BIOLOGY OF REPRODUCTION 58, 302–311 (1998)

Yeast Two-Hybrid: So Many Interactions, (in) So Little Time . . .

K.H. Young[1]

Molecular Genetic Screen Design, American Cyanamid Co., Princeton, New Jersey 08543

ABSTRACT

Protein-protein interactions are essential to cellular mechanisms at all levels in biologically responsive systems. These interactions occur extracellularly and include ligand-receptor interactions, cell adhesion, antigen recognition, and virus-host recognition. Intracellular protein-protein interactions occur in the formation of multi-protein complexes, during the assembly of cytoskeletal elements, and between receptor-effector, as well as effector-effector, molecules of signal transduction pathways. Finally, assembly of transcriptional machinery involves protein interactions. The yeast two-hybrid method is a powerful technique for analyzing these protein-protein interactions. Since the publication of this technique in the late 1980s, the robust nature and far-reaching utility of yeast two-hybrid systems for functional expression library cloning has led to the identification of many novel proteins in all areas of biological life science research. Additionally, two-hybrid techniques provide a rapid and versatile system for the further characterization of discrete protein-protein interactions. Recent variations on the basic system have enabled application well beyond protein pairs, to investigate multi-protein complexes and protein-nucleotide interactions. Yeast two-hybrid methods necessitate expression and subsequent interaction between a "protein of interest" functional pair within the yeast cell, ultimately driving reporter gene expression and thus effectively linking protein-protein interaction(s) to a change in yeast cell phenotype. Functional protein-protein interactions using the two-hybrid techniques have been demonstrated for all levels of cellular biology; however, until recently, extracellular protein-protein interactions were excluded from investigations using this technique. Investigations from several labs have now demonstrated that extracellular proteins can be studied using two-hybrid methods, thereby enabling intense study of extracellular protein partners using the robust nature and the genetic power of yeast.

INTRODUCTION

Many important cellular events are triggered by protein-protein interactions that occur at the cell surface; these include ligand-receptor interaction, cell adhesion, antigen recognition, immune response, and viral infection. These interactions can be more narrowly defined as the interaction between specific amino acid regions or interaction pockets of the two proteins. Protein interactions can be precisely and efficiently studied using the yeast two-hybrid system. Two-hybrid studies involve the expression of chimeric proteins, designed to maintain the integrity of the proteins, that must interact and generate a functional reaction within the yeast cell nucleus to drive the expression of a reporter gene. This review will highlight interactions of extracellular proteins that had previously been excluded from investigations using two-hybrid methods. This exclusion was due to the belief that complex protein folding motifs, often involving intricate cysteine bond formation, would not adequately pair to attain functional shape when expressed intracellularly in yeast cells. Many proteins, however, have been expressed and properly folded, using yeast systems [1, 2]. In addition, the interaction of several classes of peptide ligands and receptors [3–5] as well as interactions of antigen-antibody complexes [6] have been reported using standard two-hybrid methods and variations thereof. A variety of extracellular interactions have been established using two-hybrid methods, thereby demonstrating that they are not precluded from investigation using this amenable and powerful yeast method.

BASIS OF TWO-HYBRID METHOD

The modular basis of eukaryotic transcription factors provided the biological flexibility contributing to the development of the yeast two-hybrid technique. The base components of yeast two-hybrid hinge on transcription activator proteins. The yeast Gal4 transcription activator protein is an endogenously expressed protein of 881 amino acids (aa) [7] and contains DNA binding (aa 1–147) [8] and activation (aa 771–881) domains [9]. In a notable finding, Fields and Song [10] determined that the two separate and independent Gal4 domains, which lack function alone, could be synthesized as chimeric proteins with each Gal4 domain fused to one protein of interacting partner proteins (X and Y). The productive interaction between the partner proteins (X and Y) brings the separate activation domain (AD) into close proximity to the DNA binding domain (BD), thereby reconstituting the function of the Gal4 transcription activator protein and driving expression of a downstream reporter gene. This concept employed two yeast proteins, Snf1 and Snf4, in the successful demonstration of the utility of this system [10]. Functional expression library screening subsequently demonstrated the utility of this system for genetic selection using a small domain of SIR4 protein expressed as a DNA BD fusion with a library of AD fusion proteins [11].

Independent experiments by Ptashne and coworkers [12] also demonstrated the modular aspect of transcription factors. The *Escherichia coli* protein LexA was fused directly to the yeast Gal4 protein and expressed in yeast cells containing LexA operator sequences [9]. The *E. coli*/yeast fusion protein garnered the DNA BD from LexA and the AD from Gal4. In the two-hybrid LexA system [13]; the "proteins of interest" are generated as a LexA-BD fusion and a (herpes simplex virus) VP16-AD fusion. The flexibility afforded by the modular nature of eukaryotic transcription factors enables other ADs to be substituted for VP16 [14, 15]. The LexA system has been designed with a galactose-inducible promoter that has utility in studies of heterologous fusion proteins that are toxic or lethal in yeast. Yeast cells carrying recombinant plasmids are grown and maintained on glucose medium, which represses the expression of problematic fusion proteins. Plating the yeast strain on galactose induces the expression of the fusion proteins for experimental purposes. The LexA system used in this two-hybrid format is often referred to as an "interaction trap" [15, 16]. Additionally, the LexA two-hybrid system was used in an exhaustive study of the sensitivity of the two-

[1]Correspondence: K.H. Young, American Cyanamid Co., P.O. Box 400, Princeton, NJ 08543. FAX: (609) 275-3519.

hybrid system with regard to the affinity of the protein-protein interaction [17]. The system can sense protein-protein interactions with a dissociation constant (K_d) of 10^6, and this has been substantiated in the Gal4 two-hybrid system [18]. Proteins as small as 8–10 aa [19] and as large as 750 aa have been investigated in a two-hybrid format [20]. Hydrophobic domains may affect expression but may be less problematic when expressed as a small percentage of the overall protein and when contained within the protein itself.

Reporter Genes

A myriad of protein-protein interactions are amenable to investigation by two-hybrid methods [16, 20–23]. Fusion proteins are generated from recombinant plasmids and expressed inside the yeast cell. The proteins of interest are carried to the yeast cell nucleus, usually by inclusion of a nuclear localization signal, to drive expression of a reporter gene. The reporter gene contains an upstream activator sequence (UAS) complementary to the employed DNA BD fusion. The downstream reporter gene relays information regarding the protein-protein interaction to produce a change in yeast cell phenotype. Prototrophic markers (*HIS3* or *LEU2*) are commonly used and provide a positive selection scheme on media deficient for the specific amino acid encoded by the reporter gene, wherein productive protein-protein interactions result in cell growth. Other positive selection markers, like *lacZ*, enable colorimetric readout after an enzymatic assay. Reporter genes resulting in a sensitivity phenotype, such as *CYH2* [24, 25], G418 [26], or *CAN1* [27], enable counter selection, wherein the two-hybrid interaction abrogates cell growth. Many two-hybrid systems commonly use dual reporter systems to substantiate the identification of a protein-protein interaction and/or putative novel interaction partner isolated via a genetic screen. Regardless of the specific reporter system, the final effect links protein-protein interactions to an observable change in yeast cell phenotype.

VARIATIONS ON THE TWO-HYBRID METHOD

This two-hybrid system provides a powerful basic research tool. Early publications focused on identification of novel partner proteins for a known protein via functional expression library screening. More recent publications describe the many adaptations of the basic two-hybrid method, expanding these techniques to address an ever-growing list of scientific questions.

The Gal4 system, described by Fields and coworkers [10, 11], and the LexA system, described by Brent and coworkers [13], serve as the foundation for the two methods used most prevalently in the identification, confirmation, and characterization of protein-protein interactions. In a genetic screen, the respective DNA BD fusion protein serves as the "bait" and is coexpressed with a library of AD fusion proteins ("prey"). These systems link productive protein-protein interaction to a change in yeast cell phenotype. Most vectors used in two-hybrid systems generate heterologous proteins as 3′ fusions to the Gal4 domains. The wide variety of proteins that function in the system suggests that this orientation may not be detrimental. Some proteins, however, may contain critical residues at or near the N-terminus, and expression as a 3′ fusion to the Gal4 region may restrict protein function. Reversed polarity vectors have been established for the LexA BD fusion [28] and the LexA system-AD fusion [29]. The sensitivity of the interaction can be increased for proteins having critical amino acids that may be too close to the DNA BD portion when in a 3′ fusion orientation [28].

Identification of new protein partners via genetic screens have used cDNA libraries generated as fusions to the AD. There is increasing evidence that small protein regions, 8–10 aa [19], or 16–20 aa [30, 31], can be investigated/identified using two-hybrid methods. These small peptides are generated through construction of directed peptide fusions, or as a commercially available random peptide library. Colas et al. [31] generated a two-hybrid peptide library in which the peptide aptamers were embedded between paired cysteine residues within the sequence for the *E. coli* protein thioredoxin A (TrxA). This "tethers" the peptide ends and presents the peptide on the platform of TrxA [32]. Using this system, peptides were identified that interact with Cdk2 to inhibit kinase activity [31].

The two-hybrid framework has been extended beyond protein-protein interactions to protein-nucleotide interactions. One such variation focuses on the identification of proteins that recognize a specific DNA target sequence through the use of a "one-hybrid" system [33]. A unique reporter construct is engineered that contains the particular DNA consensus sequence within the promoter region upstream to the yeast reporter gene. A specific fusion or cDNA library of random protein segments is generated as AD fusions. Candidate proteins contain a protein sequence that recognizes the unique UAS in the promoter, and reporter gene activity is driven by an endogenous or engineered activation domain within the single fusion protein. By using the one-hybrid system, ORC6 was identified as a unique protein that recognizes yeast replication origins [33]. Investigation of RNA-protein interactions was demonstrated by SenGupta et al. [34] through a "three hybrid system." A hybrid RNA molecule is used as a linker for two RNA binding proteins, or domains, each generated as one half of the two-hybrid system. A LexA-MS2 coat protein fusion was linked to an IRP1-AD protein using IRE-MS2 hybrid RNA. The HIV-1 Tat protein was shown to interact with its RNA target TAR. The RNA-based system depends on physical properties rather than the in vivo biological activities of the molecular reagents. This emphasis on the physical properties of the three interacting molecules suggests that there will be broad utility of this hybrid variation [34].

Progressive modifications have since generated a "one-two-hybrid" approach for library screening to identify new transcription factors [35]. The yeast host strains developed for this system allow screening for protein-protein interaction (using a Gal4 UAS reporter gene) as well as DNA-protein (using the p53 consensus-reporter gene) interactions. Simultaneous use of these screening criteria is expected to increase the overall screen stringency and is likely to reduce the number of false positives [35].

The concept of a linker molecule was taken to the next level by using a synthetic chemical, rather than a biological molecule, as the molecular linker. Investigation of the immunosuppressive drug FK506 and its biological target immunophilin FKBP12 were exploited to establish this system. FK506 was then chemically modified, enabling its dimerization to produce FK1012 [36], which was then used to link two FKBP12 chimeric fusion proteins (FKBP12-BD and FKBP12-AD) and drive expression of the *lacZ* and *HIS3* reporter genes in yeast [37]. The FK1012 chemical molecule used in this study provides an example of a "chemical inducer of dimerization" or CID [38].

Investigation of multi-protein (ternary) complexes provided the means for an additional adaptation of the yeast two-hybrid system. Studies on peptide ligand/receptor interactions for members of the hematopoietic receptor family necessitated the coexpression of a third protein ([3]; detailed later). Coexpression of nonfusion ligand reversed the interaction of the ligand and receptor fusion proteins. Additionally, the utility of a third simultaneously expressed nonfusion protein was demonstrated in studies showing ligand-dependent dimerization for growth hormone/growth hormone receptor (GH/GHR) interactions, as well as vascular endothelial growth factor (VEGF) interaction with its tyrosine kinase receptor [3]. Investigation of extracellular protein interactions and the use of a third protein was named PRIMES, an acronym for Peptide Receptor Interaction in a Microbial Expression System. In addition to the use of a three-protein system for investigation of ligand/receptor interactions, a two-hybrid system using three proteins has been used to address other scientific questions.

A system similar to the PRIMES system was employed by Zhang and Lauter [39] to investigate intracellular associations of proteins during initiation of the epidermal growth factor receptor (EGFR) signal transduction cascade. Within the yeast cells, the EGFR cytoplasmic domain was expressed as a BD fusion protein and Sos as an AD fusion protein, and Grb2 was expressed with an SV40 T-antigen nuclear localization signal (NLS). Yeast cells expressing all three proteins showed *HIS3* and *lacZ* reporter gene activation, whereas yeast cells expressing only the EGFR and Sos fusion proteins in the absence of Grb2 were unable to drive reporter gene activity. To identify new interacting proteins, a mouse brain library was coexpressed from a third (SV40 NLS) plasmid in conjunction with the EGFR-BD and the Sos-AD fusion proteins, and subsequently identified the mouse Grb2 protein [39].

An alternative use of a third protein coexpressed with the two-hybrid fusion proteins (AD-X and BD-Y) is the third protein's ability to activate or modify one of the fused proteins and facilitate the protein-protein interaction. Tyrosine phosphorylation-dependent protein-protein interactions have been studied by the coexpression of a facilitator protein tyrosine kinase (PTK) in yeast. *S. cerevisiae* have endogenous serine/threonine kinases, but lack tyrosine kinase [40]. Despite this deficiency, molecular supplementation via coexpression of a PTK enabled studies of tyrosine phosphorylated proteins using two-hybrid methods. Osbourne et al. [41] demonstrated the utility of their "tribred" system using the cytoplasmic domain of the FcεRIγ subunit of the IgE receptor (BD) fusion and the SH2 domains of Syk (AD) fusion proteins. The PTKs Lck or Lyn were then independently coexpressed with the fusion proteins. Productive phosphorylation of the FcεRIγ region of the fusion protein resulted in interaction with Syk SH2 domains. A genetic screen employing the FcεRIγ cytoplasmic domain BD fusion, coexpressed Lck (PTK), and a mast cell AD library led to the identification of a novel SH2-containing family member [41].

Counter-Selection Systems

Because of the robust nature of *S. cerevisiae* and the powerful knowledge base on yeast genetics, many variations on the two-hybrid theme have emerged to address new scientific questions and provide desirable modifications in assay readout (yeast phenotype). Prototrophic markers such as *HIS3* or *LEU2* enable positive selection via yeast cell growth after establishing productive protein-protein interaction(s). However, this is not useful for all applications because the identification of very low-affinity, mutant, or disrupter proteins or molecules would require a failure in driving expression of downstream positive selection reporter genes. To circumvent this potential false negative readout of scientifically interesting proteins or molecules, modifications of the reporter gene have been made to provide counter selection two-hybrid systems and enable identification of proteins or synthetic molecules that function as interaction disrupters. One approach to counter selection is the use of reporter genes that confer sensitivity to agents added to the yeast media. The *CYH2* gene product provides sensitivity to cycloheximide in a phenotypic reversal from that observed with the *HIS3* reporter gene [24, 25]. The *CYH2* gene [42] encodes the L29 ribosomal protein and confers cycloheximide sensitivity and is dominant over the *cyh2* allele, which produces cycloheximide resistance. Using this reporter gene, a productive protein-protein interaction drives expression of the *CYH2* gene, and the yeast strain becomes sensitive to cycloheximide, resulting in abrogation of cell growth when cells are plated on selective media containing cycloheximide. Disruption of the protein-protein interaction results in a failure of *CYH2* gene transcription, and cells retain the cycloheximide-resistant phenotype; the strain is effectively "rescued" and able to grow on media containing cycloheximide. Leanna and Hannick [25] used the *CYH2* reporter as a counter selection to investigate the interaction between cREL and p40 proteins. Further mutagenesis studies of p40 led to the identification of proteins able to ablate the cREL/p40 protein-protein interaction [25].

The *URA3* gene product has been used for both positive and negative selection [43]. The *URA3* gene product encodes an enzyme involved in uracil biosynthesis that confers uracil prototrophy, enabling yeast cell growth on media deficient in uracil. The same enzyme, orotidine-5′-phosphate decarboxylase [44], also catalyzes the production of a toxic compound from the artificial substrate 5-flourooroic acid (5-FOA). Cells grown on media containing 5-FOA must lose the *URA3* gene or die. Combining the uracil prototrophy phenotype and the FOA-sensitive phenotype enables both positive and negative selection criteria, respectively. Additionally, Vidal et al. [43] placed the *URA3* gene under tight regulation by the cell stage-specific promoter, SPO1. In these studies, the *URA3* reporter system was used in a two-step manner, first using the FOA^s phenotype to identify mutations affecting the interaction; and second, using the uracil prototrophy phenotype to identify a subset of mutants that maintained full-length protein expression. Vidal et al. [45] went on to identify E2F transcription factor mutations that impair heterodimerization with DP1.

An alternative approach to counter selection uses the *E. coli tet*-repressor (TetR) and operator as a coupled two-step sequential reporter gene system, which the authors term the "split-hybrid" system [46] The first reporter gene of this system contains the LexA UAS upstream of the *TetR* gene, while the second reporter gene contains the *TetR* operator binding site upstream of the *HIS3* gene. The system's utility was demonstrated by testing interactions of cAMP response element binding protein (CREB) and its coactivator protein CREB binding protein (CBP). The interaction of CREB and CBP depends on phosphorylation of a critical serine residue in CREB. Coexpression of wild-type CREB and CBP fusion proteins results in the activation of the *TetR* gene and production of TetR protein. TetR protein binds to the *tet*

operator of the sequential reporter gene, and effectively prevents *HIS3* gene activity. No growth was observed on histidine-deficient media. Prevention of the CREB/CBP interaction via mutation of the critical serine, or other disruptive mutations, resulted in histidine prototrophy, since the *TetR* gene product is not produced and hence the *HIS3* gene is not repressed. Control of the intrinsic activity of the BD fusion protein as well as differences in growth rates can be delineated by the addition of tetracycline, which relieves the repression in a dose-dependent manner, to the selection media. A mutagenesis screen strategy was then adopted to identify CREB proteins defective for interaction with CBP. Mutations in the protein kinase A phosphorylation motif of CREB constituted the major class of disruption mutations [46]. Counter selection can also be achieved by pairing transcriptional activators with specific negative regulators [47]. Some examples include the yeast GAL80 protein, which binds and inactivates the transcriptional activator region of GAL4 [48], or the oncoprotein *mdm2*, which binds to the trans-activation domain of the p53 tumor suppressor [49, 50].

APPLICATIONS TO REPRODUCTIVE BIOLOGY

The methods and variations of two-hybrid systems used in one scientific discipline influence research in other fields. For example, the reproductive biologist's interest in vascular growth factors [51, 52] may focus on ovarian, uterine, or placental biology, or fetal development, while the oncologist's interest focuses on tumor biology, and the vascular biologist focuses on restenosis, diabetic retinopathy, or other vascular abnormalities. Signal transduction cascades and gene regulatory machinery also provide a common focal point. Applications of two-hybrid methods toward specific or discrete research fields have been reviewed ([53, 54]; general review [55, 56]). The following examples illustrate the increasing application of two-hybrid methods to reproductive biology.

The nuclear receptor family constitutes a conglomerate of ligand-dependent transcription factors [57, 58]. The mechanism of action of these receptors involves interactions among small steroid ligands, receptors, and DNA, and also between receptors and with transcription factors, and coactivators/repressors [58–60] proteins. Estrogen receptors (ER) are no strangers to investigation in *S. cerevisiae* [61–63]. More recently, however, two-hybrid methods have been used to further delineate the interacting players within the steroid receptor superfamily. Ligand effects on the estrogen receptor dimerization that is independent of the mammalian estrogen response element were investigated via the two-hybrid system using chimeric ER fusion proteins [64]. Using estrogen, tamoxifin, and ICI 182,780, Wang and coworkers [64] demonstrated ligand dependence of ER dimerization. The ligand binding domain of the ER was investigated using a modified one-hybrid system [65] that employed the commonly used UAS_{gal}-reporter system and the Gal4 BD fused to various ER constructs. Transcriptional activation of this one-hybrid system was dependent on the application of estrogen and provided a rapid genetic screen for the investigation of various ER mutants. Additionally, the transactivation ability of the ER mutants as identified in this yeast screen correlated with transcriptional activity in a mammalian system [65].

The control of transcriptional initiation at the chromatin level also involves a highly conserved multi-protein complex. This complex, initially characterized in yeast (SWI/SNF) [66], has homologues in *Drosophila*, humans [67] and mice [68]. The human proteins have been shown to interact with other members of the steroid receptor family and may interact via the AF2s. Ichinose et al. [69] demonstrated an interaction between the N-terminal regions of the hSNF2 subtypes and the ER, as well as estrogen enhancement of this interaction.

Functional interactions between androgen receptor cognate ligand binding domains and transactivation domains were investigated using the two-hybrid system [70]. This provided an in vivo assessment of the interaction between these domains of the androgen receptor. The protein domain interactions observed were androgen-dependent and were blocked by anti-androgens. Low-affinity ligands had no effect on the interaction of the androgen receptor domains. Whether the interaction between androgen receptor subdomains is intra- or inter-molecular requires further investigation [70].

Identification of novel proteins using a two-hybrid library screen resulted in the identification of the steroid hormone coactivator, SRC-1 [71]. The cDNA encoding the hinge and ligand binding regions of the human progesterone receptor was used as "bait" in a genetic screen with a B-lymphocyte expression library to identify a progesterone-specific coactivator. In further biochemical and mammalian transcription experiments, this coactivator (SRC-1) affected the activity of other steroid receptor family members and other transcription factors; however, the authors suggest that it is not a general coactivator for all transactivator classes [71]. Many members of this coactivator/repressor family have been identified [58] using two-hybrid genetic screens [59, 60, 72]. Additionally, two-hybrid techniques were used to dissect the signature motif [19] for transcriptional coactivators of nuclear proteins.

Sex determination in mammals is precipitated by expression of the Y-linked testis-determining gene, SRY. The 204-aa SRY gene product contains a central region of 78 aa which has a DNA BD with homology to the high-mobility group box family. Transcriptional activator activity has been identified for the C-terminal region of mouse SRY protein [73]. Coexpression of a human SRY-Gal BD fusion with a HeLa cell library led to the identification of SIP1. This interaction was further evaluated using two-hybrid methods, which demonstrated the importance of the C-terminal region of SRY for this association. The Müllerian inhibitor substance (MIS) receptor and cytoplasmic interaction components have also been studied [74] using the two-hybrid system. The localized expression of the type I transforming growth factor β (TGFβ) receptor mRNA suggested that it may be the receptor for MIS. Two-hybrid analysis using the receptor cytoplasmic domain of TGFβ demonstrated interactions with the FKBP12 pathway [74].

Receptor signal transduction systems are paramount to reproductive biology systems. Signal transduction cascades that integrate hormonal signals for insulin-like growth factors (IGFs), interferons, interleukins (IL), cytokines, GH, placental lactogens, prolactin (PRL), and others are extensively investigated. These pathways and others have been investigated using two-hybrid methods to characterize specific protein-protein interactions, or to identify new protein components within signal cascades such as Jak/STAT [75, 76], map kinase [77], insulin and IGF receptors, cellular components and insulin receptor substrate 1 [78, 79], leucine zipper proteins [80], helix-loop-helix proteins [81], and g-proteins [82].

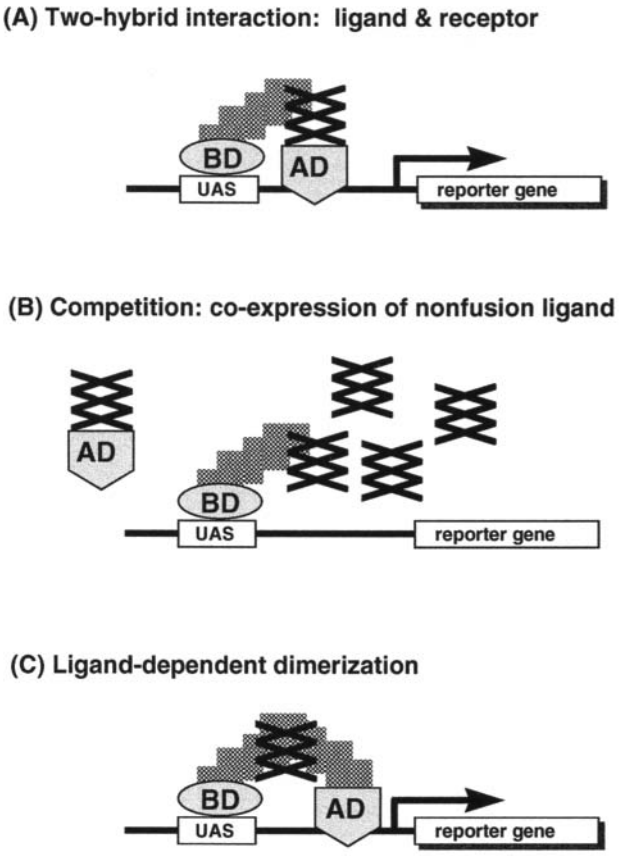

FIG. 1. Schematic of protein interactions used in yeast two-hybrid systems, and variations for ligand-receptor interactions. **A**) Interaction between two proteins. The extracellular domain of the receptor is expressed fused to the yeast Gal4 DNA BD. The full-length peptide hormone is expressed fused to the yeast Gal4 activation domain (AD). The reporter gene contains a UAS recognized by the Gal4 BD, and targets the receptor-BD fusion protein. Interaction between the receptor and ligand regions of the fusion proteins brings the BD and AD regions into close proximity, reconstitutes the function of the Gal4 domains, and drives expression of a downstream reporter gene. **B**) The ligand is coexpressed as a nonfusion protein and effectively competes with the ligand-AD fusion protein for interaction with the receptor-BD fusion protein. The nonfusion ligand lacks the AD domain, and its interaction with the receptor-BD fusion does not drive expression from the reporter gene. **C**) The extracellular domain of the receptor is expressed as both the BD fusion and AD fusion proteins. Coexpression of a nonfusion ligand results in ligand-dependent dimerization of the two receptor fusion proteins and drives expression of the reporter gene. The expressed proteins are generated from independent plasmids via the ADH promoter (BD, AD) or CUP1 promoter (nonfusion). Adapted from B.A. Ozenberger and K.H. Young, Functional interaction of ligands and receptors of the hematopoietic superfamily in yeast. Mol Endocrinol 1995; 9:1321–1329. ©The Endocrine Society.

EXTRACELLULAR PROTEIN-PROTEIN INTERACTIONS

The two-hybrid system has been used extensively for investigation of protein-protein interactions of many cytoplasmic and nuclear proteins, as described. Extracellular protein-protein interactions, however, were generally thought to be inaccessible to study using this yeast system. In an effort to apply two-hybrid methods to protein-protein interactions between extracellular proteins, Ozenberger and Young [3] focused on the interaction between GH and its receptor. The interaction of GH with its receptor has been extensively characterized [83, 84]. The GHR extracellular domain contains several Ig-like loops and ultimately functions as a receptor dimer whereby GH, via two different contact sites (high- and low-affinity), interacts with identical sites on two GHRs [85]. To establish a two-hybrid interaction, the extracellular domain of the rat GHR was expressed as a Gal4 DNA BD fusion, while the cDNA encoding the GH ligand was expressed as a Gal4 DNA AD fusion (Fig. 1A). Yeast strains expressing both fusion proteins, or a single fusion protein and a companion vector containing no heterologous DNA, were tested for functional protein-protein interaction through activation of an *HIS3* reporter gene. While all strains grew vigorously on nonselective media, only the strain that expressed both the GHR and GH fusion proteins grew well on selective histidine-deficient media (Fig. 2, top panels). In a similar experimental design, PRL and the PRL receptor (PRLR) extracellular domain were generated as Gal4 DNA BD and AD fusion proteins, respectively, and analogous yeast strains expressing both the PRL and PRLR fusion proteins, or a single fusion protein with a companion empty vector, were generated and tested for *HIS3* reporter gene activity and growth on selective media. Only the strain that expressed the PRLR and PRL fusion proteins grew well on selective media (Table 1).

Competition

Having established the interactions between a peptide ligand and a single transmembrane receptor extracellular domain using the two-hybrid paradigm, experiments then focused on the reversibility and specificity of receptor binding, but inside the yeast cell. The parent yeast strain was modified to accommodate a third plasmid for coexpression of a nonfusion protein as a potential competitor of the two fusion proteins (Fig. 1B). To test the specificity of the ligand-receptor interaction, the identical ligand to that used in the ligand fusion protein was coexpressed with the ligand and receptor fusion proteins. Simultaneous expression of nonfusion GH abrogated yeast cell growth in comparison to a similar strain in which nonfusion GH was absent (Fig. 2, middle panels, and Table 1). Similarly, coexpression of nonfusion PRL abrogated yeast cell growth when compared to a yeast strain that expressed the PRL fusion protein, the PRLR fusion protein, and the third plasmid containing no heterologous cDNA (Table 1). PRL is related to GH; however, in nonprimate species, PRL does not display reasonable affinity for the GHR. To test for selectivity in these peptide ligand/receptor interactions, the heterologous ligand was expressed as a nonfusion protein with the ligand/receptor fusion proteins (GH/GHR/PRL or PRL/PRLR/GH). Functional interaction of the ligand/receptor fusion proteins was not affected by the coexpression of heterologous peptide ligand (Table 1).

Through adaptation of the yeast strain to accommodate three plasmids, many multiprotein interactions can be established in yeast. Ligand-dependent receptor dimerization is a common mechanism for generating an appropriate signal transduction pathway(s) and subsequent biological response(s). Homo- and heterodimerization systems have been well-described for many receptor systems, including tyrosine kinase receptors, growth factor receptors, insulin, and IGF receptors. Members of the hematopoietic receptor family display both homodimerization (GH) [83–85] and heterodimerization (IL-6, Oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor) with the transducer molecule gp130 [86]. Coexpression of three proteins in yeast now enables investigation of ligand-dependent dimerization. Receptor extracellular domains have been expressed as both the BD fusion and the AD fusion proteins,

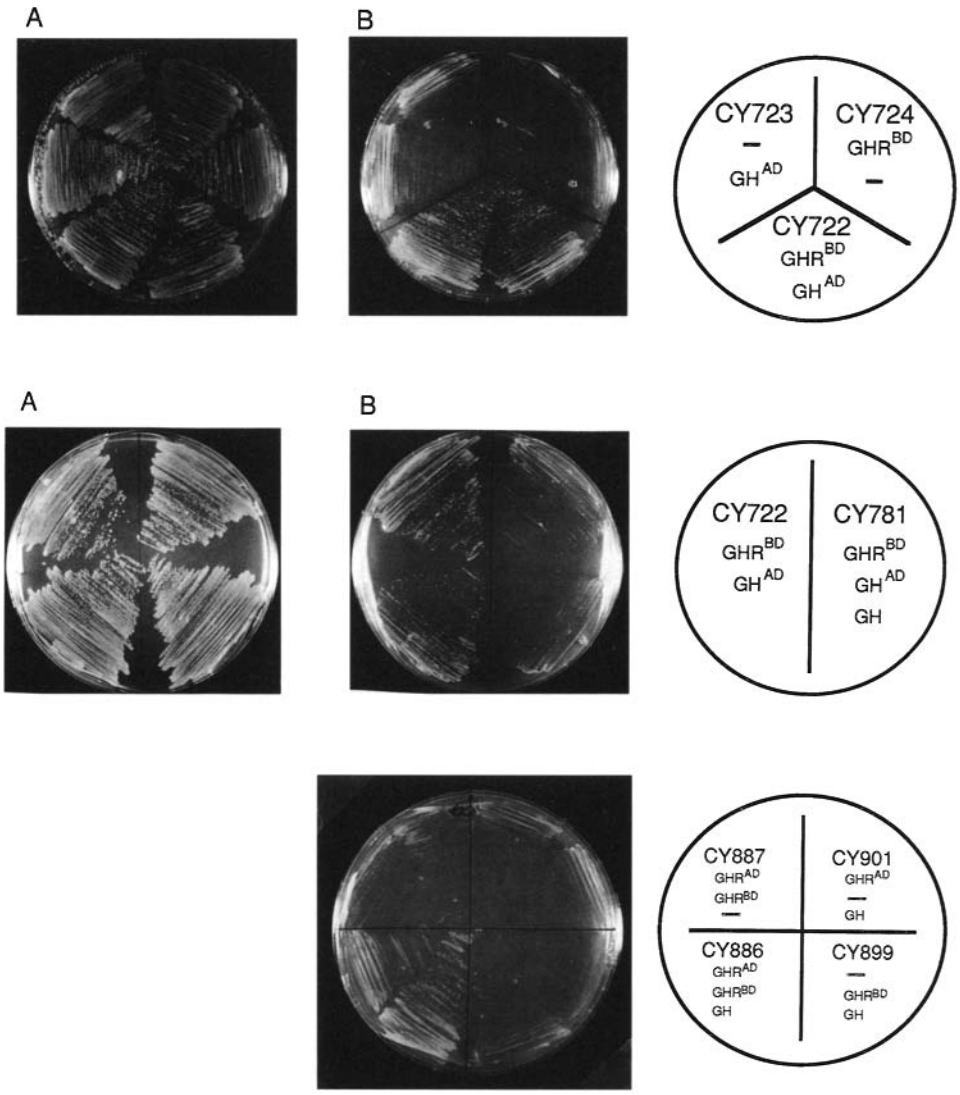

FIG. 2. Investigation of GH ligand and GHR interaction in a yeast two-hybrid system. **Top panels**) Investigation of GH and GHR interaction. Yeast strains CY723 and CY724 express a single fusion protein and serve as controls, CY722 expresses both the GH and GHR fusion proteins. Two isolates of each strain were tested on nonselective media (A) and selective media (B) deficient in histidine. **Middle panels**) Investigation of reversible ligand-receptor interaction. Yeast strain CY722 is as described above. CY781 coexpresses nonfusion GH in conjunction with the GH and GHR fusion proteins. The nonfusion GH competes with the GHR-AD and effectively abrogates cell growth. Two isolates of each strain are plated on nonselective media (A) and selective media (B) deficient in histidine. **Bottom panels**) Ligand-dependent dimerization. Yeast strains CY899 and CY901 express a single GHR fusion protein with the nonfusion GH, and serve as control strains. CY887 expresses the two GHR fusions in the absence of nonfusion GH, while CY886 expresses two GHR fusions in the presence of nonfusion GH. Two isolates of each strain are plated on selective media deficient in histidine. For all diagrams, a dash denotes the presence of a companion vector containing no heterologous DNA; Superscript AD denotes a Gal4 activation domain fusion, while superscript BD denotes a Gal4 DNA BD fusion. Adapted from B.A. Ozenberger and K.H. Young, Functional interaction of ligands and receptors of the hematopoietic superfamily in yeast. Mol Endocrinol 1995; 9:1321–1329. ©The Endocrine Society.

TABLE 1. Summary of growth and competition assay for peptide ligand and receptor interactions using modifications on the yeast two-hybrid method.[a]

Strain[b]	AD	BD	Nonfusion	Growth
Y190	—	—	—	0
CY722	GH	GHR	—	+++
CY723	Vector	GHR	—	0
CY724	GH	Vector	—	0
CY726	PRL	PRLR	—	++
CY770	—	—	—	0
CY781	GH	GHR	GH	+
CY784	GH	GHR	Vector	+++
CY785	GH	GHR	PRL	+++
CY786	PRL	PRLR	PRL	+
CY787	PRL	PRLR	Vector	++
CY788	PRL	PRLR	GH	++

[a] Adapted from BA Ozenberger and KH Young, Functional interaction of ligands and receptors of the hematopoietic superfamily in yeast. Mol Endocrinol 1995; 9:1321–1329. ©The Endocrine Society.

[b] Each yeast strain expresses those proteins indicated as Gal4 activation domain (AD) or DNA binding domain (BD) fusions or as nonfusion proteins; a dash indicates that the strain does not contain the denoted plasmid; growth on histidine deficient media is scored in relation to the parent strain (Y190 or CY770) (see text and [3] for details).

while the ligand is expressed as a "free" nonfusion protein (Fig. 1C) and has been established for GH dimerization of GHRs. Yeast strains expressing various combinations of two GHR fusion proteins and free GH were compared for growth on selective media. Yeast cells containing only one receptor with nonfusion GH failed to grow on selective media, while yeast cells containing the two receptor fusions in the absence of GH demonstrated growth that was faintly detectable. Robust growth was observed, however, for strains that contained both receptor fusions in the presence of GH (Fig. 2, bottom panels) [3].

Ligand-mediated receptor dimerization was also established for VEGF and the extracellular domain of its receptor [3]. VEGF is a cysteine-knot protein [87]; its activity depends on proper folding via both intra- and inter-molecular disulfide bonds to form a functional ligand dimer that is necessary for proper receptor binding. Homodimerization of VEGF enables identical sites on the ligand to engage the homodimer receptor, using a 2:2 stoichiometry. Cysteine mutagenesis studies determined that interfering with the disulfide pattern is detrimental to ligand-receptor interaction [88], probably because of disruption of ligand-ligand dimerization, which thereby cripples receptor binding. Extracellular ligand-ligand interactions using yeast two-hybrid studies were conducted with VEGF expressed as both the

BD and AD fusions [4]. Growth of yeast cells on selective media was observed only for cells expressing both VEGF fusion proteins, in comparison to cells expressing a single VEGF fusion protein and an unrelated fusion protein. On the basis of structural similarities to platelet-derived growth factor, specific cysteine residues in VEGF were mutated and tested in the yeast two-hybrid system for their ability to interact with a wild-type fusion protein. Cysteine residues that are essential for in vivo activity in mammalian expression systems [88] were also essential for activity in a two-hybrid interactions [4]. In addition, mutated VEGF expressed as nonfusion proteins in conjunction with receptor fusion proteins failed to engage the receptor fusions to drive reporter gene expression.

Other protein-protein interactions that normally occur at the extracellular surface have been studied using the two-hybrid system. Members of the G-protein-coupled receptor family that contain long N-terminal regions important for ligand association and include GH-releasing hormone (GHRH), vasoactive intestinal protein, and IL-8 are probably amenable to study using these methods. Kajkowski et al. [5] reported expression of the N-terminal region of the porcine GHRH receptor with the GHRH ligand in a two-hybrid system and demonstrated functional interaction of fusion proteins. Mutation of a critical cysteine ablated the response [5], in agreement with data from other studies using a mammalian system [89]. Single-chain variable regions with antigen-binding properties have been identified using the two-hybrid method [6]. Initial experiments focused on identification of variable domains targeted to CREB. The authors speculate that the system can be used to isolate combinatorial antigen-binding regions.

The proper folding of peptide ligand or receptor domains that are generated by using recombinant technologies is paramount to maintaining the integrity of protein-protein interactions and promoting functional interactions between peptide ligands and cognate receptors. Recent observations [3–6] suggest that the yeast cell interior is suitable for the formation of tertiary protein structures. The yeast cell interior is maintained at a neutral pH, despite the acidic environment of the media preferred for optimal yeast cell growth. Maintaining this pH gradient via proton movement across the yeast cell membrane could result in an enhanced ability for disulfide bond formation within the yeast cell's internal milieu. Other cytosolic buffering factors, such as glutathione and its prevalence in a reduced state [90], may play a role in an oxidative environment of the yeast cell internal milieu. In further studies [91], functional interactions between proteins dependent on complex cysteine bond formation were compared to interactions between proteins for which disulfide bond formation was inconsequential. The ability to drive reporter gene expression and growth on selective media was observed with a stepwise increase in the pH of the media. Only those yeast strains expressing the disulfide bond-dependent protein pairs were adversely affected by the increase in pH [91].

OTHER APPLICATIONS OF TWO-HYBRID SYSTEMS

Extension of yeast two-hybrid systems from a basic tool of exploratory research to a tool for directed basic research and drug discovery takes advantage of several key aspects of yeast. *S. cerevisiae* provides a strong genetic tool within a robust organism that can easily be manipulated by molecular techniques. The complete sequence of the yeast genome is available [92–94], and many genes from other organisms have homologues in yeast. Yeast can serve as a molecular tool that may not have been previously considered for investigation of mammalian systems. Irrespective of direct homologues in yeast, a remarkably diverse collection of non-yeast proteins has been studied in this organism. In drug discovery applications, yeast provides an organism easily manipulated for the development of high-throughput screens for rapid identification of pharmacophores [95].

Drug Discovery

Protein-protein interactions are often the target of therapeutic intervention [96–100] because of the specificity of individual molecular contacts and the pervasiveness of these protein-protein interactions. The ligand-receptor interaction that triggers a signaling pathway is often the earliest specific event leading to a cellular response, thus providing a desirable target for therapeutic intervention. Intervention near the apex of a signal transduction pathway may avoid cross-talk between signal transduction components, since signals are often amplified when transduced from the cell membrane to the nucleus. Although often portrayed as linear sequences, cross-talk occurs between molecules in signal cascade [101, 102]. Targeting specific protein-protein interactions and using available crystallography data contribute to the "rational drug design" approach for peptidomimetics. Additionally, large compound banks—comprising random chemical structures, structures generated by combinatorial chemistry, and natural products—are evaluated for affects on biological targets. These compound banks provide the foundation for the "random drug discovery" approach, which necessitates screening large numbers of compounds. Identification of initial structures then provides a scaffold for chemical possibilities generated through molecular modeling and analogue synthesis. Several screening platforms are used to identify structures of initial active compounds. Yeast provides advantages as a front-line screening organism and is amenable to high-throughput screening of various compound sources.

High-Throughput Screening

Protein dysfunction, via over-activity, malfunction, or dysregulation, can be problematic for interacting proteins and can be manifested in disease. The biological components involved can be engineered into yeast and developed as a primary screen. The discovery of new therapeutics often hinges on screening large numbers of compounds against a molecular target. The molecular target of choice can be configured in several formats, including, but not limited to, ligand binding assays, cell-based reporter assays, and biochemical enzyme assays. Increasingly, *S. cerevisiae* is used as a screening tool background to provide an in vivo-based screen. The yeast cells are specifically engineered to mimic an intricate molecular aspect targeted for intervention and then used for the identification of novel human or animal therapeutic, or crop-protection, agents. Yeast cell systems are more tolerant of diverse culture conditions and harsh chemicals or natural products. These compound sources often contain novel therapeutics, yet the initial source would not be tolerated in the exacting conditions of mammalian tissue culture. Yeast provides a robust organism and a relatively economical system for high-throughput screen design. The genome information is available, reagents are inexpensive, and assays are easily configured to automation. Protein-protein interactions as therapeutic targets using two-hybrid methodologies avail spe-

cific design for drug discovery research. Counter or negative selection reporter genes enable a "rescue" format as preferred in (yeast) drug screen design. For example, a productive protein-protein interaction drives a reporter gene whose product results in cell arrest; disruption of this interaction by an applied compound would prevent reporter gene activity, leading to rescue growth of the yeast cell [24, 95, 100].

Protein Linkage Maps

The strong genomics initiative resulted in the completion of genomic sequencing of several organisms, with others to be completed over the next few years. The human genome sequence is projected for completion early in the next decade. These initiatives will result in DNA sequence information encoding vast numbers of new proteins. Characterization of the biological role of these proteins lies ahead in functional genomics [103], and defining the interaction partners of new proteins can assist in protein classification by using a proposed biological role. Analysis of large protein networks has been modeled using two-hybrid scheme in the analysis of the *E. coli* bacteriophage T7 genome. The T7 genome of 39 937 base pairs is estimated to encode 55 proteins [104]. In a classical two-hybrid genetic screen, the known protein is supplied as a DNA BD fusion protein, while the cDNAs encoding all possible proteins are supplied as the activation domain fusion protein. Through two-hybrid protein linkage, the DNA binding domain fusion is also a library of all possible proteins; thereby one is able to screen a library-BD against a library-AD [105]. Technical modifications using a mating strategy were applied in screening library versus library, as described by Fromont-Racine and coworkers [106] in a functional analysis of the yeast genome using two-hybrid methods.

CONCLUSION

Since the initial establishment of two-hybrid methods for functional expression cloning of a single novel protein partner, many applications and variations have been reported. Additionally, the functional expression and interaction of peptide ligands and cognate receptors within a yeast two-hybrid paradigm have linked peptide ligand-receptor interactions that normally occur at the cell surface to a change in yeast cell phenotype. This has enabled a rapid analysis of protein domains, interaction domains, and identification partners for orphan ligand and/or receptors. Use of three proteins as a modification of two-hybrid methods now enables investigation of multi-protein complexes, ligand-dependent dimerization, and identification of novel components of multi-protein complexes, well beyond simple pairwise investigation or identification. Using chemical linkers, RNA hybrids, and steroids expands this method to other avenues, such as investigation of steroids or chemical ligands, and the potential identification of novel receptors for chemical ligands. The adaptations and variations on the theme of two-hybrid methods will continue to advance as long as scientists remain creative in applying these methods to new questions and the investigation of new systems. Incorporation of new information and understanding of gene function and protein mechanisms can only continue to build on the productivity afforded by this system.

ACKNOWLEDGMENTS

The author thanks S. Baig-Gyan for excellent technical information support. The author also thanks D. Kirsch for continued support and critical review of this manuscript, and members of Molecular Genetic Screen Design at American Cyanamid for valuable interactions.

REFERENCES

1. Sudbery PE. The expression of recombinant proteins in yeasts. Curr Opin Biotech 1996; 7:517–524.
2. Eckart MR, Bussineau CM. Quality and authenticity of heterologous proteins synthesized in yeast. Curr Opin Biotech 1996; 7:525–530.
3. Ozenberger BA, Young KH. Functional interaction of ligands and receptors of the hematopoietic superfamily in yeast. Mol Endocrinol 1995; 9:1321–1329.
4. Ozenberger BA, Hellings S, Kajkowski E, Young KH. Interactions between ligands and receptors expressed in a two-hybrid system. In: 77th meeting of the Endocrine Society; 1995; Washington DC. Abstract P3–259.
5. Kajkowski E, Price LA, Pausch MH, Young KH, Ozenberger BA. Investigation of growth hormone releasing hormone receptor structure and activity using yeast expression technologies. J Recept Signal Transduction Res 1997; 17:293–303.
6. Hoeffer JP, Bilir N, Marcell T, Schneider JK, Dunkelberg JC, Jackson SM. Isolation of single chain variable domains with high affinity antigen-binding characteristics using the two-hybrid system in yeast. J Cell Biochem 1994; Suppl 0 (1BD):190 (abstract T103).
7. Laughon A, Gesteland R. Isolation and preliminary characterization of the *GAL4* gene, a positive regulator of transcription in yeast. Proc Natl Acad Sci USA 1982; 79:6827–6831.
8. Keegan L, Gill G, Ptashne M. Separation of DNA binding from transcription-activating function of a eukaryotic regulatory protein. Science 1986; 231:699–704.
9. Brent R, Ptashne M. A eukaryotic transcriptional activator bearing the DNA specificity of prokaryotic repressor. Cell 1985; 43:729–736.
10. Fields SJ, Song O. A novel genetic system to detect protein-protein interactions. Nature 1989; 340:245–246.
11. Chien C-T, Bartel PL, Sternglanz R, Fields SJ. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA 1991; 88:9578–9582.
12. Ma J, Ptashne M. Converting an eukaryotic transcriptional inhibitor into an activator. Cell 1988; 55:443–446.
13. Golemis EA, Brent RA. Fused protein domains inhibit DNA binding by LexA. Mol Cell Biol 1992; 12:3006–3014.
14. Ruden DM, Ma J, Li Y, Wood K, Ptashne M. Generating yeast transcriptional activators containing no yeast protein sequences. Nature 1991; 350:250–252.
15. Golemis EA, Khazak V. Alternate yeast two-hybrid systems. Methods Mol Biol 1997; 63:197–218.
16. Mendelson AR, Brent R. Applications of interaction traps/two-hybrid systems to biotechnology research. Curr Opin Biotech 1994; 5:482–486.
17. Estojak J, Brent R, Golemis EA. Correlation of two-hybrid affinity data with *in vitro* measurements. Mol Cell Biol 1995; 15:5820–5829.
18. Durfee T, Becherer K, Chen P-L, Yeh S-H, Yang Y, Kilburn AE, Lee W-H, Elledge SJ. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes & Dev 1993; 7: 555–569.
19. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997; 387:733–736.
20. Golemis EA, Serebriishii I, Law SF. Adjustment of parameters in the yeast two-hybrid system: criteria for detecting physiologically significant protein-protein interaction. In: Schaefer B (ed.), Gene Cloning and Analysis: Current Innovations. Wymondham, UK: Horizon Scientific Press; 1997: 11–28.
21. Luban J, Goff SP. The yeast two-hybrid system for studying protein-protein interactions. Curr Biol 1995; 6:59–64.
22. Fields S, Sternglanz R. The two-hybrid system; an assay for protein-protein interactions. Trends Genet 1994; 10:286–292.
23. Warbrick E. Two's company, three's a crowd: the yeast two-hybrid system for mapping molecular interactions. Structure 1997; 5:13–17.
24. Young KH, Ozenberger BA. Novel cell systems having specific interaction of peptide binding pairs. 1995; International Publication Number WO95/34646 (patent).
25. Leanna CA, Hannick M. The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions. Nucleic Acids Res 1996; 24:3341–3347.
26. Hadfield C. G418 resistance as a dominant marker and reporter for

gene expression in *Saccharomyces cerevisiae*. Curr Genet 1990; 18: 303–313.
27. Ahmad M. Yeast arginine permease: nucleotide sequence of the Can1 gene. Curr Genet 1986; 10:587–592.
28. Beranger F, Aresta S, de Gunzburg J, Camonis J. Getting more from the two hybrid system: N-terminal fusions to LexA are efficient and sensitive baits for two-hybrid studies. Nucleic Acids Res 1997; 25: 2035–2036.
29. Brown MA, MacGillivray RT. Vectors for expressing proteins at the amino terminus of an activation domain for use in the yeast two-hybrid system. Anal Biochem 1997; 247:451–452.
30. Yang M, Wu Z, Fields S. Protein-peptide interactions analyzed with the yeast two-hybrid system. Nucleci Acids Res 1995; 7:1152–1156.
31. Colas P, Cohen B, Jessen T, Grishina I, McCoy J, Brent R. Genetic selection of peptide aptamers that recognize and inhibit cyclin dependent kinase 2. Nature 1996; 380:548–550.
32. LaVallie ER, DiBlasio EA, Kovacic S, Grant KL, Schendel PF, McCoy JM. A thioredoxin gene fusion expression system that circumvents inclusion body formation in *E. coli* cytoplasm. Biotechnology 1993; 11:187–193.
33. Li JJ, Herskowitz I. Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science 1993; 262:1870–1874.
34. SenGupta DJ, Zhang B, Kraemer B, Pochart P, Fields SJ. A three-hybrid system to detect RNA-protein interaction *in vivo*. Proc Natl Acad Sci USA 1996; 93:8490–8501.
35. Luo Y, Vijaychander S, Stile J, Zhu L. Cloning and analysis of DNA-binding proteins by yeast one-hybrid and one-two-hybrid systems. Biotechniques 1996; 20:564–568.
36. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Controlling signal transduction with synthetic ligands. Science 1993; 262:1019–1024.
37. Ho SN, Biggar SR, Spencer DM, Screiber SL, Crabtree GM. Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature 1996; 382:822–826.
38. Crabtree GR, Schreiber SL. Three part inventions: intracellular signaling and induced proximity. Trends Biol Sci 1996; 21:418–422.
39. Zhang J, Lauter S. A yeast three hybrid method to clone ternary protein complex components. Anal Biochem 1996; 242:68–72.
40. Lim M-Y, Dailey D, Martin GS, Thorner H. Yeast *MCK1* protein kinase autophosphorylated at tyrosine and serine but phosphorylates exogenous substrates at serine and threonine. J Biol Chem 1993; 268: 21155–21164.
41. Osbourne MA, Dalton S, Kochan JP. The yeast tribred system—genetic detection of trans-phosphorylated ITAM-SH2-interactions. Biotechniques 1995; 13:1474–1478.
42. Kaufer NF, Fried HM, Schwindinger WF, Jasin M, Warner JR. Cycloheximide resistance in yeast; the gene and its protein. Nucleic Acids Res 1983; 11:3123–3135.
43. Vidal M, Brachman RK, Fattaey A, Harlow E, Boeke JD. Reverse two-hybrid and one hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. Proc Natl Acad Sci USA 1996; 93:10315–10320.
44. Rose MD, Botstein D. Structure and function of the yeast *URA3* gene differentially regulated expression of hybrid beta-galactosidase form overlapping coding sequenced in yeast. J Mol Biol 1983; 170:883–904.
45. Vidal M, Braun P, Chen E, Boeke JD, Harlow E. Genetic characterization of a mammalian protein-protein interaction by using a yeast reverse two-hybrid system. Proc Natl Acad Sci USA 1996; 93: 10321–10326.
46. Shih H-M, Goldman PS, DeMaggio AJ, Hollenberg SM, Goodman RH, Hoekstra MF. A positive genetic selection for disrupting protein-protein interactions: identification of CREB mutations that prevent association with the coactivator CBP. Proc Natl Acad Sci USA 1997; 93:13896–13901.
47. Erickson JR, Powers S. Reverse two-hybrid method. 1995; International Publication Number WO95/26400 (patent).
48. Ma J, Ptashne M. The carboxy-terminal 30 amino acids of Gal4 are recognized by GAL80. Cell 1987; 137–142.
49. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992; 69:1237–1245.
50. Oliner JD, Peitenpol JA, Thagalingam S, Gyuris J, Kinzler KW, Volgelstein B. Oncoprotein mdm2 conceals the activation domain of tumour suppressor p53. Nature 1993; 362:857–860.
51. Klagsburn M, D'Amore PA. Vascular endothelial growth factor and its receptors. Cytokine & Growth Factor Rev 1996; 7:259–270.
52. Hanahan D. Signaling vascular morphogenesis and maintenance. Science 1997; 277:38–40.
53. Nishimune A, Nash SR, Nakanishi S, Henley JM. Detection of protein-protein interactions in the nervous system using the two-hybrid system. Trends Neurosci 1996; 19:261–266.
54. Bemis L, Geske TJ, Strange R. Use of the yeast two-hybrid system for identifying the cascade of protein interaction resulting in apoptotic cell death. Methods Cell Biol 1995; 46:139–151.
55. Phizicky EM, Fields S. Protein-protein interaction: methods for detection and analysis. Microbiol Rev 1995; 59:94–123.
56. McNabb DS, Guarente L. Genetic and biochemical probes for protein-protein interactions. Curr Opin Biotech 1996; 7:554–559.
57. Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. Cell 1995; 83:851–857.
58. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L. Nuclear receptor coactivators and corepressors. Mol Endocrinol 1996; 10:1167–1177.
59. Halachmi S, Marden E, Martin G, Mackay H, Abbodanza C, Brown M. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 1994; 264:1455–1458.
60. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfield MG. The transcriptional co-activator p/CIP binds CBP and mediates nuclear receptor function. Nature 1997; 387:677–684.
61. Metzger D, Lossen R, Bornet J-M, Lemoine Y, Chambon P. Promoter specificity of the two transcriptional activation functions of the human oestrogen receptor in yeast. Nucleic Acids Res 1992; 20:2813–2817.
62. Wrenn CK, Katzenellenbogen BS. Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. J Biol Chem 1993; 268:24089–24098.
63. Butt TR, Walfish PG. Human nuclear receptor heterodimers: opportunities for detecting targets of transcriptional regulation using yeast. Gene Exp 1996; 5:225–268.
64. Wang H, Peters GA, Zeng X, Tang M, Ip W, Khan SA. Yeast two-hybrid system demonstrates that estrogen receptor dimerization is ligand-dependent *in vivo*. J Biol Chem 1995; 270:23322–23329.
65. Bush SM, Folta S, Lannigan DA. Use of the yeast two-hybrid system to screen for mutations in the ligand-binding domain of the estrogen receptor. Steroids 1996; 61:102–109.
66. Winston F, Carlson M. Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. Trend Genet 1992; 8:387–391.
67. Peterson CL, Tamkun W. The SWI-SNF complex: a chromatin remodeling machine? Trends Biochem Sci 1995; 20:143–146.
68. La Douarin B, Nielsen AL, Garnier J-M, Ichinose H, Jeanmougin F, Losson R, Chambon P. A possible involvement by nuclear receptors. EMBO J 1996; 15:6701–6715.
69. Ichinose H, Garnier J-M, Chambon P, Losson R. Ligand-dependent interaction between the estrogen receptor and the human homologues of SWI2/SNF2. Gene 1997; 188:95–100.
70. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J. Functional *in vivo* interactions between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 1997; 36: 1052–1064.
71. Onate SA, Tsai SY, Tsai M-J, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270:1354–1357.
72. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate. Proc Natl Acad Sci USA 1996; 93:5517–5521.
73. Poulet F, Santa Barbara P, Desclozeaux M, Soullier S, Moniot B, Bonneaud N, Boizet B, Berta P. The human testis determining factor SRY binds a nuclear factor containing PDZ protein interaction domains. J Biol Chem 1997; 272:7167–7172.
74. Wang T, Donohoe PK, Zervos A. Specific interaction of type I receptors of the TGF-β family with immunophilin FKBP-12. Science 1994; 265:674–676.
75. Horvath CM, Darnell JE. The state of the STATs: recent developments in the study of signal transduction to the nucleus. Curr Opin Cell Biol 1997; 9:233–239.
76. Fujitani Y, Hibi M, Fukada T, Takahashi-Tezuka M, Yoshida H, Yamaguchi T, Sugiyama K, Nakajima K, Hirano T. An alternate pathway for STAT activation that is mediated by direct interaction between JAK and STAT. Oncogene 1997; 14:751–61.

77. Yang X, Hubbard JA, Carlson M. A protein kinase substrate identified by the two-hybrid system. Science 1992; 257:680–682.
78. O'Neill TJ, Craparo A, Gustafson TA. Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. Mol Cell Biol 1994; 14:6433–6442.
79. Dey BR, Frick K, Lopancznski W, Nissley SP, Furlanetto RW. Evidence for the direct interaction of the insulin-like growth factor I receptor with IRS1, Shc, and Grb10. Mol Endocrinol 1996; 10:631–641.
80. Chevrey PM, Nathans D. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci USA 1992; 89:5789–5793.
81. Staudinger J, Perry M, Elledge SJ, Olson EN. Interactions among vertebrate helix-loop-helix proteins in yeast using the two-hybrid system. J Biol Chem 1993; 268:4608–4611.
82. Yan K, Kalyanaraman V, Gautam N. Differential ability to form the G protein betagamma complex among members of the beta and gamma subunit families. J Biol Chem 1996; 271:7141–7146.
83. Cunningham BC, Ultsch M, DeVos AM, Mulkerrin MG, Clauser KR, Wells JA. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 1991; 254:821–825.
84. DeVos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 1992; 255:306–312.
85. Fuh G, Cunningham BC, Fukunaga R, Nagata S, Goeddel DV, Wells JA. Rational design of potent antagonists to the human growth hormone receptor. Science 1992; 256:1677–1680.
86. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994; 76:253–262.
87. MacDonald NQ, Hendrickson WA. A structural superfamily of growth factors containing a cysteine knot motif. Cell 1993; 73:421–424.
88. Potgens A, Lubsen N, van Altena M, Vermeulen R, Baker A, Schoenmakers J, Ruiter D, de Waal R. Covalent dimerization of vascular permeability factor/vascular endothelial growth factor is essential for its biological activity. J Biol Chem 1994; 269:32879–32885.
89. Lin S-C, Gukovsky I, Lusis AJ, Sawchenko PE, Rosenfield MG, Molecular basis of *little* mouse phenotype and implications for cell type specific growth. Nature 1993; 364:208–213.
90. Gilbert H. Molecular and cellular aspects of thiol-disulfide exchange. Adv Enzymol 1990; 63:69–172.
91. Ozenebrger BA, Young KH. Investigation of ligand/receptor interaction and the formation of tertiary complexes. In: Bartel P, Field S (eds.), The Yeast Two-Hybrid System. New York: Oxford University Press; 1997: 158–172.
92. Goffeau A, Barrell BG, Bussey H, Davis HG, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. Life with 6000 genes. Science 1996; 274:546–567.
93. Dujon B. The yeast genome project: what did we learn? Trends Genet 1996; 12:263–270.
94. Johnston M. Genome sequencing: the complete code for a eukaryotic cell. Curr Biol 1996; 6:500–503.
95. Broach JR, Thorner J. High throughput screening for drug discovery. Nature 1996; 384(suppl 7):14–16.
96. Levitzki A. Signal transduction interception as a novel approach to disease management. Ann NY Acad Sci 1995; 766:363–368.
97. Saltiel AR, Sawyer TK. Targeting signal transduction in the discovery of antiproliferative drugs. Chem Biol 1996; 3:887–893.
98. Feng S, Kapoor TM, Shirai F, Combs AP, Schreiber SL. Molecular basis for the binding of SH3 ligands with non-peptide elements identified by combinatorial synthesis. Chem Biol 1996; 3:661–670.
99. Cwirla SE, Balasubramanian P, Duffin DJ, Wagstrom CR, Gates CM, Singer SC, Davis AM, Tansik RL, Mattheakis LC, Boytos CM, Schatz PJ, Baccanari DP, Wrighton NC, Barrett RW, Dower WJ. Peptide agonist of the thrombopoietin receptor as potent as a natural cytokine. Science 1997; 276:1696–1699.
100. Young KH, Hellings S, Modi M, Lin S, Husbands M, Franco R, Haskins JT. N-type calcium channel subunit association in yeast using two-hybrid methods: identification of inhibitors. In: 27th annual meeting of the Society of Neuroscience; 1997; New Orleans, LA. Abstract 783.12.
101. Cook SJ, McCormick F. Inhibition of cAMP by Ras-dependent activation of Raf. Science 1993; 262:1069–1072.
102. Dunn JJ, Studier FW. Complete nucleotide sequence of bacteriophage T7 DNA and the location of T7 genetic elements. J Mol Biol 1983; 166:477–535.
103. De Meyts P, Urso B, Christoffersen CT, Shymko RM. Mechanism of insulin and IGF-I receptor activation and signal transduction specificity. Ann NY Acad Sci 1995; 766:388–401.
104. Oliver SG. From DNA sequence to biological function. Science 1996; 379:597–600.
105. Bartel PL, Roecklein JA, SenGupta D, Fields S. A protein linkage map of *Escherichia coli* bacteriophage T7. Nat Genet 1996; 12:72–77.
106. Fromont-Racine M, Rain J-C, Legrain P. Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nat Genet 1997; 16:277–282.