT can be used to supplement TetR to completely shut off histidine production. This dual modulation of the system expands the number of protein interactions that can be studied.

There are several different classes of factors that might disrupt protein-protein interactions. Mutations that disrupt the binding of one protein with its interacting protein can be screened efficiently in the split-hybrid system. CREB was randomly mutagenized to identify residues involved in the binding to its coactivator CBP (10). The majority of mutations identified in this screen are located in the CREB Ser-133 protein kinase A phosphorylation motif, which had previously been demonstrated to be necessary for CBP binding (20–22). The other mutations identified are located at hydrophobic residues 137, 138, and 141. Subsequent nuclear magnetic resonance studies have identified these hydrophobic CREB residues as points of contact with CBP (22). These results highlight the extraordinary precision of using the split-hybrid system for mutagenesis screening.

The reverse two-hybrid system has also successfully been used for mutagenesis screens. Mutations in E2F1 were identified that disrupted its interaction with its dimerization partner DP1 (12). In addition, the reverse two-hybrid assay was used to identify dominant negative mutations of p53 (23), whereas the split-hybrid system was used to identify an altered specificity mutant in the GAT-1:FOG complex (19).

The split-hybrid and reverse two-hybrid systems can also be used to screen cDNA libraries for protein disrupters of protein-protein interactions. These screens may identify enzymes such as kinases, phosphatases, or proteases that disrupt a protein-protein interaction by covalently modifying one of the interacting proteins. Proteins that act as competitive inhibitors or inhibitors that

require the presence of both interacting proteins may also be identified. The utility of using the reverse two-hybrid system to screen for protein inhibitors of protein-protein interactions was demonstrated by the ability of E1A to inhibit the interaction of pRB and p107 with E2F (*11*). Large peptide libraries can also be screened in the split-hybrid or reverse two-hybrid systems to identify peptides capable of disrupting a protein-protein interaction.

A potentially important use of the split-hybrid and reverse two-hybrid systems is as a screen for small molecules that disrupt clinically important protein-protein interactions. Because the disruption event occurs *in vivo*, small molecules that are permeable and stable are preferentially selected. Furthermore, because the readout of the disruption is yeast growth, toxic small molecules are screened against. The ability to weed out impermeable, unstable, and toxic small molecules in the primary screen reduces the time consumed performing secondary screens in living cells that eliminate undesirable small molecule candidates. As an example, the reverse two-hybrid assay was modified to accommodate screens for small molecules that disrupt protein-protein interactions (*14*). In this system, the expression of two interacting proteins is controlled with an inducible promoter (*GALI*), ensuring that the small molecules are present in the yeast before the interacting proteins activate the expression of the toxic reporter gene. Inducible expression of the interacting proteins is not necessary in the split-hybrid system because TetR produced prior to the presence of drug can be suppressed with tetracycline (unpublished observations).

Over time, the split-hybrid and reverse two-hybrid systems will likely undergo improvements and modifications in much the same way as the original yeast two-hybrid system. Logical extensions include one-hybrid systems that screen for factors that disrupt protein-DNA binding and adapting the systems to mammalian cells. These screens should provide valuable insights into the understanding of cellular protein networks.

#### 4. Notes

1. All plasmids and yeast strains discussed are available from Bio101.
2. See Chapter 5 for detailed protocols for the transformation of yeast with plasmid DNA.
3. We recommend sequential transformations that introduce target plasmid and bait plasmids in independent experiments.
4. It is recommended that uracil-containing and uracil omission media be tested here because differences are observed for certain interactions.

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## Three-Hybrid Screens

### *Inducible Third-Party Systems*

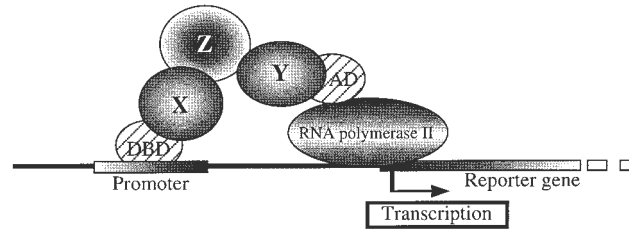
**Björn Sandrock, Franck Tirode, and Jean-Marc Egly**

#### **1. Introduction**

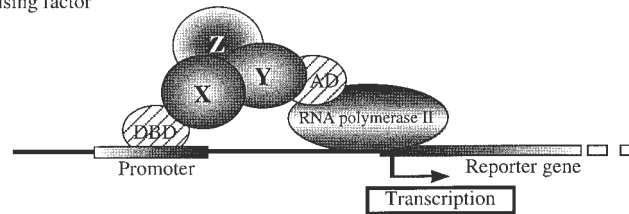
Recent studies have revealed the existence of several molecular complexes either stable, like the RNA polymerase II holoenzyme, transcription factor TFIID, and mediators, or transient as could be observed in the various steps of the transcription process (e.g., initiation, elongation, and termination). These observations point out the fact that regulation of different cellular mechanisms is orchestrated by interactions between molecular species either to modify proteins or to position one of them within a complex that then will be functional. Several techniques such as affinity precipitation, glycerol gradient sedimentation, and the yeast two-hybrid system are currently used to study protein-protein interactions (1). These various methods, usually investigating the connection between two partners, account for only the strong (and stable) interactions and neglect the weaker ones. Considering the complexes studied so far, it is suspected and sometimes shown that interactions often occur between more than two proteins (e.g., to stabilize the complex). In an effort to understand the various biologic mechanisms—and in our case gene expression regulation—we were interested in developing the yeast three-hybrid (or tri-hybrid) system.

The three-hybrid system, as illustrated in **Fig. 1**, is based on the reconstitution of a transcriptional activator complex either to search for or to study a protein that interacts with two others and to acquire information about ternary complex assembly (2). This technique detects direct or mediated interactions between two fusion proteins that contain either a DNA-binding domain (DBD)

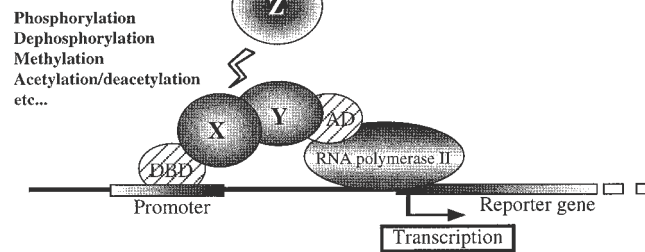
**A** Z = Bridging factor



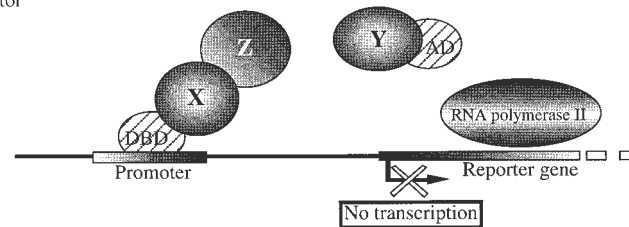
**B** Z = Stabilising factor



**C** Z = Regulating factor



**D** Z = Inhibitor



(the DBD-X protein) or an activation domain (AD) (the AD-Y protein). In some cases, when these two-hybrid proteins interact weakly or not at all, a third partner (the protein Z) is necessary to promote (to induce) the formation of the transcriptional activator, allowing the transcription of the reporter genes. Thus, specific and stable protein-protein interactions among X, Y, and Z lead to the activation of the reporter genes that are integrated in the yeast genome. The *HIS3* reporter gene contains a specific DNA sequence that can be recognized by the DBD of the transcriptional activator. Activation of the *HIS3* gene permits the endogenous synthesis of histidine allowing the yeast to grow on histidine-lacking medium. Activation of the *LacZ* gene, another reporter gene containing the same DNA-binding element, will lead to the synthesis of the  $\beta$ -galactosidase ( $\beta$ -Gal) that catalyzes the transformation of either (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (X-Gal) or *o*-nitrophenyl- $\beta$ -D-galactopyranoside) (ONPG) into a detectable blue or yellow product, respectively.

There are several options to reconstitute the transcriptional activator. First, the third partner Z can act as a bridging factor (**Fig. 1A**) by interacting with both the DBD-X and AD-Y, thus allowing the DNA-binding protein X to target the basal transcription machinery. Second, in the case of weak interactions, Z may function as a stabilizing factor (**Fig. 1B**) that strengthens the interaction between X and Y. Third, in some other cases, reconstitution of the transcriptional activator requires some post-transcriptional modifications of one of the two partners, in order to allow interaction between X and Y. The third partner Z will then be a regulating factor (**Fig. 1C**), an enzyme that will not necessarily be part of the reconstituted transcriptional activator.

When DBD-X and AD-Y are sufficient to reconstitute a stable transcriptional activator, the three-hybrid system can also be used for the search of inhibitors. In this case, the Z partner can act as an inhibitor (**Fig. 1D**) by interacting with, or enzymatically modifying, one of the two main partners, thus preventing their interactions. This will result in the inhibition of the expression of the

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Fig. 1. (*previous page*) Three-hybrid system. The three-hybrid system can be used to characterize or detect new factors such as (A) a bridging factor Z, which interacts with both proteins X and Y, which do not interact with each other; (B) a stabilizing factor Z, which strengthens a weak interaction between the two proteins X and Y; (C) a regulating factor Z, which stimulates the interaction between the two fused proteins X and Y by modifying one of them; and (D) an inhibitor Z, which prevents the interaction between two interacting proteins X and Y. In the three first cases, on expression of the third partner (Z) the transcriptional activator formed stimulates the transcription of the reporter genes, whereas in the last case, the third polypeptide inhibits the formation of transcriptional activator, thus preventing the transcription of the reporter genes.

reporter genes followed by growth restriction on histidine-lacking medium as well as repression of the  $\beta$ -Gal activity.

To control the specificity of the ternary complex formation that depends on the expression of one of its components, we have set up an inducible three-hybrid system, in which the expression of the third partner Z can be easily switched on or off. Therefore, we have used the already characterized yeast Met25 promoter (3). Excess methionine in the culture medium leads to the transcriptional repression of a cDNA cloned downstream of this promoter. Thus, we have designed two plasmids, pBridge and pLexA9-3H (Fig. 2, left), containing the DBDs of GAL4 and LexA, respectively. These can be fused to the cDNA of the X protein. Both vectors contain methionine-regulated expression cassette, under the control of the Met25 promoter, that can be fused to the cDNA of the third partner Z.

### 1.1. Inducible Third Partner Z as Activator

To reconstitute the transcriptional activator, the third partner Z (as shown in Fig. 1) can be overexpressed in the absence of methionine. We have illustrated this feature with the formation of the cdk-activating kinase (CAK) that contains three subunits: a cyclin-dependent kinase, cdk7; the cyclin H; and MAT1, a stimulatory factor (4). Although the role of CAK, a subcomplex of the multiprotein complex TFIIF, is not well known in the various events that regulate the cell-cycle cascade, it seems to be involved in protein coding gene transcription through the phosphorylation of the carboxy-terminal domain of RNA polymerase II (5). It has been shown that cdk7 and cyclin H interact with each other, but the role of MAT1 in the assembly of CAK is still unknown (6,7). Using the three-hybrid system, we demonstrated that the weak interaction between the two fused proteins DBD-cdk7 (X) and AD-MAT1 (Y) was stabilized on expression of the third partner, cyclin H (Z), cloned in the Met25 expression cassette (8). This assembly does not occur in the presence of methionine, which prevents the expression of cyclin H. This assay shows that MAT1 contacts cdk7 or integrates the CAK complex in the presence of cyclin H.

The inducible three-hybrid system could be used to screen a cDNA library in order to search for some activating partners. Therefore, two different screenings are possible. First, to identify proteins (Y) that may target an already formed binary complex (X-Z; see Fig. 1A), the screening is made with a cDNA library inserted downstream of the AD sequence like most of the commercially available cDNA two-hybrid libraries. In this case, to isolate positive clones, yeast growth is performed on a medium lacking methionine; the colonies are then replica plated on a medium containing both methionine and X-Gal, on which they will no longer grow or stay white. Second, to identify proteins (Z)

that stabilize or regulate a weak interaction (X-Y; *see* **Fig. 1B,C**), the cDNA library must be cloned downstream of the Met25 promoter. The screening is then made on a histidine-lacking medium in the absence of methionine. Positive clones are identified through a replica plating on methionine-containing medium as just discussed.

### 1.2. Inducible Partner Z as Inhibitor

As an example to investigate Z components that target either X or Y proteins and consequently prevent the reconstitution of the transcriptional activator, we studied two oncogenic proteins, Raf and Ras (**9**), involved in signal transduction. The fused DBD-Ras (X) and AD-Raf (Y) proteins specifically interact with each other in a two-hybrid system (**9**). An inhibition of the transcription of the reporter gene can be observed (**8**) in the absence of interaction between DBD-Ras (X) and AD-Raf (Y). This may occur when the cDNA of a nonfused cRaf is cloned downstream of the Met25 promoter and further expressed as the third partner (Z) in the absence of methionine. In this case, cRaf (Z) competes with AD-Raf (Y) for targeting DBD-Ras (X). Because the Met25 promoter is stronger than the hybrid gene promoter (**3**), its activation (in the absence of methionine) leads to an excess of cRaf titrating out the DBD-Ras. Thus, the transcriptional activator will not be reconstituted, resulting in the repression of the reporter genes.

## 2. Materials

### 2.1. Yeast Strains

The strains used in the three-hybrid system are the same as the ones used for the two hybrid. There are different yeast strains that are either more or less sensitive for the detection of protein-protein interactions, depending on the vectors, transcriptional activator (Gal4 or LexA-VP16), and protein used as the bait (*see* **Note 1**). The most common yeast strains are the L40 strain (MATa, trp1, his3, leu2, ade2, *LYS2*: : [LexAop]<sub>4</sub>-*HIS3*, *URA3*: : [LexAop]<sub>8</sub>-*LacZ*) for the LexA-VP16 system, and the HF7c strain (ura3, his3, lys2, ade2, trp1, leu2, *LYS2*: : Gal1<sub>UAS</sub>-Gal1<sub>TATA</sub>-*HIS3*, *URA3*: : GAL4<sub>17mersx3</sub>, CyC1<sub>TATA</sub>-*lacZ*) or the Y190 (ura3, his3, lys2, ade2, trp1, leu2, cyh<sup>r</sup>2, *LYS2*: : Gal1<sub>UAS</sub>-*HIS3*<sub>TATA</sub>-*HIS3*, *URA3*: : GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-*lacZ*) when using the GAL4 system. These strains are commercially available.

### 2.2. Plasmid Constructs

For the activation experiments, we used the LexA-VP16 system. Thus, the bait (cdk7) is linked to the DBD of LexA in pLex9-3H (**Fig. 2, left**) carrying the *TRP1* selection gene that allows growth on tryptophan lacking (minimal

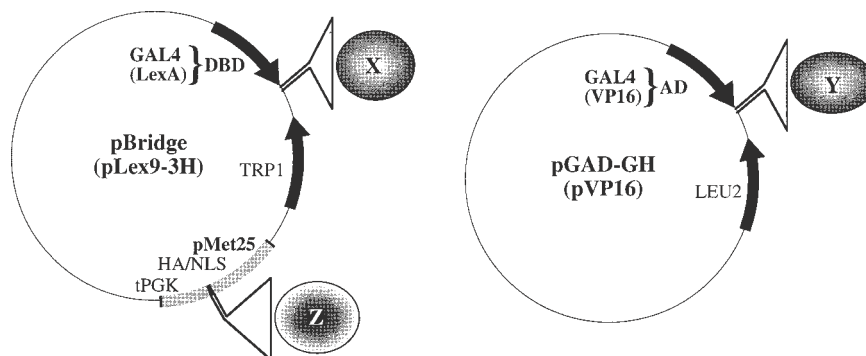


Fig. 2. Three-hybrid vectors. (**Left**) pBridge, derived from the pGBT9 vector, contains the *TRP1* selective gene, the DBD of GAL4, and the Met25 expression cassette (see **Note 2**) with the *NotI* and *BglIII* cloning sites. pLex9-3H, derived from the pBTM116 vector, contains the *TRP1* selective gene, the DBD of LexA, and the Met25 expression cassette with the *NotI* and *SrfI* cloning sites. The Met25 cassette contains a hemagglutinin (HA) tag sequence, a nuclear localization signal (NLS) sequence, and the terminator of the phosphoglycerate kinase (tPGK). (**Right**) The plasmids pGAD-GH and pVP16 contain the *LEU2* selective gene and the AD of GAL4 and VP16, respectively.

medium) (MM). The second partner (MAT1), which has no significant interaction properties with the bait, is fused to the AD of VP16 in pVP16 (**Fig. 2, right**), containing the *LEU2* selection gene that allows growth on leucine lacking MM. In addition, the cDNA of the third partner (cyclin H) is cloned in the Met25 expression cassette (see **Note 2**), which is on the same plasmid as the bait (pLex9-3H).

For the inhibition experiments, we used the GAL4 system, in which the bait (H-Ras[V12]) is fused to the DBD of GAL4 in pBridge and the interacting partner (cRaf1) is added behind the AD of GAL4 in pGAD-GH. The cDNA of the third partner (cRAF1) is cloned into the Met25 expression cassette, which is on the same plasmid as the bait (pBridge).

### 2.3. Growth and Maintenance of Yeast

1. Yeast extract, peptone, dextrose (YPD) medium: 20 g/L of Bacto™ Peptone (Difco, Detroit, MI), 10 g/L of yeast extract (Difco), 20 g/L of D(+)-glucose (Riedel-deHaën, Germany), and water to 1 L. Autoclave at 121°C for 15 min (or add water to 950 mL, autoclave for 30 min, and then add 50 mL of sterile filtrated 40% D[+]-glucose). For culture plates add 20 g/L of agar (Difco).
2. MM: 6.7 g/L of yeast nitrogen base without amino acids (Difco), 20 g/L of D(+)-glucose, water to 1 L. Autoclave at 121°C for 15 min (or add water to 950 mL; autoclave for 30 min, and then add 50 mL of 40% D[+]-glucose). For culture

plates add 20 g/L of agar. Depending on the different strains, the following amounts of specific nutrients should be added (either as a 10-fold stock for small volumes or directly as powder for higher volumes) except the ones for which the medium should select: 0.2 g/L of L-tryptophan, 0.2 g/L of L-histidine, 0.2 g/L of L-adenine, 1.0 g/L of L-leucine, 0.3 g/L of L-lysine, 0.2 g/L of uracil (Sigma, St. Louis, MO).

3. 90-mm (Greiner) and 245 × 245 mm (PolyLabo) culture plates.

#### **2.4. Transformation**

1. 50% Polyethylene glycol (PEG) 4000 (sterile filtrated) (MERCK).
2. 100% Dimethylsulfoxide (DMSO) (MERCK).
3. 10X Lithium acetate (10X LiAc): 1 M LiAc (Sigma), pH 7.5. Sterilize by autoclaving.
4. 10X TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5; autoclave.
5. 10 mg/mL of herring testis carrier DNA (Sigma). Boil just prior to use for 5 min, and then cool on ice.
6. TE/LiAc: 1X TE, 1X LiAc.
7. PEG/LiAc: 40% PEG, 1X TE, 1X LiAc.
8. Appropriate minimal medium containing plate.

#### **2.5. Methionine Selection**

1. MM (*see Subheading 2.3., item 2*).
2. Methionine stock: We prepare a 0.2-μm filtered 1 M L-methionine (Sigma) in deionized H<sub>2</sub>O. This solution can be frozen to -20°C and kept for years. Minimal medium is supplemented with 1 mM L-methionine.
3. 3-Amino-1,2,4-triazole (3-AT) (Sigma).

#### **2.6. Protein Extraction**

1. Extraction buffer: 50 mM Tris-HCl, pH 7.8, 10% glycerol, 0.1 mM EDTA, 100–300 mM KCl, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1X protease inhibitor cocktail (contains leupeptin, pepstatin, aproptinin, antipain, and chymostatin).
2. Acid-treated glass beads (Sigma).
3. A 0.45-μm microspin-column (Ultrafree-MC; Millipore, Bedford, MA). This is used to remove the insoluble particles from the extracts.

#### **2.7. Immunopurification**

The following reagents are required to covalently crosslink the antibodies to protein A-Sepharose.

1. Protein A-Sepharose CL-4B (AP Biotech).
2. 5 M NaCl.
3. 1M Sodium borate, pH 9.0.
4. 0.2 M ethanolamine, pH 8.0.



5. Bovine serum albumin (BSA) (10 mg/ml).
6. Sodium azide (Sigma).
7. Dimethyl pimelidate (DMP) (ICN).
8. TG<sub>10</sub>EK<sub>X</sub>: 20 mM Tris-HCl, pH 7.8, 10% glycerol, 0.1 mM EDTA, *X* mM KCl (*X* = 50–500).
9. Crosslink wash buffer: 3 M NaCl, 50 mM Na borate, pH 9.0.
10. Crosslinking buffer: 3 M NaCl, 200 mM Na borate, pH 9.0, 20 mM DMP.
11. Elution buffer: TG<sub>10</sub>EK<sub>50</sub> containing 0.01% NP40, 0.2 mg/mL of insulin, 2 mg/mL of peptide corresponding to the epitope.

## 2.8. $\beta$ -Galactosidase Assays

1. Z buffer: 21.5 g/L of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 5.5 g/L of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.75 g/L of KCl, 0.246 g/L of MgSO<sub>4</sub>·7H<sub>2</sub>O. Adjust to pH 7.0 and autoclave.

### 2.8.1. Colony Lift Filter Assays

For LexA transformants, use plates containing MM with 2% D(+)-galactose and 1% raffinose instead of D(+)-glucose.

1. Hybond™ nylon membranes (Amersham).
2. 3MM Whatman paper.
3. Liquid nitrogen.
4. X-Gal (Merck) solution: 20 mg of X-Gal/mL in *N,N*-dimethylformamide (Sigma). Store in the dark at –20°C.
5. Z-M-X buffer: 100 mL of Z buffer, 0.27 mL of  $\beta$ -mercaptoethanol, 1.67 mL of X-Gal solution.

### 2.8.2. Liquid Culture Assay

1. Z-M buffer: 100 mL of Z buffer, 0.27 mL of  $\beta$ -mercaptoethanol.
2. Z-O solution: Dissolve 4 mg of ONPG (Sigma)/mL of Z buffer (prepare about 1 h before each use).
3. 1 M Na<sub>2</sub>CO<sub>3</sub>.

### 2.8.3. On-Plate Assay

1. 10X S buffer (for 500 mL): 68 g of KH<sub>2</sub>PO<sub>4</sub>, 9.9 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 21 g of KOH, pH ~ 8.0.
2. X-Gal (*see Subheading 2.8.1., item 4*).
3. MM (*see Subheading 2.3., item 2*).

## 3. Methods

### 3.1. Growth and Maintenance of Yeast

Working with yeast should be done under sterile conditions, because the different culture media do not contain antibiotics. Thus, contaminations should be avoided by using sterile glass and plasticware. It is recommended that work

should be done at a clean bench or beside a burning flame. Freshly streaked yeast grows at 28°C for 4 to 5 d until colonies are 1 to 2 mm in diameter. One such colony can then be used for amplification in liquid culture. For the maintenance of yeast strains, a colony resuspended in 0.5 mL of sterile water should be restreaked every 6–8 wk. For long-term storage, yeast strains can be stored in the appropriate medium containing 15% glycerol at –70°C.

### 3.2. Transformation

To introduce plasmid DNA into yeast, the LiAc-mediated transformation is very easy, fast, and reproducible. The method described here allows single transformation or simultaneous cotransformation of two different plasmids carrying different selection genes. Our protocol can be used for up to 10 parallel transformations but can easily be scaled up. Furthermore, with this method a cDNA library transformation can be performed; the culture or buffer volumes are indicated in parentheses. A positive and a negative control should be incorporated to demonstrate the efficiency of the transformation. This efficiency should be about  $10^5$  colonies/ $\mu$ g of plasmid for a single transformation.

1. Inoculate 10 mL of YPD or the appropriate MM with a single colony of about 2 mm in diameter and incubate overnight at 28°C. (For library screening, further inoculate 150 mL of the same medium with the 10 mL of the preculture and let grow overnight.) Dilute the suspension to an OD<sub>600</sub> of 0.2–0.3, and incubate the culture for approx 3 h to get an OD<sub>600</sub> of about  $0.5 \pm 0.1$  (the log phase).
2. Centrifuge 50 mL (1000 mL) of the culture for 5 min at 1000g. Discard the supernatant and wash the cells in 30 mL (500 mL) of sterile water. After a second centrifugation step, resuspend the cells in 1 mL (8 mL) of sterile TE/LiAc.
3. Mix 0.1 mg (20 mg) of herring sperm DNA with 0.1  $\mu$ g of each plasmid—for simultaneous cotransformation use 0.2  $\mu$ g of each different plasmid—(0.5 mg of cDNA library) in a sterile 1.5-mL (500-mL) tube and add 100  $\mu$ L (8 mL) of the yeast solution. Mix vigorously and add 600  $\mu$ L (60 mL) of PEG/LiAc. Vortex and incubate for 30 min at 30°C with shaking (20,000g).
4. Add 70  $\mu$ L (7 mL) of DMSO, mix carefully by inverting the tubes, and incubate for 15 min at 42°C. Centrifuge for 20 s (5 min) at 13,000 rpm and remove the supernatant. Dissolve the yeast pellet in 500  $\mu$ L (10 mL) of TE and plate 100  $\mu$ L (200  $\mu$ L) of this suspension on a 90-mm (245 · 245 mm) plate, which allows selection for the desired transformants.
5. Incubate the plates at 30°C until the colonies are 1 to 2 mm in diameter (4 to 5 d).

### 3.3. Methionine Selection

The advantage of the three-hybrid system using the Met25 inducible promoter resides in the on or off switch for the expression of the third protein (*see Note 3*). A simple replica-plating experiment allows one to check quickly the interaction specificity among the various partners:

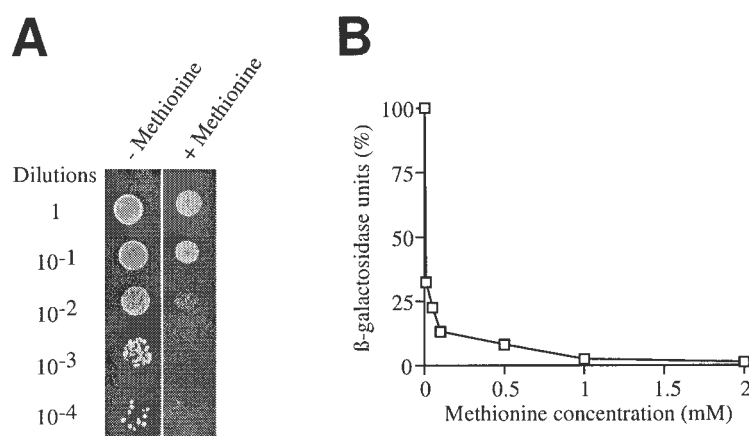


Fig. 3. **(A)** L40 yeast cells cotransformed with pLex9-3H, containing LexA-cdk7 and cyclin H under the dependence of the Met25 promoter, and with MAT1pVP16 were spotted at various dilutions, as indicated on the left, on a selective minimal medium lacking histidine. Yeast growth was performed during 4 d at 30°C, in the absence (–) or presence (+) of 1 mM methionine, controlling the expression of the third partner, cyclin H. In the presence of the third partner, we can observe a yeast growth for the highest dilutions, whereas in the presence of methionine, and thus in the absence of the cyclin H, we can observe yeast growth only for the first dilutions. **(B)**  $\beta$ -Galactosidase assay from liquid culture of pLex9-3H/cdk7/Met-cyclinH-pVP16/MAT1 cotransformed yeast was done as described in **Subheading 3.6.2**. The  $\beta$ -Gal activity was measured as a function of the methionine concentration and thus as a function of the presence of the third partner. The interaction, reflected by the  $\beta$ -Gal activity, was no more detectable in the presence of 1 mM methionine. One hundred percent corresponds to the  $\beta$ -Gal activity of the yeast grown on a medium lacking methionine, and 0% to the activity of yeast grown on a 10 mM medium containing methionine.

1. Select at least three different clones from the leucine- and tryptophan-lacking plates selective for both plasmids. Scrape the whole colony and resuspend in 100  $\mu$ L of sterile H<sub>2</sub>O.
2. Make 1–10 dilutions in sterile H<sub>2</sub>O up to 10<sup>–4</sup>.
3. Drop 10  $\mu$ L of each dilution on selective MM plates either containing or lacking 1 mM methionine.
4. Let the yeast grow for 4 d at 28°C. The duration of the growth may be dependent on the proteins expressed and can take up to 10 d.

An example of the methionine-dependent reconstitution of the transcriptional activator is shown in **Fig. 3A**. The three proteins of the TFIIH CAK complex (constructions described in **Subheading 2.2.**) were expressed in yeast.

Cyclin H is conditionally expressed from the Met25 promoter. The complex is then formed only if the three proteins are present. Thus, the yeasts are able to grow in the absence of methionine because the cyclin H is expressed and the ternary complex is reconstituted, leading to the transcriptional activation of the reporter genes. The colonies that are plated on selective MM lacking 1 mM methionine grow faster than colonies plated on MM containing methionine. Thus, the methionine repression is observed for the  $10^{-2}$ – $10^{-4}$  dilutions. Because of a weak interaction between DBD-cdk7 and AD-MAT1, background growth can be observed for the lower dilutions. Furthermore, nonspecific interactions can take place between one of the hybrid proteins and the fused DBD directly. To remove these background growths, 3AT (between 5 and 50 mM) can be added to the medium. Moreover, the Met25 promoter is known to be leaky, meaning that the expression of the third partner, cyclin H, is not completely repressed, resulting in some detection of yeast growth on a methionine-containing medium.

As shown in **Fig. 3A**, the Met25 promoter is downregulated on the addition of methionine. To determine the optimum conditions of the methionine repression, several concentrations have been tested using the liquid  $\beta$ -galactosidase assay (*see Subheading 3.5.2.*) with the system containing the three proteins of the CAK complex, as described above. Thus, the resulting  $\beta$ -Gal activity, corresponding to the concentration of the third partner, was measured as a function of the methionine concentration. As shown in **Fig. 3B**, the  $\beta$ -Gal activity, reflecting the cyclin H expression, is about 30% of the activity without methionine for 0.01 mM methionine, 10% for a concentration of 0.5 mM, and completely repressed for 1 mM. This system can also be useful for the control of the expression of toxic proteins, in order to decrease the amount of these proteins in the cell.

### **3.4. Protein Extraction and Immunopurification**

#### **3.4.1. Protein Extraction**

To investigate whether or not the hybrid proteins are well expressed, it is necessary to prepare yeast protein extracts and to perform Western-blotting assays.

1. Inoculate 50 mL of selective medium for the plasmids (*see Subheading 2.3.*) to an OD<sub>600</sub> of 0.05. Grow at 28°C with shaking until reaching an OD<sub>600</sub> between 0.8 and 1. Depending on the protein expressed, growth can take between overnight and 3 d.
2. Transfer the culture in a 50-mL tube and cool the cells by placing the tube on ice for a few minutes. Centrifuge for 5 min at 1000g at 4°C.
3. Resuspend the cell pellet in 1 mL of cold H<sub>2</sub>O, transfer to a microfuge tube,

- centrifuge for 30 s at maximum speed, remove the supernatant, and add 300  $\mu$ L of extraction buffer. Different buffers can be used for the extraction but usually a high-salt buffer (300 mM KCl) is recommended for higher recovery. To analyze the *in vitro* interaction between the hybrid proteins using immunoprecipitation or immunopurification assays, the cell pellet should be resuspended at this stage in a low-salt extraction buffer (100 mM KCl), to retain potential weak interactions.
4. Add glass beads to 1 to 2 mm below the surface and keep on ice for 10 min.
  5. Vortex vigorously in a cold room three times for 1 min each with intervals of 1 min on ice.
  6. Centrifuge at maximum speed (20,000g) for 15 min at 4°C.
  7. Transfer the supernatant to a microspin column (Ultrafree-MC; Millipore) to filter residual big particles (*see Note 4*). Centrifuge for 30 s at maximum speed. Samples can be frozen at  $-80^{\circ}\text{C}$  and kept for several months.
  8. Estimate the total protein concentration by performing a Bradford assay.
  9. Run 10–100  $\mu$ g of protein on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8–15%) and perform a Western blot.

### 3.4.2. Crosslinking Antibody to Protein A-Sepharose

The purification of a new protein, identified as an interacting partner during a cDNA library screening, is important for further analysis. It is therefore useful that the proteins expressed by the vectors containing the cDNA library be tagged.

We have introduced the hemagglutinin sequence corresponding to the hemagglutinin (HA) tag (SYPYDVDPDYASLGGPSS, one amino acid code) upstream of the cloning site (**Fig. 2**) in the methionine-regulated expression cassette. Thus, the expressed protein can be immunoprecipitated or immunopurified directly from a yeast crude extract with an HA tag specific antibody (commercially available). Instead of the HA antibody, a specific antibody can be used as well.

1. Wash the protein A-Sepharose three times with 10 vol of crosslink wash buffer.
2. Add 0.7 vol of purified anti-HA monoclonal antibody with 4.3 vol of crosslink wash buffer. Incubate at room temperature for 1 h under gentle shaking.
3. Wash two times with 10 vol of crosslink wash buffer.
4. Add 10 vol of crosslink buffer. DMP should be added immediately before use. Incubate for 30 min at room temperature under gentle shaking.
5. Wash once with 10 vol of 0.2 M ethanolamine, and incubate with 10 vol of 0.2 M ethanolamine at room temperature for 2.5 h or at 4°C overnight.
6. Wash two times with 10 vol of TG<sub>10</sub>EK<sub>300</sub>/0.05% NP40. The beads are then ready to use. If you wish to keep the antibody crosslinked to protein A-Sepharose for several weeks, add 1 mg/mL of BSA and 0.2% Na azide. Before use, wash three times with 10 vol of TG<sub>10</sub>EK<sub>300</sub>/0.05% NP40.

### 3.4.3. Immunoprecipitation of HA-Tagged Protein from Yeast

For a pulldown assay, very little yeast extract is needed. Remember that if the strength of the interaction is not known, it is recommended that one make the yeast extract at a low-salt concentration or dialyze the extract against a low-salt buffer.

1. Incubate 300  $\mu$ L of the yeast extract with 50  $\mu$ L of the antibody sample crosslinked to the protein A-Sepharose for at least 4 h at 4°C under gentle shaking.
2. Wash the beads three times with 1 mL of TG<sub>10</sub>EK<sub>100-500</sub> (usually 300 mM KCl is preferred, once more depending on the strength of the interaction).
3. Add 50  $\mu$ L of Laemmli sample buffer to the beads removed from the supernatant and load (5–20  $\mu$ L) on an SDS-polyacrylamide gel.

### 3.4.4. Immunopurification of HA-Tagged Protein

Using the three-hybrid system, it is possible to directly purify the protein under the control of the Met25 promoter as well as the tripartite complex. The immunoprecipitation procedure can be scaled up to 2 L of yeast culture or even more, if necessary. We describe the method for a 20-mL culture:

1. Proceed as in **Subheading 3.4.3.** until **step 2.** After the TG<sub>10</sub>EK<sub>100-500</sub> washings, wash two times more with 1 mL of TG<sub>10</sub>EK<sub>50</sub>.
2. Remove the supernatant and add 50  $\mu$ L of elution buffer containing the oligopeptide corresponding to the HA or the specific antibody epitope. Incubate at 4°C overnight with gentle shaking.
3. Centrifuge at low speed (1,000g) for a few seconds and transfer the supernatant to a microfuge tube. Centrifuge and transfer once more to discard all the remaining beads. Most of the time the oligopeptide does not interfere with the activity of the purified protein; if necessary, dialyze against TG<sub>10</sub>EK<sub>50</sub> for 4 h at 4°C.
4. Verify by Western blot the presence of the protein in the elution fraction using the HA or the specific antibody. Compare with a yeast extract transfected with a control expression vector.

## 3.5. $\beta$ -Galactosidase Assays

Three different  $\beta$ -galactosidase assays are described. The colony lift filter assay with the substrate X-Gal is a qualitative method similar to the on-plate assay. Both techniques allow the analysis of a large number of transformants appearing on the plates. However, they are less sensitive than the liquid culture assay with the substrate ONPG. The liquid culture assay allows the quantification of  $\beta$ -Gal activity. It can then be used to compare the relative strength of

protein-protein interactions of different proteins or different mutations of the same protein.

The colony lift filter assay and the ONPG assay use the freeze/thaw cycle in liquid nitrogen for the lysis of the cell walls. This is a rapid step for the accurate analysis of  $\beta$ -Gal activity.

For the on-plate assay, X-Gal is added directly to the selective MM, leading to a slight growth restriction because of a shift in pH.

### 3.5.1. Colony Lift Filter Assay

It is recommended that fresh colonies (1 to 2 mm in diameter) growing on the selective MM be used. When colonies grown in the presence of methionine have to be analyzed, it is necessary that these transformants not be plated on a 3AT-containing medium (*see Subheading 3.3.*).

1. For every plate to be assayed, presoak a sterile 3MM Whatman paper in 2.5 mL (5 mL) of Z-M-X buffer in a 90-mm (150-mm) plate. Lay, under sterile conditions, a dry nylon membrane filter on the yeast plate for a few seconds to transfer the colonies onto the filter. Poke holes through the filter into the agar using hot needles in three asymmetric locations to orientate the filter compared to the plate for later assignments of positive clones.
2. Carefully remove the filter from the surface and lay it colony side up on an aluminum boat, which floats on liquid nitrogen. After 10 s, submerge the boat with the filter for 1 min and thaw it at room temperature. Place the filter colony side up on the presoaked Whatman filter avoiding air bubbles under the filter.
3. Incubate at 30°C until blue colonies appear (from 30 min to 12 h).

### 3.5.2. Liquid Culture Assay

Compare the results of different experiments in a statistical manner, we suggest the analysis of at least three separate colonies of each transformant.

1. Inoculate one colony in 10 mL of the appropriate MM with or without 1 mM methionine, and incubate at 30°C under gentle shaking until the stationary phase (*see Note 5*). For further comparative studies, it is important to have all cultures in the same growth phase.
2. Add approx 16 mL of MM ( $\pm$ methionine) to 2 mL of each culture to achieve an OD<sub>600</sub> of 0.2–0.3, and incubate for 3 h at 30°C until the OD<sub>600</sub> is in the range of 0.5–0.8. Note the OD<sub>600</sub> and the volume of the culture.
3. After centrifugation (5 min at 1000g), resuspend the cells in 1 mL of Z buffer. Transfer to a 1.5-mL microfuge tube and centrifuge at high speed for 20 s. Discard the supernatant and dissolve the pellet in 250  $\mu$ L of Z buffer. Divide the cell suspension into two 100- $\mu$ L aliquots, and set up a standard reaction tube with 100  $\mu$ L of Z buffer.
4. Freeze the tubes three times for 1 min in liquid nitrogen and thaw them each time in a water bath.

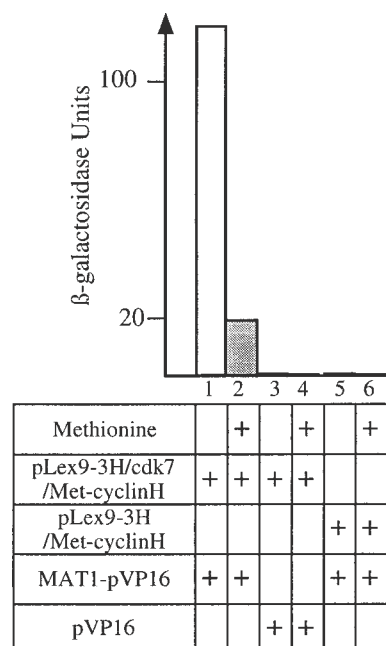


Fig. 4. Liquid culture assay. L40 cells were cotransformed with the plasmids as indicated and analyzed as described in **Subheading 3.6.2.** with  $V = 20$  mL and  $t = 1$  min. In addition, the controls with vectors without fused protein were performed (lanes 3–6).

5. Add to each tube 700  $\mu$ L of Z-M buffer and 160  $\mu$ L of freshly prepared Z-O solution, and incubate at 30°C until a yellow color develops. It is important to note the time of this reaction.
6. Stop the reaction by adding 400  $\mu$ L of 1 M  $\text{Na}_2\text{CO}_3$  solution. Centrifuge for 10 min at maximum speed to clear the supernatant, and measure the  $\text{OD}_{420}$  against the standard.

The linear range of this assay lies in the area from 0.02 to 1.0 for an  $\text{OD}_{420}$ . Calculate the  $\beta$ -Gal units using the following formula:

$$\beta\text{-Gal} = 1000 \times \text{OD}_{420} / (\text{OD}_{600} \times V \times t)$$

in which  $V$  = volume of the culture and  $t$  = reaction time. With both  $V$  and  $t$  constant, you can simplify the formula to

$$\beta\text{-Gal} = 1000 \times \text{OD}_{420} / \text{OD}_{600}$$

Using this technique, we could demonstrate that the formation of the CAK-complex (**Fig. 4**, lanes 1 and 2) is highly dependent on cyclin H. After cotransformation of the L40 strain with both pLex9-3H, containing LexA-cdk7



and Met25-cyclinH, and pVP16-MAT1, the  $\beta$ -Gal activity of such clones grown in a methionine-containing medium is <20% of the activity of colonies grown in a methionine-lacking one, owing to the repression of the cyclin H. The observed basal activity could be owing to, e.g., the leakiness of the Met25 promoter, as discussed under **Subheading 3.3**. In control experiments, yeast cotransformed with pLex9-3H containing LexA-cdk7 and Met25-cyclin H, and native pVP16 (**Fig. 4**, lanes 3 and 4), or with pLex9-3H containing only Met25-cyclin H, and pVP16-MAT1 (**Fig. 4**, lanes 5 and 6) does not present any  $\beta$ -Gal activity.

### 3.5.3. On-Plate Assay

$\beta$ -Gal activity can be directly detected on the plates if the colonies are plated or replica plated on a selective MM containing 1X S buffer and 50–250  $\mu$ L of X-Gal solution and incubated at 28°C. Blue color will appear with the colonies after 4–8 d.

### 3.6. Inducible Three-Hybrid System: Three Verifications for Positive Clones During Library Screening

The three-hybrid system described here possesses an inducible promoter for the conditional expression of the third partner. This allows one to perform an additional control of interaction specificity, especially in a screening experiment for an activator. Once the positive clones are identified first by the appearance of the histidine prototrophy and second by their  $\beta$ -gal activity (*see Note 6*), a third control can be done by transferring these positive clones onto a methionine-containing medium as described under **Subheading 3.3**. (*see Note 7*). Only the clones whose growth will be repressed by methionine will be considered as true positives. For screening a cDNA library, look for an inhibitor of a stable binary complex (*see Note 8*).

## 4. Notes

1. It is difficult to recommend a particular yeast strain for a random system. Because of the different properties concerning the activator system (GAL4 or LexA) and the promoter strength of the HIS3 or the other reporter genes, the appropriate strain has to be tested. The choice of the plasmids is also important because there are many differences in the number of copies per cell (high or low copy number). In addition, the promoter length of the alcohol-dehydrogenase I gene (*ADHI*), which controls the expression of the GAL4, LexA, and VP16 fusion polypeptides, varies among most of the commonly used plasmids. This leads to a different expression level of the fused proteins (**10**). There are also other vectors expressing the protein of interest fused to the N-terminus of LexA (**11**).
2. The Met25 expression cassette, which contains the region from –474 to –1 of the Met25 promoter (**3,12**), the HA epitope, the nuclear localization sequence, and

two rare restriction sites (*NotI* and *SrfI* for pLexA, *NotI* and *BglIII* for pBridge) was cloned in the unique *PvuII* site of the plasmid vectors pBTM116 and pGBT9 to give the plasmids pLexA and pBridge, respectively. The transcription arrest signal is provided by the addition of the phosphoglycerate kinase termination region corresponding to the *BglIII-HindIII* terminator fragment from plasmid pEMBLYe30/2 (**Fig. 2**, left panel) (8). It should be taken into account that the Met25 promoter is a strong promoter in the absence of methionine, compared with the ADH1 promoter. Therefore, the third polypeptide is much more expressed than the two other fused proteins.

3. There are several other three-hybrid systems using various inducible promoters:
  - a. Galactokinase/galactose epimerase promoter (GAL1/GAL10), which is repressed by glucose and induced by galactose (13,14). This promoter cannot be used in a GAL4-based two-hybrid system because the GAL4 activator binds the sequence between GAL1 and GAL10 and induces transcription.
  - b. Acid phosphatase promoter (PHO5), regulated by the presence or absence of inorganic phosphate (15).
  - c. Metallothionein protein promoter (CUP1), induced by the addition of copper to the medium (16,17).

Some other promoters can be used for the regulated expression of the third partner in a three-hybrid system:

- a. Sorbitol dehydrogenase protein (SOR1), an enzyme responsible for sugar metabolism, is upregulated in the presence of sorbitol. McGonigal et al. (18) described some inducible protein expression vectors that can be used in a two- or three-hybrid system.
  - b. Aromatic aminotransferase II (ARO9), which catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism in *Saccharomyces cerevisiae*, is upregulated by those amino acids (19).
4. Sometimes yeast extract immunoblot can be “smearlike” independently of the antibodies used. For optimum detection, we found that filtering the yeast extract on a 0.45- $\mu$ m spin column can efficiently overcome this problem. Several techniques can be used such as affinity chromatography; however, the advantages of the filter method reside mainly in the low cost and in time savings.
5. To analyze the strength of an interaction or the amount of hybrid proteins in the liquid ONPG assay, it can be useful to test different volumes of yeast culture. We have noticed that using a low culture volume, the  $\beta$ -gal activity measured was not in the linear range ( $OD_{600}$  between 0.02 and 1.0). Thus, further analyses should be done under the conditions found to be the most suitable. It must be observed that in the liquid ONPG assay, weak (or background) interactions yield no yellow color. The reaction can then be stopped if no yellow color has appeared after 30 min.
6. During the selection of positive clones by performing the on-plate and the colony lift filter assays in a yeast three-hybrid screening, only strong interactions are identified using the Y190 and L40 strains. Thus, the liquid culture assay is more sensitive for the identification of the weakest interactions in these strains.

7. The two-hybrid system is used for detection of interaction between two proteins. Many interactions could not be detected because of the lack of specificity or because the correct positioning of the interacting domains was not achieved. The three-hybrid system can overcome these problems by increasing the specificity of an interaction. Thus, the presence of the third polypeptide is necessary to detect ternary complexes. One can imagine that a four-hybrid system can be used to detect quaternary complexes. Because many complexes involved in the different cellular processes contain more than three proteins, adding one more polypeptides to the bait increases specificity.
8. To screen a cDNA library to search for a binary complex inhibitor, the methionine-regulated system presented here is not appropriate. In the presence of methionine, the repression of the inhibitor will lead to the formation of the transcriptional activator complex. Because each clone will express the reporter genes and will then be able to grow, replica plating of all the corresponding colonies (approx  $2 \times 10^6$ ) will not be practical. Therefore, a reverse three-hybrid system can be adapted from the system presented here (*see* Chapter 18). However, once a candidate is identified as an inhibitor, our system can be used to rapidly verify the inhibitory properties of the new protein.

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## Three-Hybrid Screens for RNA-Binding Proteins

### *Proteins Binding 3' End of Histone mRNA*

**Zbigniew Dominski and William F. Marzluff**

#### **1. Introduction**

In metazoans, replication-dependent histone mRNAs, unlike all other mRNAs, are not polyadenylated but instead terminate with a unique, highly conserved sequence containing a 6-bp stem and a 4-base loop (**1**). This relatively short, 26-nucleotide sequence has multiple functions in metabolism of all replication-dependent histone mRNAs, including their nuclear export (**2,3**) and localization to polyribosomes (**4,5**), as well as regulation of their half-life in the cytoplasm (**6**). In metabolism of all other mRNAs, these functions are mediated by the poly(A) tail. Additionally, the histone stem-loop sequence is required for efficient 3' end cleavage of replication-dependent histone pre-mRNAs, leading to the formation of mature histone mRNAs (**7,8**). Critical features of the 16-nucleotide stem-loop structure and the 5-nucleotide flanking sequences are shown in **Fig. 1A**. The two GC base pairs at the base of the stem and the UA base pair at the top of the stem are invariant. Moreover, there are virtually always uridines in the first and third nucleotides of the loop. The only known exception is the loop of *Caenorhabditis elegans* histone mRNA, which in the first position contains cytidine rather than uridine (**9**). Both flanking sequences contain mostly adenines and cytidines, with CCAA consensus sequence on the 5' side and ACCA or ACCCA the consensus on the 3' side.

Ultraviolet-crosslinking experiments and mobility shift (band-shift) assays revealed that the histone stem-loop sequence tightly interacts with a protein called the stem-loop binding protein (SLBP) (**Fig. 1B**) (**10,11**). This protein is

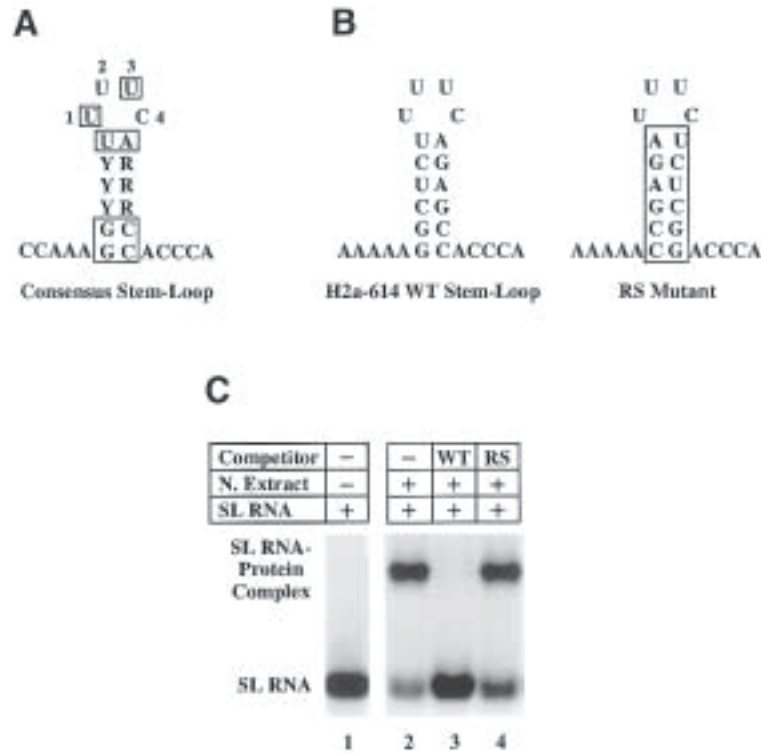


Fig. 1. The last 26 nucleotides of replication-dependent histone mRNAs are recognized by the SLBP. (A) Consensus sequence for the 16-nucleotide stem-loop structure and the 5-nucleotide flanking regions at the 3' end of histone mRNAs. The invariant nucleotides are boxed. (B) The 3' end of the mouse H2a-614 histone mRNA and the reverse-stem mutant (RS) unable to bind SLBP. Altered nucleotides are boxed. (C) Binding of SLBP from the nuclear extract of the mouse myeloma cells to the radio-labeled RNA containing the H2a -614 wild-type stem-loop sequence, as detected by mobility shift assay. A slowly migrating complex of SLBP and the RNA probe (lane 2) can be competed by 50-fold excess of the cold wild-type RNA (lane 3) but not by a 1000-fold excess of the RS mutant, which is unable to bind SLBP (lane 4). The probe is shown in lane 1.

found in both the nuclear and cytoplasmic fractions of mammalian cells (12), indicating that SLBP shuttles between the two compartments. Mobility shift assays were used to determine the sequence requirements for binding of SLBP to the stem-loop structure (13). Substitutions of individual nucleotides in the loop are relatively well tolerated and reduce affinity to SLBP by a factor of 5. Mutations of the conserved stem nucleotides have a more severe effect. Changing the two base pairs of the stem from GC/GC to CG/CG reduces the binding

affinity more than 30-fold, and reversing the entire stem leaving the loop unchanged (thus creating the reverse stem mutant used in many of our studies) virtually abolishes binding to SLBP (**13**). These mutations, retaining the overall structure and stability of the stem-loop structure, revealed an unusual property of SLBP-RNA interaction, i.e., a requirement for the wild-type sequence of the stem. This is in contrast to the majority of other RNA-binding proteins that rely on specificity determinants in the single-stranded regions of loops or internal bulges (**14,15**). In addition to the stem loop, five nucleotides on either side of this structure play an important role in binding to SLBP. Deleting or mutating the flanking sequences reduces the binding affinity 20- to 30-fold (**13**). Presumably, the 26 nucleotides of the stem-loop and flanking sequences represent a unique RNA motif, collectively contributing to the three-dimensional structure recognized by SLBP. SLBP has a very high affinity ( $5 \times 10^{-10} M$ ) for this RNA motif; once bound to the radiolabeled stem-loop RNA, SLBP persists in the complex for hours and cannot be competed by several hundredfold excess of subsequently added cold stem-loop RNA (**13**). The low dissociation rate of the SLBP-RNA complex is consistent with the role of SLBP as a shuttling protein that remains associated with the stem loop for the lifetime of histone mRNA.

Replication-dependent histone mRNAs acquire their unusual 3' end by endonucleolytic cleavage of longer histone pre-mRNAs, four to five nucleotides downstream from the stem loop (**16,17**). Because histone pre-mRNAs do not contain introns, formation of the 3' end is the only processing event in biosynthesis of histone mRNAs. The 3' processing depends on two *cis*-elements present in histone pre-mRNAs: the stem-loop sequence and a purine-rich sequence located about 10 nucleotides downstream of the cleavage site and referred to as the histone downstream element (HDE). The cleavage occurs in a multicomponent complex containing at least three *trans*-acting factors: the SLBP, the U7 small nuclear ribonucleoprotein (snRNP) particle, and the heat-labile factor (HLF) (**Fig. 2**). SLBP, also called the hairpin-binding factor (HBF), binds the stem-loop structure and greatly stimulates the efficiency of cleavage, but it is not absolutely required for processing of most histone pre-mRNAs *in vitro* (**18**). The U7 snRNP contains one molecule of 63-nucleotide U7 snRNA that base-pairs through its 5' end with the HDE of histone pre-mRNA (**19,20**). In addition to the RNA component, the U7 snRNP contains a complete set of common Sm proteins and an as yet unknown number of U7-specific proteins (**21,22**). Cleavage of the histone pre-mRNA occurs between the stem-loop structure and the HDE and requires a third factor, HLF (**23**). The role of HLF in 3' end processing and sites of its interaction with the histone pre-mRNA are unknown. In contrast to SLBP, both HLF and the U7 snRNP are indispensable components of the processing machinery. The 3' end processing of histone pre-mRNAs may also require additional factors. It is pos-



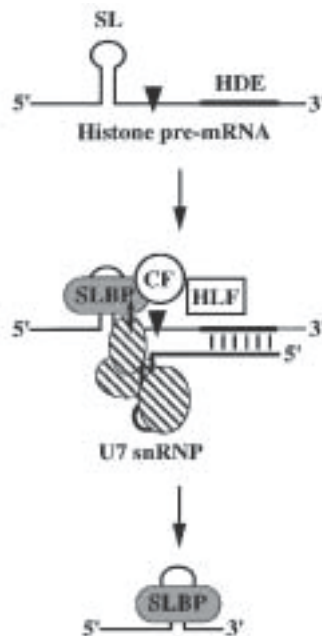


Fig. 2. The 3' end processing of histone pre-mRNAs. *cis*-Elements required for 3' end processing are depicted at the top and include the stem-loop structure (SL) and the histone downstream element (HDE), indicated by a heavy line. The inverted triangle represents a site of cleavage. *trans*-Acting factors include the SLBP, the U7 snRNP, the heat-labile factor (HLF), and a hypothetical cleavage factor (CF). Cross-hatched ovals represent protein components of the U7 snRNP, and the thick line forming the stem-loop structure represents the U7 snRNA. A potential to form base pairs between the histone downstream element (HDE) and the U7 snRNA 5' end is indicated by vertical lines. The double-headed arrow indicates putative interaction between a protein component of the U7 snRNP and SLBP.

sible that endonucleolytic cleavage of histone pre-mRNA is mediated by one of the accessory proteins of the U7 snRNP or by the poorly characterized HLF.

Following cleavage of the pre-mRNA, SLBP remains associated with the newly formed end of the histone mRNA and most likely mediates all functions of the stem-loop sequence, including nuclear export of histone mRNAs (2), localization of histone mRNAs to the polyribosomes (5), and regulation of histone mRNA stability (6). In the cytoplasm, SLBP may assume many of the roles played in general mRNA metabolism by poly(A) binding protein. SLBP also likely plays a key role in restricting accumulation of histone mRNAs to S-phase and, hence, may be a key protein that couples histone gene expression to DNA replication. The stem-loop sequence and SLBP provide a target for



coordinate regulation of expression of the mRNAs for all five classes of histones and allow evolution of unique regulatory mechanisms to control this set of mRNAs. During the cell cycle there is regulation of both histone pre-mRNA processing and histone mRNA half-life (24). The efficiency of processing increases dramatically as cells progress from G1- to S-phase (24,25). At the end of S-phase, the efficiency of processing as well as the histone mRNA half-life rapidly decline, significantly contributing to the disappearance of histone mRNA from the cytoplasm on completion of DNA replication. The decrease in stability of replication-dependent histone mRNA can also be induced by treating S-phase cells with inhibitors of DNA replication (26). A key role of the terminal stem-loop structure associated with SLBP in cell-cycle regulation of histone mRNA levels is supported by the lack of this structure in mRNAs encoding replication-independent (or replacement-variant) histone proteins used for chromatin remodeling and repair. These mRNAs are polyadenylated and synthesized at the basal level throughout the cell cycle (27).

Because the stem-loop structure plays a critical role in biogenesis and metabolism of replication-dependent histone mRNAs, including participating in their cell-cycle regulation, we and others have attempted to purify SLBP as the likely *trans*-acting regulatory factor. Paradoxically, in addition to low abundance, the strong affinity of SLBP to the histone mRNA 3' end contributed to the failure to purify this protein from various sources using chromatographic methods (11,28). The majority of SLBP exists in the cell in the complex with the endogenous histone mRNA and was difficult to solubilize. The stable complex between SLBP and the histone mRNA 3' end prevented utilization of mobility shift assay to trace the activity during fractionation.

Here we describe isolation of cDNA encoding SLBP by utilization of the yeast three-hybrid system. The three-hybrid system is a modification of the two-hybrid system and has been specifically designed to detect protein-RNA interactions (29). Isolation of a cDNA for SLBP was the first successful attempt to use this system in cloning an RNA-binding protein and was achieved simultaneously in our (30) and Dani Schümperli's laboratories (31). We also describe a modified version of the yeast two-hybrid system designed for cloning proteins interacting with SLBP in the presence of the stem-loop RNA. This approach should be generally applicable for identification of proteins that interact with RNA-protein complexes.

## 2. Materials

### 2.1. Transformation of Yeast Cells

1. 50% polyethylene glycol (PEG) 4000 solution (average mol wt of 3350) (cat. no. P-3640; Sigma, St. Louis, MO): Dissolve 50 g of PEG 4000 in distilled water to give a final volume of 100 mL and autoclave (*see Note 1*).

2. 10X TE buffer, pH 7.5: 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 7.5.
3. 10X LiAc (1 M LiAc): Dissolve 10.2 g of LiAc (cat. no. L-4158, Sigma) in 100 mL of distilled water, adjust pH to 7.5 with acetic acid, and autoclave.
4. Carrier DNA at 10 mg/mL (denatured herring testes DNA; cat. no. 1606-A, Clontech).
5. 40% Dextrose (glucose): Dissolve 200 g of dextrose (cat. no. D-9434, Sigma) in distilled water to give a final volume of 500 mL and filter sterilize.
6. 1 M Solution of 3-amino-1,2,4-triazole, (3-AT) (cat. no. A-8056, Sigma): Dissolve 8.4 g of 3-AT in 100 mL of distilled water and filter sterilize.
7. Yeast extract, peptone, dextrose (YPD) medium: Dissolve 20 g of peptone (cat. no. 0118-17-0; Difco, Detroit, MI) and 10 g of yeast extract (cat. no. 0127-17-9; Difco) in 950 mL of water. Autoclave, cool to ~55°C, and add 50 mL of 40% solution of dextrose to a final concentration of 2%. For YPD plates, add 25 g of agar (cat. no. 0140-01; Difco) before autoclaving.
8. 10X Synthetic defined (SD)-His/-Leu/-Trp/-Ura (10X synthetic defined solution lacking histidine, leucine, tryptophan, and uracil): Dissolve the following in 1 L of distilled water (all from Sigma): 0.3 g of L-isoleucine (cat. no. I-7383), 1.5 g of L-valine (cat. no. V-0500), 0.2 g of L-adenine hemisulfate salt (cat. no. A-9126), 0.2 g of L-arginine HCl (cat. no. A-5131), 0.3 g of L-lysine HCl (cat. no. L-1262), 0.2 g of L-methionine (cat. no. M-9625), 0.5 g of L-phenylalanine (cat. no. P-5030), 2.0 g of L-threonine (cat. no. T-8625), and 0.3 g of L-tyrosine (cat. no. T-3754). Filter sterilize and store refrigerated.
9. 100X Histidine: Dissolve 0.2 g of L-histidine HCl monohydrate (cat. no. H-9511; Sigma) in 100 mL of water and filter sterilize. Store refrigerated.
10. 100X Leucine: Dissolve 1.0 g of L-leucine (cat. no. L-1512; Sigma) in 100 mL of water and filter sterilize. Store refrigerated.
11. 100X Tryptophan: Dissolve 0.2 g of L-tryptophan (cat. no. T-0254; Sigma) in 100 mL of water and filter sterilize. Store refrigerated.
12. 100X Uracil: Dissolve 0.2 g of uracil (cat. no. U-750; Sigma) in 100 mL of water and filter sterilize. Store refrigerated.
13. SD minimal medium: In 850 mL of water, dissolve 6.7 g of yeast nitrogen base without amino acids (cat. no. 0919-15-3; Difco) and autoclave. Cool to ~55°C, add 100 mL of 10X SD-His/-Leu/-Trp/-Ura, and 50 mL of 40% solution of dextrose, and adjust the pH to ~6.0 by adding 1.0 mL of 1 N NaOH. When required, supplement with 10 mL of 100X solution of omitted components (histidine, leucine, tryptophan, and uracil) and with 1 M solution of 3-AT to the appropriate concentration. For SD plates, add 25 g of agar (cat. no. 0140-01; Difco) before autoclaving.

## 2.2. Liquid Assay for $\beta$ -Galactosidase Activity

1. Z buffer ( $\beta$ -galactosidase assay buffer): Dissolve the following in 100 mL of distilled water: 1.6 g of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.55 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.075 g of KCl, and 0.025 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . Adjust the pH to 7.0 and autoclave.

2. 5-Bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) stock solution (20 mg/mL): Dissolve 100 mg of X-gal (cat. no. 15520-034; Gibco-BRL, Gaithersburg, MD) in 5 mL of *N,N*-dimethylformamide (cat. no. 27054-7; Sigma-Aldrich) and store at  $-20^{\circ}\text{C}$ .

### 2.3. Isolation of Library Plasmid from Yeast Transformants

1. *Escherichia coli* HB 101 competent cells (cat. no. 1011; Promega, Madison, WI); *F*<sup>-</sup>, *thi*-1, *hsd* S20 (*r*<sub>B</sub><sup>-</sup>, *m*<sub>B</sub><sup>-</sup>), *supE44*, *recA13*, *ara14*, *leuB6*, *proA2*, *lacY1*, *galK2*, *rpsL20* (*str*<sup>R</sup>), *xyl*-5, *mtl*-1,  $\lambda$ <sup>-</sup>.
2. Zymolyase-20T (20,000 U/g) (cat. no. 320921; ICN).
3. 5X M9 salts: Dissolve in 1 L of distilled water the following: 64 g of Na<sub>2</sub>HPO<sub>4</sub> · 7 H<sub>2</sub>O, 15 g of KH<sub>2</sub>PO<sub>4</sub>, 2.5 g of NaCl, and 5 g of NH<sub>4</sub>Cl. Sterilize by autoclaving.
4. M9 minimal medium: Autoclave 650 mL of distilled water, cool to  $\sim 55^{\circ}\text{C}$ , and add the following components while stirring: 100 mL of 10X SD-His/-Leu/-Trp/-Ura, 10 mL of 100X histidine, 10 mL of 100X tryptophan, 10 mL of 100X uracil, 200 mL of 5X M9 salts, 10 mL of 40% solution of dextrose (glucose), 2 mL of 1 M MgSO<sub>4</sub>, 1 mL of 0.1 M CaCl<sub>2</sub>, 1 mL of 1 M thiamine-HCl (cat. no. T-4625; Sigma), 4 mL of 10 mg/mL solution of proline (cat. no. P-8449; Sigma), and 1 mL of 1000X ampicillin (100 mg/mL). For M9 plates, add 25 g of agar (cat. no. 0140-01; Difco) to water before autoclaving.
5. LB/amp medium: Prepare liquid LB medium by dissolving in 1 L of distilled water the following: 10 g of tryptone (cat. no. 0123-17-3; Difco), 5 g of yeast extract (cat. no. 0127-17-9; Difco), and 5 g of NaCl. Adjust the pH with concentrated NaOH to 7.0 and autoclave. Before using, add a stock solution of ampicillin (100 mg/mL, stored frozen in  $-20^{\circ}\text{C}$ ) to a final concentration of 100  $\mu\text{g/mL}$  (1000X dilution).

### 2.4. Cloning of SLBP Using Three-Hybrid System

1. Yeast strain L40-coat: *MATa*, *ura3-52*, *leu2-3,112*, *his3 $\Delta$ 200*, *trp1 $\Delta$ 1*, *ade2*, *LYS2:: (LexAop)-HIS3*, *ura3:: (LexAop)-LacZ*, *LexA-MS2 coat (TRP1)*. This strain was constructed by transforming the L40 yeast cells with a hybrid gene that is under control of the constitutive yeast *ADHI* promoter and encodes a fusion protein consisting of the LexA DNA-binding domain (DBD) at the N-terminus and the MS2 coat protein at the C-terminus (29). This gene was stably integrated into the chromosomal DNA of the L40 along with the *TRP1* selective marker allowing L40-coat cells to grow on minimal medium lacking tryptophan.
2. Plasmids pMS2-1 and pMS2-2 (see Note 2): These plasmids contain a region encoding two identical, closely spaced RNA-binding sites for the MS2 coat protein. Each site bears a specific point mutation that increases the affinity of the interaction between RNA and the protein. Immediately downstream (pMS2-1) or upstream (pMS2-2) of this region there are two unique restriction sites for *Xma*I (*Sma*I) and *Spe*I allowing subcloning of a DNA fragment encoding the histone

stem-loop sequence (or any other RNA sequence of interest). The position of the MS2 sites relative to both restriction sites (and hence to the sequence of interest) is the only difference between pMS2-1 and pMS2-2 (29). The entire insert encoding the MS2 binding sites and the stem-loop sequence (or any other RNA sequence of interest) can be recovered from both pMS2-1 and pMS2-2 by cleaving with *EcoRI*. pMS2-1 and pMS2-2 contain the tetracycline resistance gene as a selective marker and can be propagated only in *E. coli* cells.

3. Plasmid pIIIEx426RPR: This plasmid, constructed by Good and Engelke (32), is a shuttle vector that can be transformed into both *E. coli* and yeast cells using as a selection marker the ampicillin resistance gene or the *URA3* gene, respectively. pIIIEx426RPR contains the RNA polymerase III-specific promoter of the yeast RNase P gene and a unique *EcoRI* site 84 nucleotides downstream from the transcription start point. This site can be used to insert the *EcoRI* fragment recovered from the pMS2-1 or pMS2-2 plasmids that encode the MS2 binding sites and the sequence specifically recognized by the RNA-binding protein of interest (see Note 3).
4. Two pairs of complementary oligonucleotides, each 39 nucleotides long: On annealing these oligonucleotides generate a 35-nucleotide double-stranded region and 5' overhangs for *XmaI* and *SpeI* on the left and right end, respectively. The double-stranded region formed by the first pair of oligonucleotides encodes the histone RNA stem-loop sequence (SL<sub>WT</sub>) recognized by the SLBP, and a stretch of six uridines located five nucleotides downstream, serving as a termination site for the RNA polymerase III (Fig. 3B) (see Note 4). The double-stranded region formed by the other pair of oligonucleotides encodes, in addition to a stretch of six uridines, a mutant version of the stem-loop sequence (SL<sub>RM</sub>) that is unable to bind SLBP (Fig. 3B) (see Note 5).
5. A two-hybrid cDNA library from the organism of interest: The human SLBP was cloned using an HeLa cDNA library constructed in pGAD GH vector (cat. no. 6182-1; Clontech) expressing the GAL4 activation domain (AD) (amino acids 768–881) (see Note 6).
6. Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were from either New England Biolabs or Promega.
7. LB/amp medium (see Subheading 2.3.): When making LB/amp plates, before autoclaving add 15 g of agar to liquid LB medium, cool to ~55°C, add stock solution of ampicillin to a final concentration of 100 µg/mL, gently mix, and pour plates.
8. LB/tet medium: To cold liquid LB medium (see Subheading 2.3.) add a stock solution of tetracycline in ethanol (5 mg/mL, stored at –20°C) to a final concentration of 10 µg/mL (500X dilution). When making LB/tet, before autoclaving add 15 g of agar to liquid LB medium, cool to ~55°C, add stock solution of tetracycline to the same final concentration, gently mix, and pour plates.

## 2.5. Cloning of Proteins Interacting with SLBP-RNA Complex

1. Yeast strain CG-1945: *MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4-542*, *gal80-538*, *cyh2*, *LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3*,

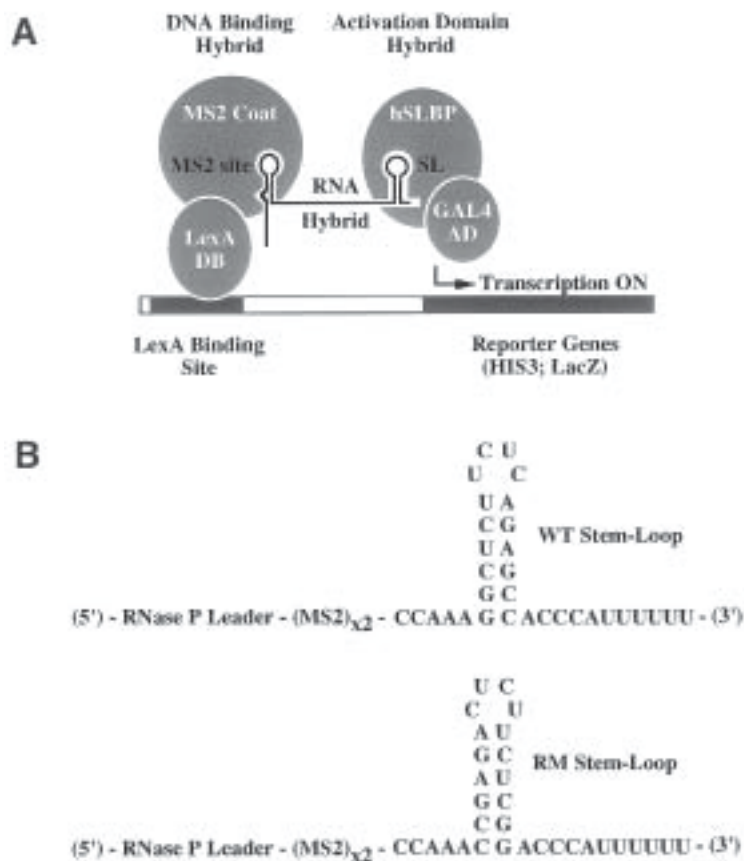


Fig. 3. The three-hybrid system developed in laboratories of Wickens and Fields (29), as specifically modified for cloning proteins interacting with the wild-type histone stem-loop RNA. (A) Components of the three-hybrid system. The DNA-binding hybrid consists of the LexA DBD (DB) at the N-terminus and the coat protein of the MS2 phage at the C-terminus. The AD hybrid consists of SLBP expressed from a cDNA library and the GAL4 AD at the N-terminus. The RNA hybrid contains the RNase P leader at the 5' end, the two binding sites for the MS2 coat protein, and the histone stem loop at the 3' end. Binding of the two protein hybrids to their RNA targets results in reconstitution of the transcriptional activator and expression of two reporter genes controlled by the LexA-binding site, *HIS3* and *LacZ*. (B) Sequence of the wild-type (WT) stem-loop structure in the RNA hybrid used as a bait for cloning SLBP and of the reversed mutant (RM), unable to bind SLBP and used as a negative control in the secondary screen.

- URA3::GAL4<sub>17-mer(x3)</sub>-CYC1<sub>TATA</sub>-lacZ*. This strain, commonly used as a host strain in the two-hybrid screen, contains the *HIS3* and the *LacZ* reporter genes, both under the control of the GAL4-binding site.
2. pGBT8: This plasmid is a modified version of the commercially available pGBT9 plasmid (cat. no. K1605-A; Clontech) (*see Note 7*). Both pGBT8 and its parental pGBT9 are autonomously replicating plasmids that can be transformed into *E. coli* cells using the ampicillin resistance gene and into yeast cells using the *TRP1* gene. Both shuttle vectors contain a region of the *GAL4* gene encoding the N-terminal DBD (amino acids 1–147) from the constitutive *ADHI* promoter. Immediately downstream from this region is a multiple cloning site that allows in-frame insertion of a cDNA encoding the protein of interest that will be expressed as a fusion protein with the N-terminal GAL4 DBD.
  3. pIII/MS-SL<sub>WT</sub>: This plasmid expresses the hybrid RNA consisting of the two MS2-binding sites and the histone stem-loop structure at the 3' end and has been used in the three-hybrid screen (*see Subheading 3.4.1.*).
  4. A two-hybrid cDNA library: A human cDNA library from HeLa cells used in this study was constructed in the pGAD GH vector (cat. no. 6182-1; Clontech) expressing the GAL4 AD (amino acids 768–881) (*see Note 8*).
  5. Forward primer for sequencing and polymerase chain reaction (PCR) amplification of the cDNA insert in the pGAD GH vector: 5' CTATTCGATGATGA AGATACCCACCAAACCC 3'.
  6. Reverse primer for sequencing and PCR amplification of the cDNA insert in the pGAD GH vector: 5' GTGAACTTGCGGGGTTTTTCAGTATCTACGAT 3'.
  7. Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, T4 DNA polymerase and Klenow fragment of the *E. coli* DNA polymerase I were from New England Biolabs or Promega.
  8. LB/amp medium (*see Subheading 2.3.*).

### 3. Methods

#### 3.1. Transformation of Yeast Cells

Successful cloning of rare sequences from cDNA libraries requires screening a high number of transformants. Therefore, only protocols that result in high-efficiency transformation should be used. The following protocol is a combination of the procedure developed by Gietz et al. (33) and the large-scale transformation procedure recommended by Clontech. This protocol routinely yields approx  $5 \times 10^4$  transformants/ $\mu$ g of DNA (*see Note 9*).

##### 3.1.1. High-Efficiency, Large-Scale Yeast Transformation

1. Use a frozen stock of the yeast cells that have been carefully checked for nutritional requirements prior to freezing. Scrape some of the frozen stock and inoculate 5 mL of SD minimal medium selecting against loss of any plasmid previously transformed into these cells.

2. Grow at 30°C with constant shaking or rotation for approx 48 h to stationary phase. Dilute appropriately in the same medium to get a culture in the mid to late logarithmic phase ( $OD_{600}$  at 0.25–1.0) the night before performing transformation.
3. Inoculate 1 L of prewarmed YPD with a sufficient amount of the refreshed culture to get an  $OD_{600}$  at 0.5–0.6 the next morning (*see Note 10*).
4. Centrifuge the cells at 1000g for 5 min at room temperature in a GSA rotor (Sorvall).
5. Discard the supernatant and rinse the pellet twice with 100 mL of sterile water, each time centrifuging the cells as in **step 4**.
6. Resuspend the cell pellet in 10.0 mL of 1X TE/LiAc freshly prepared from 1 mL of 10X TE, 1 mL of 10X LiAc, and 8 mL of sterile water.
7. Mix the following in a large 250-mL centrifuge tube: 2 mL of denatured herring testes carrier DNA (10  $\mu$ g/ $\mu$ L), 200  $\mu$ g of cDNA library, and 10 mL of the yeast competent cells (*see Note 11*).
8. Incubate at 30°C for 30 min with gentle shaking.
9. Add 50 mL of sterile 40% PEG 4000 in 1X LiAc/TE freshly prepared by combining the following components: 40 mL of 50% PEG 4000, 5 mL of 10X TE buffer, and 5 mL of 10X LiAc (1 M). Mix well and incubate with shaking for an additional 30 min.
10. Add 6.5 mL of dimethylsulfoxide (cat. no. D-5879; Sigma) and mix well by inverting the tube several times.
11. Transfer the tube to a 42°C water bath and incubate for 15 min with occasional mixing. Chill on ice for 15 min.
12. Centrifuge the cells at 1000g for 5 min at room temperature and remove the supernatant. Rinse the pellet with 100 mL of sterile water and centrifuge again.
13. Resuspend the pellet in 15 mL of sterile water and spread on 30–35 large (150-mm diameter) plates with the selective medium and, if necessary, supplemented with 3-AT. Estimate the efficiency of transformation by plating sequentially diluted cells on the selective medium containing histidine.

### 3.1.2. Low-Efficiency, Small-Scale Yeast Transformation

Generation of a host strain for cDNA library screening by introducing a bait plasmid does not require a large number of transformants and can be achieved using frozen competent cells. Compared with freshly prepared cells, frozen cells have significantly reduced competency (approx 100-fold) but yield several hundred colonies/ $\mu$ g of autonomously replicating plasmid DNA. Frozen stocks of competent cells can be prepared by following the protocol described in **Subheading 3.1.1.** up to **step 6**. The cells are suspended in 1X LiAc/TE and then 100% glycerol is added to a final concentration of 10%. The cell suspension is divided into small aliquots and stored at –80°C for future use. Competent cells for introducing the bait plasmid can also be prepared fresh each time by appropriately scaling down the large-scale protocol. Small-scale transformation of yeast cells is performed as follows (*see Note 12*):



1. Mix 150  $\mu\text{L}$  of yeast cells suspended in 1X LiAc/TE (also containing 10% glycerol if an aliquot of frozen cells is used) with 1  $\mu\text{g}$  of plasmid DNA and 200  $\mu\text{g}$  of herring testes carrier DNA (20  $\mu\text{L}$  of 10  $\mu\text{g}/\mu\text{L}$ ) and vortex (*see Note 11*).
2. Add 500  $\mu\text{L}$  of freshly prepared 40% PEG in 1X LiAc/TE and vortex vigorously.
3. Incubate with shaking for 30 min at 30°C.
4. Centrifuge for a few seconds in a microcentrifuge and carefully aspirate off the supernatant.
5. Resuspend in 200  $\mu\text{L}$  of sterile water and spread on two plates containing the selective SD minimal medium.
6. Analyze several colonies for self-activation of *HIS3* and *LacZ* reporter genes, by replating on SD minimal medium lacking histidine and performing liquid assay for  $\beta$ -galactosidase activity, respectively.

### 3.2. Liquid Assay for $\beta$ -Galactosidase Activity

In addition to the routinely used filter assay, we often test  $\beta$ -galactosidase activity of His<sup>+</sup> colonies using a modified version of the liquid assay. Although more laborious than the filter assay, the liquid assay described below is reproducible and allows identification of colonies with relatively weak  $\beta$ -galactosidase activity.

1. Grow individual His<sup>+</sup> colonies in small patches (~0.5 in.<sup>2</sup>) on plates with the selective medium.
2. Scrape a full loop of yeast cells (approx one-third of the patch), transfer to an Eppendorf tube containing 100  $\mu\text{L}$  of Z buffer, and vortex vigorously. Keep the plates for subsequent isolation of plasmid DNA from the  $\beta$ -galactosidase positive colonies.
3. Freeze/thaw the cell suspension several times to permeabilize the cells by sequentially placing the tubes in dry ice and a 37°C water bath.
4. Leave at room temperature until a blue color develops (*see Note 13*).

### 3.3. Isolation of AD/Library Plasmid from Yeast Transformants

Two different plasmids reside in yeast transformants that have been positively selected for *HIS3* and *LacZ* expression: the bait plasmid and the library plasmid. The library plasmid typically contains the yeast *LEU2* gene that can complement the *leuB6* mutation of the *E. coli* HB101 strain. Thus, the library plasmid can be easily separated from the other residing plasmid by transforming HB101 bacterial cells with a crude yeast DNA preparation and selecting for Leu<sup>+</sup> colonies on minimal M9 medium lacking leucine. Here we present a method routinely used in our laboratory for isolating plasmid DNA from double-positive yeast colonies. This method results in high-quality DNA preparations that are useful for both transformation of *E. coli* and PCR. Transformation of moderately competent HB101 cells ( $5 \times 10^5$  transformants/ $\mu\text{g}$  of



supercoiled DNA) with 1  $\mu\text{L}$  of this DNA preparation (1% of the total volume) routinely yields between 50 and 100  $\text{Leu}^+$  colonies of *E. coli*.

1. Scrape most of the remaining two-thirds of the yeast patch from colonies positively assayed for  $\beta$ -galactosidase activity and transfer to 500  $\mu\text{L}$  of solution containing 1 *M* sorbitol (cat. no. S-1876; Sigma), 0.1 *M* EDTA, pH 7.5, and 150  $\mu\text{g}$  of zymolyase-20T.
2. Vortex until the yeast cells are completely resuspended and incubate for 1 h at 37°C.
3. Briefly spin in the microcentrifuge to pellet spheroplasts and remove the supernatant.
4. Resuspend in 500  $\mu\text{L}$  of solution containing 50 mM Tris-HCl and 20 mM EDTA, pH 7.4.
5. Add 50  $\mu\text{L}$  of 10% sodium dodecyl sulfate, mix well, and incubate for 30 min at 65°C.
6. Add 200  $\mu\text{L}$  of 5 *M* potassium acetate and chill on ice for 15 min.
7. Centrifuge for 15 min in a microcentrifuge at the maximum speed.
8. Transfer the supernatant to a new tube and add 1 vol of isopropanol. Mix well and incubate for 5 min at room temperature.
9. Centrifuge for 15 min in a microcentrifuge at the maximum speed.
10. Aspirate the supernatant, wash the pellet with 70% ethanol, and air-dry.
11. Dissolve the pellet in 100  $\mu\text{L}$  of sterile water containing 0.1  $\mu\text{g}/\mu\text{L}$  of RNase A (cat. no. R-4642; Sigma).
12. Use 1  $\mu\text{L}$  of DNA solution for transformation of *E. coli* HB101, and select transformants containing the library plasmid by growing for 2 to 3 d on M9 medium lacking leucine. This DNA can also be used directly for PCR using standard protocols.
13. Grow individual transformants in 5 mL of LB/amp, and isolate the plasmid using standard miniprep procedures.

### 3.4. Cloning of SLBP Using Three-Hybrid System

The yeast three-hybrid system was developed for cloning unknown proteins with RNA-binding activity (29,34). In addition, this system can be used to analyze known RNA-protein interactions (35,36) and to identify new RNA targets for specific RNA-binding proteins (37). The strong affinity of interaction between SLBP and the RNA stem loop, which interfered with purification from mammalian cells, was a great advantage in the three-hybrid system and made SLBP an excellent candidate for using this genetic approach as a cloning strategy. Indeed, human SLBP (or HBF) was the first RNA-binding protein cloned using the three-hybrid system (30,31). This system should be generally and readily applicable for isolation of proteins that bind small, structured RNA sequences with high affinity.

The three-hybrid system is an extension of the two-hybrid system that, in addition to the two-hybrid proteins, includes a hybrid RNA (**Fig. 3**). The hybrid RNA contains two independent binding sites, each specifically recognized by a different RNA-binding protein. The first binding site is a defined RNA sequence avidly recognized by known RNA-binding protein expressed as a hybrid protein with the DBD. The second binding site of the hybrid RNA molecule, also referred to as RNA bait, is a sequence of interest that is recognized by an unknown protein fused to an AD and expressed from a cDNA library. Association of the two RNA-binding proteins with their cognate targets on the bifunctional RNA brings the DBD and the AD into proximity and reconstitutes the transcriptional activator. This in turn results in activation of engineered reporter genes controlled by the DBD allowing easy selection of yeast clones expressing the unknown RNA-binding protein. The system can be readily adapted to individual needs by introducing a desired sequence into the RNA hybrid. The remaining components of the system remain unchanged.

Two three-hybrid systems based on the same strategy and varying only in details have been independently developed and reported (**29,34**). The differences include the source of DBD and the type of RNA-binding protein and its RNA target. SLBP (or HBF) was cloned simultaneously in our (**30**) and D. Schümperli's (**31**) laboratories using the three-hybrid system developed in collaboration between the laboratories of M. Wickens and S. Fields (**29**). This variant of the three-hybrid system relies on the DBD of the bacterial protein LexA and the two reporter genes, *HIS3* and *LacZ*, containing the LexA recognition site in the promoter region (**Fig. 3**). The LexA is fused to the coat protein of MS2 phage. The gene encoding this hybrid protein is stably integrated into the yeast genome of the L40 strain, resulting in L40-coat strain. The multicopy plasmid pIIIEx426RPR carrying the *URA3* selection marker is used to express the RNA hybrid. This plasmid contains the RNA polymerase III-specific promoter of the *RNase P* gene attached to the region encoding two closely spaced MS2-binding sites, each containing a specific point mutation to enhance the interaction with the MS2 coat protein. The sequence of interest can be subcloned either upstream or downstream of this region using available restriction sites. In vivo transcription of this hybrid gene by RNA polymerase III generates the hybrid RNA that, in addition to the double MS2 site and sequence of interest, contains an 84-nucleotide leader from RNase P at the 5' end (**Fig. 3B**). The plasmid expressing the hybrid RNA is first introduced as an episome into the L40-coat yeast strain. The resulting strain is subsequently transformed with a cDNA library inserted into a high-copy LEU2 vector that carries the cDNA library fused to the GAL-4 AD.

### 3.4.1. Construction of Plasmids Expressing RNA Hybrids

1. Combine two pairs of complementary single-stranded oligonucleotides (5  $\mu$ g each) containing the sequence of either wild-type (WT) or reversed mutant (RM) histone stem-loop sequence in two Eppendorf tubes in a final volume of 100  $\mu$ L and a final NaCl concentration of 150 mM (*see Note 14*).
2. Place the Eppendorf tubes in a beaker containing 1 L of water. Boil the water for 5 min and allow to cool slowly to room temperature (approx 1.5 h). On annealing the oligonucleotides form the *Xma*I and *Spe*I sticky ends (*see Note 15*).
3. Combine 10  $\mu$ L of each annealed mixture with 4  $\mu$ L of 10X ligation buffer, 0.5  $\mu$ g of gel-purified pMS2-1 plasmid cleaved with *Xma*I and *Spe*I, 1  $\mu$ L of T4 DNA ligase (both the enzyme and buffer were from New England Biolabs, cat. no. 202S), and water to a final vol of 40  $\mu$ L. Carry out the ligation overnight at 14°C.
4. Transform 5  $\mu$ L of each ligation mixture into *E. coli* DH5 competent cells and select transformants on LB plates with tetracycline.
5. Isolate plasmid DNA from several colonies by standard techniques of DNA miniprep. Identify clones containing the wild-type (pMS-SL<sub>WT</sub>) and mutant (pMS-SL<sub>RM</sub>) inserts by sequencing. The ligation places the insert of either type downstream from the region encoding the two MS2-binding sites (*see Note 16*).
6. Recover the *Eco*RI fragment encoding the two MS2-binding sites and the stem-loop and termination sites from pMS-SL<sub>WT</sub> and pMS-SL<sub>RM</sub> and ligate into the *Eco*RI site of the pIIIEx426RPR. Treat the *Eco*RI-cleaved vector with alkaline phosphatase (cat. no. 290S; New England Biolabs) prior to ligation to prevent self-ligation. Carry out both phosphatase treatment and ligation according to the standard protocols.
7. Use a small portion of each ligation mixture for transformation of *E. coli* DH5 competent cells and select transformants on LB plates containing ampicillin. Identify clones containing the *Eco*RI fragment with either the wild-type stem-loop sequence (pIIIEx-SL<sub>WT</sub>) or the mutant sequence (pIIIEx-SL<sub>RM</sub>) by isolating plasmid DNA from several ampicillin-resistant colonies followed by DNA restriction analysis. Confirm the correct orientation of the insert by sequencing. This manipulation places the *Eco*RI fragment under the control of RNase P promoter, between the regions encoding the RNase P leader and the RNase P termination site (*see Note 17*).

### 3.4.2. Construction of Yeast Host Strain for Three-Hybrid Screen

1. Transform the yeast strain L40-coat with either pIIIEx-SL<sub>WT</sub> or pIIIEx-SL<sub>RM</sub> using a frozen aliquot of competent cells (*see Subheading 3.1.1*). Select the transformants of each type (L40-coat/WT and L40-coat/RM, respectively) on SD minimal medium lacking uracil and tryptophan.

2. Choose five independent colonies from each transformation and test for self-activation of the *HIS3* reporter gene by plating on SD medium in which histidine is omitted along with the two other nutritional markers (uracil and tryptophan).
3. Test different concentrations of 3-AT to suppress slow rates of growth on medium lacking histidine owing to residual activity of the *HIS3* gene. The addition of 5 mM 3-AT completely eliminates background growth.

### 3.4.3. Screening Human HeLa cDNA Library

1. Use an individual colony of L40-coat transformed with pIIIEx-SL<sub>WT</sub> (L40-coat/WT) to prepare competent cells for a large-scale transformation.
2. Use approx 200 µg of DNA from the human cDNA library constructed in the AD vector pGAD GH and carrying the *LEU2* selective marker to transform the L40-coat/WT competent cells. Plate the cells on SD minimal medium lacking the following components: tryptophan and uracil (to select against the loss of the two previously introduced markers), leucine (to select colonies containing pGAD GH library plasmid), and histidine (to allow growth of only those Leu<sup>+</sup> colonies that express *HIS3* gene). To suppress residual activity of *HIS3* gene conferred by pIIIEx-SL<sub>WT</sub> and to increase the stringency of selection, supplement the medium with 5 mM 3-AT. Sequentially dilute a small portion of the transformation mixture in sterile water and plate on medium containing histidine to assess the efficiency of transformation and to calculate the overall number of transformants tested (see **Note 18**).
3. Test all His<sup>+</sup> colonies for β-galactosidase activity using both the filter and the liquid assays (see **Subheading 3.2.**).
4. Isolate plasmid DNA from all double-positive colonies (see **Subheading 3.3.**) and rescue AD/library plasmid by transforming 1 µL of DNA preparation into HB101 competent cells. Select bacterial colonies on M9 medium lacking leucine and supplemented with ampicillin.
5. Isolate each of the library plasmids from individual HB101 Leu<sup>+</sup> amp<sup>R</sup> transformants and analyze in a secondary screen with the mutant RNA stem loop (see **Note 19**). Transform approx 1 µg of DNA into L40-coat/RM yeast strain using frozen competent cells. Select the transformants on SD medium lacking tryptophan, uracil, and leucine (but containing histidine).
6. Transfer individual yeast transformants onto the selective medium lacking histidine to determine expression of the *HIS3* gene in the presence of the mutant stem-loop RNA structure. In addition, assay all colonies for the β-galactosidase activity to determine expression of the *LacZ* reporter gene (see **Note 20**). Library plasmids that lead to expression of both *HIS3* and *LacZ* in the presence of the wild-type stem loop but that fail to activate the two reporter genes when assayed in the L40-coat/RM apparently encode proteins capable of specific interaction with the stem-loop RNA. Sequence and compare these plasmids to database sequences using the BLAST search (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) (see **Note 21**).

The final task is to demonstrate the ability of the protein to specifically interact with histone stem-loop RNA *in vitro* and to prove its relevance to histone mRNA biogenesis and metabolism. Details of experimental approaches leading to the functional characterization of the protein we cloned are described elsewhere (30,38,39). These experiments confirmed the identity of protein cloned by the three-hybrid system using the stem-loop bait as the previously identified SLBP. The frog homolog of the human SLBP, retaining amino acid sequence homology throughout the entire protein and called xSLBP1, was simultaneously isolated from the *Xenopus* oocyte cDNA library using the same system (30,40) (see Note 22). SLBPs are not the only RNA-binding proteins that have been cloned using the three-hybrid system. Screening of a *C. elegans* cDNA library using this system resulted in isolation of two additional RNA-binding proteins, both involved in controlling sexual development of worms (41,42). These proteins bind relatively weakly to unstructured and short sequences (used as baits) in the 3' untranslated region of some *C. elegans* mRNAs, indicating that application of the three-hybrid systems is not limited to cloning of strong RNA binders.

### 3.5. Cloning of Proteins Interacting with SLBP-RNA Complex

The yeast two-hybrid system is a widely used method for cloning proteins that interact *in vivo* with a previously cloned protein of interest (43). Here we describe utilization of this system for cloning proteins interacting with the SLBP-RNA complex. These proteins could include novel components of the processing machinery. Previous results indicate that one role of SLBP in histone pre-mRNA processing is to stabilize association of the U7 snRNP with the pre-mRNA (39), which is only weakly anchored to the substrate through the base-pairing between the U7 snRNA and the HDE (Fig. 2). This is most likely achieved by direct interaction between SLBP stably associated with the stem-loop structure and one of the protein components of the U7 snRNP (see Note 23). The *in vitro* complementation assay, in which processing activity of the nuclear extract depleted of endogenous SLBP is restored by the truncated versions of the recombinant SLBP expressed in baculovirus, revealed that this putative interaction requires only the RNA-binding domain and 20 amino acids to its C-terminus (39). Since the interaction between SLBP and a component of the U7 snRNP requires prebinding of SLBP to the histone pre-mRNA, we are likely to detect this interaction in the yeast two-hybrid system only in the presence of the stem-loop RNA. Dependence of protein-protein interaction on RNA was recently demonstrated for the yeast poly(A) binding protein (Pab1p). This protein can efficiently associate with the translation initiator factor eIF-4G only in the presence of poly(A) RNA (44). Binding of Pab1p to its

RNA target presumably triggers a conformational change in the protein, which exposes some critical amino acids for interaction with other proteins, including the eIF-4G.

The two-hybrid system in its standard design is based on reconstitution of a transcriptional activator from two-hybrid proteins, each containing a portion of the yeast transcriptional activator GAL4. In our adaptation of this system, SLBP was fused in-frame to the GAL4 DBD and coexpressed with the stem-loop RNA. This modification allowed screening of a cDNA library against the complex of SLBP with the 3' end of histone mRNA (its natural status in the cell) rather than against SLBP alone (**Fig. 4A**). To express the stem-loop RNA in yeast cells, we took advantage of an already existing construct, successfully used for cloning SLBP in the three-hybrid system. Screening for proteins interacting with SLBP-RNA complex requires any regular two-hybrid cDNA library and is carried out according to a standard two-hybrid screening protocol.

### 3.5.1. Construction of pGBT/SLBP

pGBT/SLBP allows yeast to express the fusion protein consisting of the GAL4 DBD and a truncated version of SLBP that lacks the last 27 amino acids since the full-length SLBP strongly self-activates the reporter genes (*see Note 24*).

1. Amplify the cDNA for SLBP by PCR to generate a fragment encoding the truncated version of SLBP. This fragment contains a single *NcoI* site (amino acid 1) and an *SacI* (amino acid 243).
2. Gel purify the 736-nucleotide PCR product and subsequently cleave at the *NcoI* and *SacI* sites using standard protocols.
3. Ligate the fragment containing the *NcoI* and *SacI* sticky ends into the same sites in the pGBT8 plasmid to generate pGBT/SLBP. Confirm in-frame ligation of the insert by sequencing.

### 3.5.2. Construction of pGBT/SLBP/SL<sub>WT</sub>

pGBT/SLBP/SL<sub>WT</sub> allows coexpression of SLBP and its natural RNA target, the histone stem-loop structure (SL<sub>WT</sub>) expressed as a fusion RNA with 84-nucleotide RNase P leader, and the two MS2-binding sites. The histone stem-loop structure is located downstream from the MS2-binding sites and is followed by a stretch of uridines at the termination site. Expression of the hybrid RNA is under the control of the RNA polymerase III promoter of the *RNase P* gene.

1. Cleave the polIII/SL<sub>WT</sub> plasmid with *SpeI* to generate a 534-nucleotide DNA fragment encompassing the *RNase P* promoter and the region encoding the hybrid RNA.

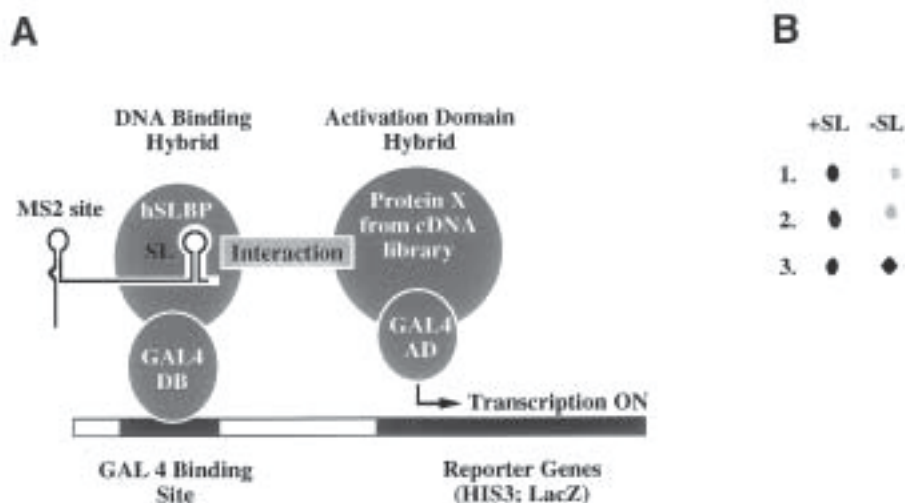


Fig. 4. The RNA-supplemented two-hybrid system designed for cloning proteins interacting with SLBP-RNA complex. **(A)** Components of the RNA-supplemented two-hybrid system. The DNA-binding hybrid is composed of the GAL4 DBD (DB) and human SLBP. The stem-loop RNA is expressed from the same gene as that used for cloning SLBP in the three-hybrid system. This RNA, in addition to the stem-loop sequence placed at the 3' end, contains the RNase P leader at the 5' end and the two MS2-binding sites in the center (**Fig. 3B**). Proteins interacting with the SLBP-RNA complex are expressed from a cDNA library constructed in the pGAD GH vector and are fused to the GAL4 AD. The interaction brings both domains of the GAL4 transcriptional activator into the proximity and results in activation of the *HIS3* and *LacZ* reporter genes controlled by the GAL4-binding site. **(B)** Ability of two proteins selected in the screen to activate the *LacZ* gene in the presence and absence of the stem-loop RNA (lanes 1 and 2). As determined by expression of the *LacZ*, interaction of both proteins with SLBP is strongly stimulated by the presence of the stem-loop RNA. For comparison, the same assay was carried out with an unknown SLBP-interacting protein selected in the standard two-hybrid screen, when the stem-loop RNA was not coexpressed with SLBP (unpublished results). Interaction of this protein with SLBP is not stimulated by the stem-loop RNA (lane 3).

This fragment ends immediately downstream from the six thymidines after the stem-loop structure, and, therefore, does not contain an additional termination site for RNA polymerase III from the *RNase P* gene.

2. Blunt-end the *SpeI* fragment with the Klenow fragment of the *E. coli* DNA polymerase I (cat. no. 210S; New England Biolabs) and gel purify using standard protocols.



3. Cleave DNA of the pGBT8/SLBP with *Aat*II at the unique site outside the ampicillin resistance gene and blunt the ends with the 3' exonuclease activity of T4 DNA polymerase (cat. no. 203S; New England Biolabs).
4. Mix an approx 10-fold molar excess of the *Spe*I fragment with the *Aat*II-cleaved pGBT8/SLBP in the presence of 1X ligation buffer and 2  $\mu$ L of T4 DNA ligase in a total volume of 20  $\mu$ L. Incubate the ligation mixture overnight at room temperature and transform into *E. coli* DH5 competent cells.
5. Isolate plasmid DNA from several ampicillin-resistant colonies and identify clones containing the insert by restriction analysis (see **Note 25**). To avoid possible introduction of mutations during PCR amplification, sequence the entire insert of one positive clone in two directions. This subcloning procedure results in the generation of the pGBT/SLBP/SL<sub>WT</sub> plasmid that allows coexpression of SLBP fused to the GAL4 AD and the hybrid RNA, identical to the RNA expressed from polIII/SL<sub>WT</sub> (see **Note 26**).

### 3.5.3. Construction of Yeast Host Strain for RNA-Supplemented Two-Hybrid Screen

1. Transform pGBT/SLBP/SL<sub>WT</sub> into CG-1945 yeast strain using an aliquot of frozen competent cells and select the transformants on SD minimal medium lacking tryptophan.
2. Test several individual colonies for self-activation of the *HIS3* reporter gene by plating on minimal medium lacking both tryptophan and histidine. Also test the same colonies for expression of the *LacZ* reporter gene using liquid assay for  $\beta$ -galactosidase activity (see **Note 27**).
3. In addition to the CG<sub>SLBP+SL</sub> strain, construct the CG<sub>SLBP-SL</sub> strain by transforming the CG-1945 competent cells with pGBT/SLBP plasmid. Test multiple colonies of this strain, expressing only SLBP and not the stem-loop RNA, for self-activation of the *HIS3* and *LacZ* reporter.

### 3.5.4. Screening of Human cDNA Library Using RNA-Supplemented Two-Hybrid System

1. Use an individual colony of the CG<sub>SLBP+SL</sub> (CG-1945 strain transformed with pGBT/SLBP/SL plasmid) to prepare competent cells for a large-scale transformation (see **Subheading 3.1.**)
2. Use approx 100  $\mu$ g of the human cDNA library constructed in the GAL4 AD vector pGAD GH carrying the *LEU3* selective marker to transform the CG<sub>SLBP+SL</sub> competent cells. Spread the transformation mixture on 40 large plates with SD minimal medium lacking tryptophan, leucine, and histidine (the medium is not supplemented with 5 mM 3-AT). To calculate the total number of transformants screened, sequentially dilute a small portion of the transformation mixture in sterile water and plate on medium containing histidine (see **Note 28**).
3. Isolate plasmid DNA from the double-positive yeast colonies (see **Subheading 3.3.**) and PCR amplify the cDNA insert using a standard PCR protocol and prim-



ers complementary to each flank of the pGAD GH multiple cloning site (*see Subheading 2.5.*).

4. Digest single-band PCR products using a combination of several restriction enzymes with frequent recognition sites. Tentatively assign the inserts that are successfully amplified by PCR to one of several groups based on the same or overlapping restriction pattern.
5. Transform one plasmid from each group and plasmids that did not generate any PCR product into HB101 competent cells and sequence. Digest all remaining PCR products from each group with rare cutting restriction enzymes, selected based on the known sequence, to confirm their identity with the sequenced member of the group (*see Note 29*).

#### 3.5.5. Secondary Screen Without Stem-Loop RNA

1. Introduce all plasmids into the CG<sub>SLBP-SL</sub> strain expressing human SLBP but not the stem-loop RNA. Select the transformants on SD medium containing histidine and lacking tryptophan and leucine.
2. Analyze three individual colonies from each transformation for expression of the *HIS3* and *LacZ* reporter genes by replating cells on SD medium lacking histidine, tryptophan, and leucine and by performing the liquid  $\beta$ -galactosidase activity assay, respectively (*see Notes 30 and 31*).

#### 4. Notes

1. All glassware used in high-efficiency library transformation should be free of any residual detergents and chemicals.
2. All plasmids and strains used in the three-hybrid system can be directly obtained from the laboratories of Drs. Marv Wickens (University of Wisconsin, Madison) and Stan Fields (University of Washington, Seattle).
3. The list of plasmids provided for the three-hybrid system by Wickens's and Field's laboratories also includes pIII/MS2-1 and pIII/MS2-2 plasmids. These plasmids allow one-step insertion of the RNA sequence of interest either at the 3' end of the hybrid RNA (pIII/MS2-1) or between the RNase P leader and the two MS2-binding sites (pIII/MS2-2) without the necessity of using the intermediate pMS2 plasmids. However, both plasmids contain the *SpeI* site within the vector precluding convenient directional cloning between the *SpeI* and *XmaI* sites.
4. The RNA sequence to be expressed from the RNase P promoter and used as a bait should be carefully inspected for the presence of four or more uridines in a row. The region encoding uninterrupted stretches of uridines can potentially be used as a termination site for the RNA polymerase III and thus can significantly decrease the expression of the RNA sequence of interest. The consensus for the stem-loop sequence contains four uridines in a row (the top base in the stem and three bases in the loop). To avoid possible problems with pretermination, this sequence was changed to UUCU by substituting the relatively less-conserved second uridine in the loop with a cytidine, frequently found at this position (**Fig.**

**1A** and **3B**). However, this did not seem to be necessary because the stem-loop sequence containing four uridines in a row was successfully used for cloning of SLBP by D. Schümperli and coworkers (**31**).

5. This mutation was created by reversing the entire sequence of the stem-loop structure (**Fig. 3B**). The RNA expressed from this mutant retains the ability to form the stem-loop structure with the same stability as the wild-type sequence but is not recognized by SLBP.
6. The HeLa cell library used for cloning SLBP was provided by Dr. Yue Xiong (University of North Carolina at Chapel Hill). This library also can be purchased from Clontech (cat. no. HL4000AA). In our experience, the quality of the cDNA library is critical, although it is very hard to evaluate. We screened three different *Drosophila* libraries, presumably prepared from the same stage embryos, and only obtained the SLBP from one of these.
7. This plasmid, provided by Dr. Yue Xiong (University of North Carolina at Chapel Hill), was constructed from pGBT9 by incorporating several additional restriction sites into the multiple cloning site, including sites for *NcoI* and *SacI*.
8. The DNA used in this study was provided by Dr. Branko Stefanovic (University of North Carolina at Chapel Hill) and contained the same cDNA library that was used for the three-hybrid screen.
9. To test the quality of reagents and the phenotype of the yeast cells, we recommend performing a small-scale transformation prior to scaling up the protocol for library transformation.
10. The growth rate varies significantly among yeast strains. Therefore, the amount of 50-mL culture necessary to produce the desired OD<sub>600</sub> the next day should be established empirically. If the OD value of the 1-L culture is too high in the morning, dilute the culture two to three times with a fresh YPD medium and incubate for an additional 2 to 3 h. Because this requires a large volume of the medium, it is always safer to use a smaller inoculum and wait several hours the following day for the culture to reach correct density without the need for dilution.
11. A small-scale transformation carried out without DNA (which should yield no colonies on the selective medium) in parallel to the library transformation is a useful control allowing detection of any contamination with other yeast cells. This control transformation is especially important when a frozen stock of competent cells is used because a relatively small number of genuine transformants can be overwhelmed by possible contamination.
12. This protocol omits a few nonessential steps that are important only for high-efficiency transformation.
13. The blue color usually appears within 2 to 3 h. However, clones with lower  $\beta$ -galactosidase activity may require overnight incubation.
14. A DNA fragment encoding an RNA sequence of interest can be inserted into either MS2-1 or MS2-2 by using standard subcloning methods. These methods, including PCR-mediated amplification or isolation of an appropriate restriction

fragment, are especially useful when the length of RNA sequence exceeds 70–80 nucleotides.

15. To facilitate cloning and screening of correct clones, it may be desirable to design the sequence of the oligonucleotides so that one of the two restriction sites will be disrupted on ligation of the insert into the pMS2-1 plasmid. The ligation mixture can be cleaved back with the enzyme, which does not cut the successful clones but will eliminate the background of self-ligating plasmids.
16. SLBP can bind the stem-loop structure located both in the middle of the RNA (i.e., as it occurs during pre-mRNA processing) and at the 3' terminus of the RNA molecule (in the mature histone mRNAs). Since binding to the terminal stem loop seems to occur with a higher affinity, we choose to place the bait downstream from the MS2-binding sites. However, SLBP was also cloned using the stem-loop structure upstream of the MS2-binding site (31). In other cases, the polarity of the MS2-binding sites and the sequence of interest may be a critical factor in screening because it could lead to less or more desirable folding of the hybrid RNA.
17. In addition to the RNA polymerase III termination site derived from the RNase P gene, there is an upstream site provided with the stem-loop insert. The upstream site should be predominantly used to terminate the transcription in vivo.
18. Of approx 2 million transformants plated, 19 grew on the selective medium with 5 mM 3-AT, indicating a high-level expression of the *HIS3* reporter gene.
19. If the activation of both reporter genes is truly dependent on a specific interaction between the RNA stem loop and SLBP, mutations in the stem loop that prevent the interaction should eliminate both the ability to grow on the medium lacking histidine and the ability to convert X-gal to a blue compound. Mutations that result in complete inhibition of binding but do not introduce gross changes to the sequence or structure of the bait RNA are ideal for the secondary screen. When sequence requirements for a specific protein-RNA interaction are unknown, an unrelated sequence can be used instead. However, in such cases failure to activate the reporter genes may result from instability of RNA rather than from requirement for specific RNA-protein interaction. Therefore, it is strongly recommended to define sequence requirements for the RNA-protein interaction prior to screening and to verify potential positives using mutant sequences unable to bind the protein of interest in vitro. Note that this assay will likely only be successful when the protein binds the RNA by itself and not as a complex with other polypeptides.
20. It is recommended that the phenotype of transformants be assayed using at least three different colonies. Individual colonies can differ in the level of expression of each reporter gene and doing the assay in triplicate allows elimination of any possible variations.
21. Our screen identified five individual clones that when sequenced were shown to contain different portions of the same cDNA. The BLAST search revealed that the cDNA encoded a novel protein lacking any previously identified RNA-binding motifs.
22. The frog homolog of human SLBP, xSLBP1, was cloned by screening a *Xenopus* oocyte two-hybrid cDNA library constructed in pGAD10 vector (cat. no.

ZL4000AB; Clontech). All other components of the three-hybrid system were the same as those used for screening the human cDNA library. Approximately 200  $\mu$ g of the *Xenopus* cDNA library was used to transform the L40-coat host strain. From more than 4 million transformants screened, 60 grew on the selective medium lacking histidine and containing 5 mM 3-AT, indicating expression of the *HIS3* reporter gene. Thirty-five also expressed  $\beta$ -galactosidase. Of these 35 clones, 30 passed a secondary screen, i.e., did not activate *LacZ* in the presence of the reverse mutant RNA. Sequencing analysis revealed that 23 contained different portions of the cDNA encoding *Xenopus* homolog of the human SLBP (xSLBP1). The remaining seven clones contained a cDNA encoding a novel SLBP, xSLBP2.

23. Although the main goal of this screen is to identify a component of the U7 snRNP, the RNA-supplemented two-hybrid system can potentially yield other proteins interacting with the SLBP-RNA complex that are involved in other steps in histone mRNA metabolism.
24. The full-length SLBP fused to the DBD of GAL4 and transformed into CG-1945 yeast strain activates both the *HIS3* and the *LacZ* reporter genes without the need for the library plasmid encoding the AD. We have established that SLBP lacking 27 amino acids from the C-terminus is the shortest protein that does not self-activate the reporter genes and, at the same time, is fully active in 3'-end processing in vitro.
25. The *SpeI* fragment contains the entire transcription unit that can correctly function in either orientation.
26. The fusion RNA consisting of the two closely spaced MS2 sites and the histone stem-loop structure at the 3' end was efficiently expressed in the three-hybrid screens. Although the presence of the MS2 binding sites is not required in the RNA-supplemented two-hybrid system, it may be important in stabilizing the entire RNA.
27. None of the selected colonies grew on the minimal medium without histidine nor turned blue in the presence of X-gal, indicating that coexpression of the truncated version of SLBP and the stem-loop RNA does not lead to self-activation of the two reporter genes.
28. From approx 4 million transformants screened, 209 grew rapidly on medium lacking histidine and 64 of these were resistant to 10 mM 3-AT, indicating high expression of the *HIS3* reporter gene. Of the AT-resistant colonies, 41 turned blue in the liquid  $\beta$ -galactosidase assay, indicating expression of the second reporter gene, *LacZ*.
29. This step is necessary because some plasmids initially assigned to one group based on the pattern of frequent restriction sites may in fact contain different inserts. Sequencing and additional restriction analysis identified 11 different cDNA inserts encoding 9 previously characterized proteins and two unknown proteins.
30. Proteins encoded by each of 11 different plasmids identified in the screen with the stem-loop RNA showed either significantly reduced or virtually no ability to activate any of the reporter genes when SLBP was expressed in the absence of the stem-

loop RNA (**Fig. 4B**). While the first screening using the RNA-supplemented two-hybrid system yielded proteins interacting efficiently only with the SLBP-RNA complex, this system should also allow cloning proteins that interact strongly with SLBP regardless of its binding status (with or without RNA). However, given that all SLBP in yeast cells is associated with the stem-loop RNA, the RNA-supplemented two-hybrid system should not allow cloning proteins that interact efficiently with SLBP itself, but not with the SLBP-RNA complex.

31. Among the nine known proteins selected, five have no apparent relevance to any cellular function of SLBP and are most likely false positives, while the remaining four proteins can potentially play a role in aspects of histone mRNA metabolism other than pre-mRNA processing. The two unknown proteins (one selected twice in the screen) are the primary candidates for the U7 snRNP component (presumably a novel protein) that interacts with SLBP during processing of histone pre-mRNAs. These proteins are being further analyzed by using various deletion mutants of SLBP bait to map the region of interaction. Proteins that make contacts with the N-terminal domain of SLBP, dispensable for processing, will be tested along with four known proteins for involvement in other functions mediated by SLBP, including cell-cycle regulation of histone mRNA levels, nuclear export of histone mRNAs, and their localization to polyribosomes.

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## Membrane Recruitment Systems for Analysis of Protein–Protein Interactions

Ami Aronheim

### 1. Introduction

Protein-protein interaction plays a major role in all biologic processes from bacteria, viruses, plants, and animal cells. Proteins are composed of modular structures that enable them to form large active units that function in the correct place and time. Understanding the multiple interaction surfaces and partners of a protein of interest is the main goal in molecular biology research today in almost all research arenas and disciplines. To this end, multiple and diverse methods have been developed and are being employed including biochemical, biophysical, and genetic systems (*1*). Among the latter, the two-hybrid system has been used successfully for the last decade (*2–4*).

Although very powerful, the two-hybrid system, which is based on a transcriptional readout, exhibits several limitations and inherent problems (*5,6*). The two-hybrid system cannot be used with proteins with intrinsic transcriptional activity. In addition, protein interaction should occur in the yeast nucleus. This results in problems of toxicity for several proteins when expressed in yeast, such as homeobox genes and cell-cycle regulators. Alternatively, proteins of nonnuclear origin may be inappropriately folded while expressed in the nucleus. Moreover, for almost a decade, the use of the two-hybrid system with different baits has generated data regarding the repetitive isolation of prey proteins following a library-screening approach. Yet, while these numerous proteins may pass bait-specificity tests, they are considered false positives and result in wasted effort and confusion. In recent years we have developed protein recruitment systems that complement the limitations and problems of the two-hybrid system.

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The protein recruitment systems are based on the original finding that the Ras guanyl nucleotide exchange factor, hSos, is activated on translocation to the plasma membrane (7). This membrane translocation naturally occurs on growth factor stimulation via the recruitment of an adapter protein, Grb2, to the autophosphorylated receptor (8). Artificially, hSos can be constitutively localized to the plasma membrane by its fusion to classic membrane localization signals such as myristoylation or farnesylation sequences. Once expressed at the plasma membrane, hSos accelerates the exchange of guanosine 5'-diphosphate by guanosine 5'-triphosphate on Ras and thereby results in its activation. This mechanism was shown to function in both mammalian and yeast cells (7). In mammalian cells, Ras activation results in induction of the mitogen-activated protein kinase (MAPK) cascade leading to increased transcription of Ras-responsive genes. Therefore, Ras activity can be monitored at multiple stages along the MAPK cascade or simply by using different reporter genes placed under the control of Ras-responsive promoter elements. In yeast cells, Ras activation results in cell growth. Therefore, Ras activation provides efficient cell growth of an otherwise temperature-sensitive yeast mutant strain (*cdc25-2*) defective in its hSos homolog, Cdc25, at the nonpermissive temperature. Naturally, hSos membrane translocation is achieved via interaction with the Grb2 protein on binding to the autophosphorylated receptor. Alternatively, hSos membrane localization can occur through protein-protein interaction. In principle, a protein of interest is fused to hSos to either its amino or carboxy termini, whereas a protein partner is placed at the plasma membrane by fusion to membrane localization sequences. Protein-protein interaction is expected to result in hSos membrane translocation and thereby Ras activation. This system, designated Sos recruitment system (SRS), is efficient in detecting protein-protein interactions between known proteins as well as in screening cDNA expression libraries fused to v-Src membrane localization signal (9,10).

Recently, an additional protein recruitment system was developed that overcomes several problems of the SRS (11). This system, designated Ras recruitment system (RRS), is based on a similar approach; however, it employs the absolute requirement for the Ras protein to be localized to the inner leaflet of the plasma membrane for its function (12). Because Ras is involved in numerous human cancers, its membrane localization is an attractive avenue in the search for an anticancer drug (13). The RRS system exhibits several advantages over the SRS, such as effector size, lower rate of self-activation, and reduced number of predicted false positives. It also provides all the advantages of the SRS over the two-hybrid approach. The RRS system, similarly to the SRS, can be used to test known and novel protein-protein interactions (14). In addition, the RRS was shown to be functional not only in yeast but also in

mammalian cells (**15**). This makes it possible to quantitate the strength of protein-protein interactions using Ras-responsive reporter gene assays.

This chapter describes in detail the technical aspects of the study of protein-protein interactions using the SRS and RRS systems.

## 2. Materials

### 2.1. Yeast Medium

For **items 1** and **2**, the following amino acids should be added to a final concentration of 50 ng/mL excluding the amino acids that are encoded by the transfected plasmid: leucine (L-8125), uracyl (U-0750), tryptophan (T-0271), methionine (M-2893), lysine (L-5626), adenine (A-3159), histidine (H-9511). All amino acids are from Sigma (St. Louis, MO).

1. YNB galactose medium (500 mL): 0.85 g of yeast nitrogen base without amino acids (0335-15-9; Difco, Detroit, MI), 2.5 g of ammonium sulfate, 15 g of galactose (G-0750; Sigma), 10 g of D-raffinose (R-0250; Sigma), 10 mL of glycerol, and 20 g of Bacto agar (0140-07-4; Difco).
2. YNB glucose medium (500 mL): 0.85 g of yeast nitrogen base without amino acids, 2.5 g of ammonium sulfate, 10 g of glucose (Sigma G8270), and 20 g of Bacto agar.
3. Yeast extract, peptone, dextrose (YPD) medium: 1% yeast extract (0127-17-9; Difco), 2% Bacto peptone (0118-17-0; Difco), 2% glucose, and 4% Bacto agar.

### 2.2. Yeast Solutions

1. LISORB: 100 mM lithium acetate (LiAc), 1 M sorbitol in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
2. LIPEG: 40% polyethylene glycol (PEG) 3350, 100 mM LiAc in TE.
3. Salmon sperm DNA: Salmon sperm DNA (D-1626; Sigma) is dissolved in distilled water at a concentration of 10 mg/mL followed by sonication for 10 min. Prior to transfection, the salmon sperm is boiled for 10 min and cooled on ice for 5 min (*see Note 1*).
4. STET: 8% sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% Triton X-100.

### 2.3. Mammalian Cell Transformation Solutions

1. 2X HEPES buffered saline (HBS): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>. Adjust pH to 7.1 with NaOH.
2. Chloramphenicol acetyltransferase (CAT) reaction mixture/reaction: 40 µL of 1 M Tris-HCl, pH 8.0, 3 µL of 5 mg/mL *n*-butyryl coenzyme A, 1 µL of C<sup>14</sup> chloramphenicol, and 6 µL of distilled water.

### 2.4. Materials

1. Biodegradable Counting Scintillant (BCS) (NBCS104; Amersham).
2. *n*-Butyryl coenzyme A (B-1508; Sigma).

3. C<sup>14</sup> Chloramphenicol (NEC-408; NEN).
4. Dimethylsulfoxide (DMSO) (D-2650; Sigma).
5. Glass beads 425–600  $\mu$  (G-8772; Sigma).
6. Luciferase assay reagent (E1500; Promega, Madison, WI).
7. Tetramethyl-pentadecane (T-7640; Sigma).

### 3. Methods

#### 3.1. General Yeast Transformation Protocol

1. Place a single *cdc25-2* yeast colony into 200 mL of YPD medium. Grow cells overnight at 24°C to  $2\text{--}10 \times 10^6$  cells/mL.
2. Pellet the cells for 5 min at 1000g and resuspend the pellet in 20 mL of LISORB. Following two washes with LISORB, resuspend the cells with LISORB to  $2\text{--}5 \times 10^8$  cells/mL and rotate the cells for 30 min at 24°C.
3. For each transfection, add 10  $\mu$ L of preboiled sheared salmon sperm DNA (10 mg/mL) and 2 to 3  $\mu$ g of plasmid DNA in a sterile 1.7-mL microfuge tube.
4. Mix DNA by vortexing and add 200  $\mu$ L of the prewashed cells.
5. Vortex the cell-DNA mixture briefly and add 1.2 mL of LIPEG. Mix well and incubate for 30 min at room temperature with constant rotation. Add 100  $\mu$ L of DMSO and mix well (*see Note 2*).
6. Heat-shock for 10 min at 42°C.
7. Centrifuge the transfection mixture in a microfuge for 1 min and discard the supernatant.
8. Respin for 30 s to completely remove the remaining PEG with a 200- $\mu$ L tip. Resuspend the pellet in 150  $\mu$ L of 1 M sorbitol, and plate the cells on appropriate medium.
9. Incubate the plates at 24°C in a humidified incubator for 4 d.

Transformants incubated at 24°C appear 3 to 4 d following transfection. Single colonies are plated using a grid plate on YNB glucose (–*leu* –*ura*) grown at 24°C for an additional 2 d before further replica plating into YNB galactose, YNB glucose, and YPD plates incubated at 36°C.

#### 3.2. Controls for Transformation

The following two controls should be included in every transfection.

##### 3.2.1. Plating Cells on YPD at 36°C.

Plate approx  $1 \times 10^6$  cells (100  $\mu$ L of the culture ready for transformation) onto a YPD plate and incubate at 37°C. This control tests the *cdc25-2* culture and provides an estimation for the rate of revertants (*see Note 3*).

##### 3.2.2. Control for Contamination and Revertants

A control transfection tube should include both pYes2 and pADNS expression plasmids. Following the transformation procedure, the control transfor-

mantants are plated on a YNB glucose (–leu –ura) plate and incubated directly at 36°C. This control gives an estimate regarding revertants and possible contamination accumulated during the transfection procedure. No colony is expected to grow on this plate.

### 3.3. Library Screening (see Fig. 1)

Library screening with the SRS/RRS systems requires the use of special libraries. The cDNA library is routinely inserted fused to the v-Src myristoylation signal through *EcoRI-XhoI* in the pYes2-(URA)-derived expression vector. To reduce the isolation of the mammalian Ras false positives, the plasmid encoding for the mGAP is coexpressed with the bait and the cDNA library expression plasmids (16). The mGAP is expressed under the control of the GAL1 promoter using the pYes2 (TRP)-based expression vector. Efficient elimination of the mammalian Ras false positives requires the expression of mGAP by a multicopy expression plasmid, since a single copy plasmid encoding for mGAP was able to eliminate only part of the mRas false positives when expressed in *cdc25-2* cells (unpublished results).

1. To obtain high transformation efficiency, first introduced the bait and mGAP expression plasmids into the *cdc25-2* yeast strain.
2. Isolate transformants and use them to inoculate a 3-mL liquid culture for overnight growth at 24°C.
3. Transfer the culture to a 200-mL liquid culture for an additional overnight growth at 24°C.
4. Pellet the culture pelleted at 1000g for 10 min and then transfer to 200 mL of YPD medium for a recovery period of 3–5 h.
5. Use these cells to transform 20 tubes with 3 µg of library plasmid, resulting in 5000–10,000 transformants on each 10-cm plate.
6. Following 4 to 5 d at 24°C, replica plate the plates to galactose (–leu –ura –trp) medium and incubate for 3 to 4 d at 36°C.
7. Select colonies that exhibit growth and place on a glucose plate marked with a grid pattern and containing the appropriate amino acids and bases. Incubate these plates at 24°C for 2 d.

These clones are tested for their ability to grow at 36°C depending on the presence of galactose in the medium. The growth is compared to the growth obtained on a YPD-glucose plate. Those clones that show preferential growth when grown on galactose medium are considered candidates. To test the specificity of the library plasmid, plasmid DNA is extracted from candidate clones and is used to cotransform *cdc25-2* cells with either the specific bait or a nonrelevant bait. Candidate clones that exhibit bait-specific growth are further analyzed.

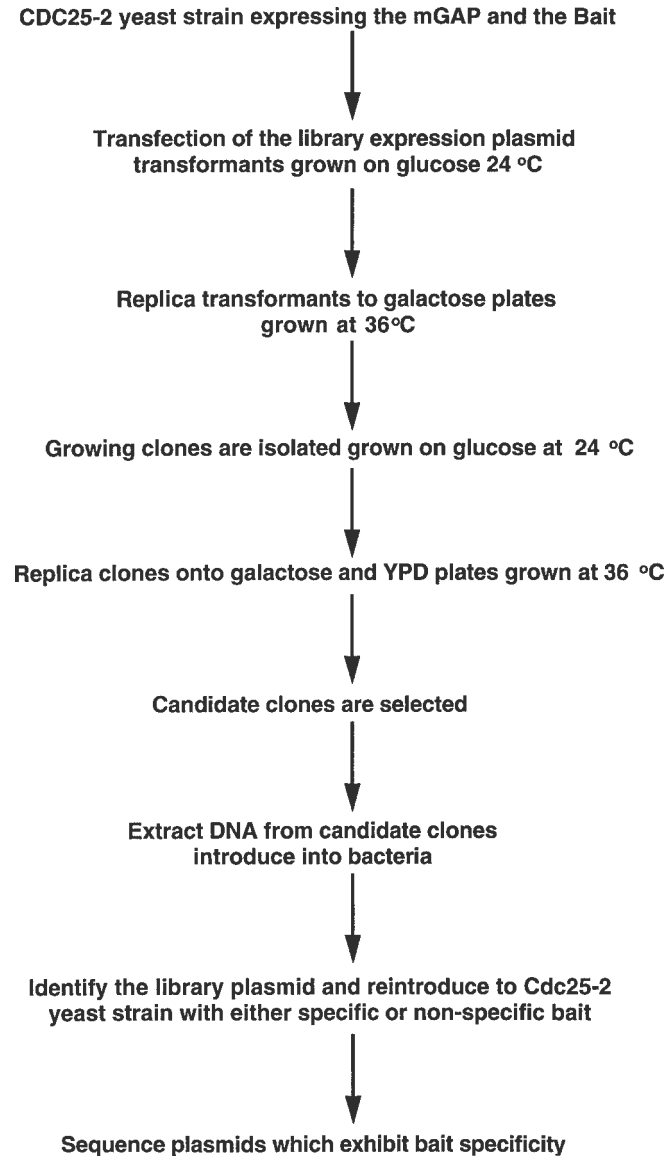


Fig. 1. SRS/RRS Library-Screening Flowchart.

### **3.4. DNA Plasmid Isolation from Yeast**

In principle, yeast candidate clones contain three different DNA plasmids (bait, prey, and GAP expression plasmids). The following protocol is designed to rescue the library plasmid from the yeast.

1. Grow galactose-dependent clones overnight at 24°C in 3 mL of –ura glucose liquid medium.
2. Pellet the cells at 1000g for 5 min and wash once with 1 mL of distilled sterile water.
3. Resuspend the pellet in 100 µL of STET. Add 0.2 g of 0.45-mm glass beads and vortex vigorously for 5 min.
4. Following the addition of another 100 µL of STET, vortex briefly and boil for 3 min (punch a hole at the lid). Cool on ice for 5 min.
5. Spin in a microfuge for 10 min at 4°C. Transfer 100 µL of the supernatant to 50 µL of 7.5 M ammonium acetate, incubate at –20°C for 1 h, and centrifuge for 10 min at 4°C.
6. Transfer 100 µL of the supernatant to 200 µL of ice-cold ethanol. Mix well and recover the DNA by centrifuging for 10 min at 4°C.
7. Wash the pellet with 150 µL of 70% ethanol and resuspend in 24 µL of distilled water.
8. To increase the yield of the library plasmid, digest the DNA mixture with *NotI* (an 8-cutter rare restriction enzyme), which linearizes the bait and GAP plasmids. The library derived expression plasmid does not have a *NotI* recognition site (see **Note 4**).
9. Following a 1-h digestion, extract the DNA by phenol/chlorophorm and recover by ethanol precipitation using 2 µL of tRNA (10 mg/mL) as carrier. Dissolve the final DNA in 10 µL of distilled water.
10. Use 1 µ of the isolated plasmid DNA to transform highly competent bacteria. Plate bacteria on LB+Amp (100 µg/mL) and select single colonies for the preparation of plasmid DNA by standard miniprep procedures (see **Note 5**). Analyze these further by digestion with *EcoRI-XhoI* restriction enzymes for identification of the cDNA inserts.
11. Use individual library plasmids to retransform *cdc25-2* yeast cells with either the specific bait or a nonspecific bait.

### 3.5. 293-HEK Cell Transfection

Following the identification and verification of a DNA plasmid that provides efficient yeast growth at the restrictive temperature only in the presence of the specific bait, it is possible to test the interaction directly in mammalian cells.

1. Plate 293 human embryonic kidney cells (300,000) onto 60-mm plates 1 d before transfection.
2. Use 12 µg of PEG-prepared DNA plasmid for each transfection. The DNA mixture contains 3 µg of each of the following plasmids: polyoma enhancer-CAT reporter gene, 4XAP-1-luciferase reporter gene, pcDNA (Invitrogen)-derived bait expression plasmid, and prey expression plasmid (see **Note 6**).
3. Adjust the volume of the DNA mixture to 450 µL with sterile distilled water and add 50 µL of 2.5 M CaCl<sub>2</sub>.

4. Slowly add the DNA- $\text{CaCl}_2$  mixture into a sterile tube containing 500  $\mu\text{L}$  of 2X HBS by air bubbling, and incubate for 15 min at room temperature.
5. Add 500  $\mu\text{L}$  of the transfection mixture to the cells. Following 5 h, replace the medium with fresh medium.
6. Harvest the cells 40 h following the addition of the DNA to the cells. Collect the cells by resuspending in 1 mL of phosphate-buffered saline followed by centrifugation.
7. Resuspend the cell pellet in 100  $\mu\text{L}$  of 100 mM potassium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol. Prepare a cell extract by three freeze/thaw cycles ( $37^\circ\text{C}$ ) followed by centrifuging at 10,000g for 5 min at  $4^\circ\text{C}$ . Transfer the supernatant to new tubes and use for further analysis.

### 3.6. Luciferase Reporter Assay

A luciferase assay is performed with 10–25  $\mu\text{L}$  of cell extract using the luciferase assay system (Promega) according to the manufacturer's instructions. The assays are measured with a TD-20/20 luminometer (Turner Designs).

### 3.7. CAT Reporter Assay

1. Dilute 10–25  $\mu\text{L}$  of cell extract to a 50- $\mu\text{L}$  volume with distilled water.
2. Add 50  $\mu\text{L}$  of CAT reaction mixture and incubate at  $37^\circ\text{C}$  for 1 h.
3. Stop the enzymatic reaction by adding 200  $\mu\text{L}$  of TMPD/xylene (2 : 1), vortexing for 1 min, followed by centrifuging for 2 min at maximum speed in a microfuge.
4. Transfer 100  $\mu\text{L}$  of the acetylated upper phase into scintillation tubes containing 1 mL of BCS. Determine the percentage of the acetylated chloramphenicol using a conventional  $\beta$ -counter.

## 4. Notes

1. Whenever high efficiency of transformation is necessary, such as for library screening, YEASTMAKER Carrier DNA from Clontech (cat. no. 1606-A) is used.
2. The addition of DMSO at this stage dramatically increases the transformation efficiency and yields increased colonies.
3. Typically 10–20 colonies exhibit growth at  $36^\circ\text{C}$ .
4. Of course, the possibility exists that the insert of the library plasmid may contain a *NotI* cleavage site. In these rare instances, such inserts would not be efficiently isolated in this procedure.
5. Commercial plasmid libraries that provide chloramphenicol resistance (Stratagene) are currently available. Thus, the isolation of the library plasmid is highly facilitated.
6. In control transfections in which either the bait or prey expression plasmids are omitted, pcDNA empty expression vector is used to adjust the total DNA content to 12  $\mu\text{g}$ .



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# Two-Hybrid Systems

## *Methods and Protocols*

Edited by

**Paul N. MacDonald***Department of Pharmacology, School of Medicine  
Case Western Reserve University, Cleveland, OH*

The yeast two-hybrid system is one of the most widely used and productive techniques available for investigating the macromolecular interactions that affect virtually all biological processes. In *Two-Hybrid Systems: Methods and Protocols*, Paul N. MacDonald has assembled a collection of these powerful molecular tools for examining and characterizing protein–protein, protein–DNA, and protein–RNA interactions. The techniques range from the most basic (introducing plasmids into yeasts, interaction assays, and recovering the plasmids from yeast) to the most advanced alternative strategies (involving one-hybrid, split two-hybrid, three-hybrid, membrane recruitment systems, and mammalian systems). Methods are also provided for dealing with the well-known problems of artifacts and false positives and for identifying the interacting partners in important biological systems, including the Smad and nuclear receptor pathways. To ensure ready reproducibility and robust results, each technique is described in step-by-step detail by researchers who employ it regularly.

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