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*Review Article*

# The Yeast Two-Hybrid System and Its Pharmaceutical Significance

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The detected phenotypes in many diseases are caused from dysfunction in protein-protein, protein-DNA and receptor-ligand interactions. Therefore, determination of these molecular interactions followed by designing or screening the compounds to target these interactions provides a significant challenge in drug development. This review aims to highlight the yeast two-hybrid system in determination of protein-protein interactions and its possible outcomes in pharmaceutical research. The variations of the basic methodology as one- and three-hybrid systems are also disussed in relation to their potential pharmaceutical applications.

**KEY WORDS:** protein-protein interaction; yeast two-hybrid system; drug screen.

## INTRODUCTION

Contemporary therapeutic inventions often target protein-protein interactions because a vast majority of the physiological processes in living cells are mediated by these interactions. Expression and regulation of genetic information, protein transport, cell adhesion, antigen recognition, viral infection, receptor-ligand binding, and signal transduction are some of the events requiring protein-protein interactions. Several human diseases occur as a consequence of inappropriate interactions due to protein dysfunction, malfunction, or dysregulation. Conventional biochemical techniques, such as coimmunoprecipitation, chromatographic copurification, and covalent crosslinking (1,2) offer limited use in determination of the protein-protein interactions. These *in vitro* techniques require the proteins in a pure form and at high concentration.

The yeast two-hybrid system (Y2H), first developed by Fields and Song in *S. cerevisiae* (baker's yeast), is a powerful technique for identifying novel protein-protein interactions (Fig. 1) (3). The system involves the expression of chimeric proteins and their subsequent interactions within the yeast cell nucleus. The interaction is detected by the expression of a reporter gene that changes the phenotype of the recipient yeast cell. The reviews on the impacts of Y2H on different fields of research (4–6) and its methodology (7,8) are available in the literature. The interactions between known proteins as well as partner proteins for a given protein can be investigated by using this system. Unlike the *in vitro* biochemical methods, Y2H can detect *in vivo* interactions. Moreover, neither protein purification nor antibody production is required for this methodology.

## THE YEAST TWO-HYBRID METHODOLOGY

### Why Yeast?

Yeast serves as an excellent organism to investigate mammalian systems. The ease and low cost of yeast, convenience of retrieving plasmids, availability of nutrition markers and presence of well-characterized reporter genes are the main advantages of yeast (5,9). Moreover, endogenous yeast proteins are unlikely to bind mammalian target proteins to prevent their interactions. The complete sequence of yeast genome is known (10) and detailed experimental protocols on manipulation of this organism are available (11). A number of yeast strains for the system have been reported (12).

### Principle of the Yeast Two-Hybrid System

The idea behind Y2H is based on the fact that most transcription factors are composed of two physically separable domains: a site-specific DNA binding domain (BD) and a transcription activation domain (AD) (3). The BD serves targeting the transcription factor to specific promoter sequences, upstream activating sequences (UAS), whereas the AD facilitates assembly of the transcription complex. These two domains need not be present in the same polypeptide to activate transcription. Thus, the interaction between two proteins can be determined by producing one construct that couples the DNA sequence encoding part or all of one protein (X) to a BD plasmid (BD-X, "bait" fusion protein) (Fig.

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**ABBREVIATIONS:** Y1H, -2H, -3H, the yeast one-, two-, three hybrid systems, respectively; AD, DNA activation domain; BD, DNA binding domain; UAS, upstream activating sequences; NLS, nuclear localization signal; β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 3-AT, 3-aminotriazole3-aminotriazole.



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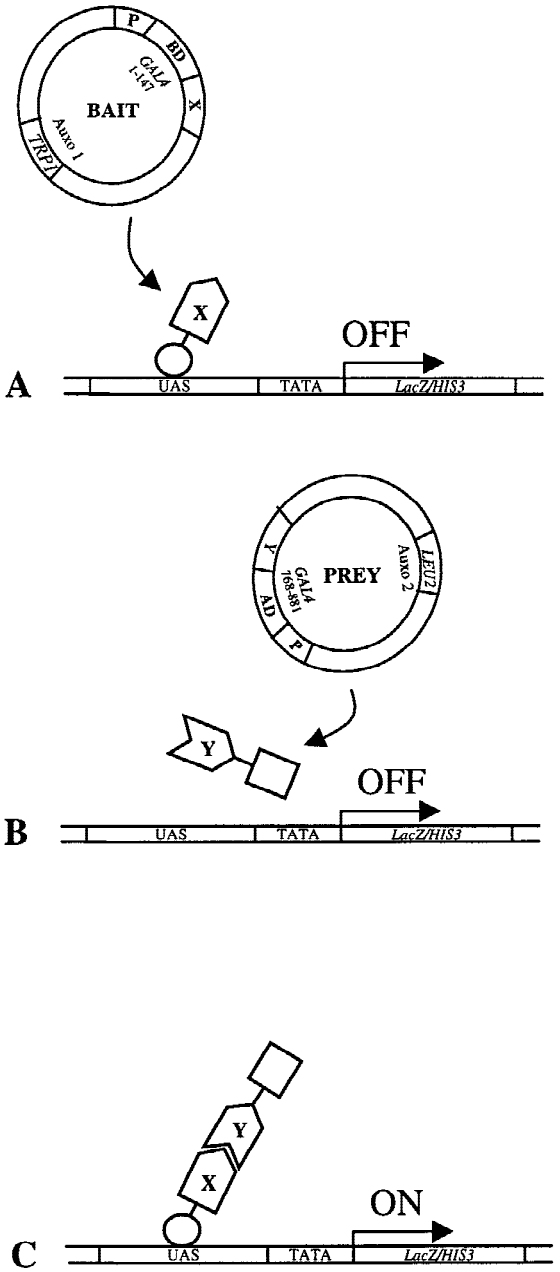


**Fig. 1.** Schematic representation of a yeast two-hybrid system. The interaction between two proteins can be tested by producing one construct that couples the DNA sequence encoding part or all of one protein (X) to a binding domain plasmid ("bait" fusion protein) (panel A), and a second construct that couples the DNA sequence encoding part or all of the other protein (Y) to an activation domain plasmid ("prey" fusion protein) (panel B). Expression of fusion proteins are controlled by the promoter, *P*. Upstream from the starting point of transcription by RNA polymerase is TATA box. The bait plasmid contains the *TRP1* gene while the prey plasmid contains *LEU2* gene. Neither of the individual constructs alone can activate transcription (panels A and B). Upon transformation, only yeast containing both plasmids can grow in the absence of Trp/Leu. Association of the two fusion proteins causes expression of two integrated reporter genes (*LacZ* and *HIS3*) and only the yeast containing a two-hybrid interaction can grow in the absence of Trp/Leu/His media (panel C).

1A), and a second construct that couples the DNA sequence encoding part or all of the other protein (Y) to an AD plasmid (AD-Y, "prey" fusion protein) (Fig. 1B). The two plasmids contain different auxotrophy markers (see Fig. 1. legend). Neither of these domains alone is able to activate the transcription machinery (Fig. 1A and B) while association between BD-X and AD-Y fusion proteins reconstitutes an active trancription factor that initiates the expression of one or more reporter genes (Fig. 1C).

### The Steps of a Yeast Two-Hybrid Experiment

The first step in a Y2H experiment is the construction of the fusion plasmids (7,8). If the bait is meant to be tested with another known protein for a possible interaction, the coding sequence of the candidate protein is inserted into AD plasmid. If the experiment aims to search for partner protein(s), a total genomic or cDNA library is constructed using the AD plasmid. Transformation of an appropriate yeast strain with bait and prey plasmids is the next step in Y2H. Among the transformation protocols, the simplest one is the lithium acetate method (13). Although transformation of bait and prey plasmids can be achieved either sequentially or simultaneously, the former strategy gives relatively higher tranformation efficiency (8). More recently, mating the haploid yeast strain, which contains the bait with the prey-containing transformants considerably increased the efficiency (14).

Some proteins other than transcription factors possess intrinsic trancriptional activation or they can activate transcription when fused to DNA binding domain. Therefore, the BD fusion plasmid in yeast must be tested for auto-activation of reporter genes before starting a Y2H experiment.

Transformed yeast cells can be selected for the activation of reporter genes. The *LacZ* gene encoding β-galactosidase (β-gal) is routinely used as a reporter (15). Expression of *LacZ* provides a screenable color phenotype in the presence of the substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (11). The sensitivity of detection is greatly increased by the use of chemiluminescent 1,2-dioxetane, which can be linked to a β-gal substrate (16). Dual selection by including a second reporter, typically histidine gene (*HIS3*) gives more reliable results as its expression permits a nutritional selection (17). The selection process of the interacting clones can be further refined with the use of 3-aminotriazole (3-AT), a competitive inhibitor of the *HIS3* gene product (7). Increasing concentration of 3-AT makes the reaction environment more stringent and therefore eliminates weak interactions. If a library screen is performed, DNA from positive clones can be isolated for sequence analysis of AD fusions (11). Engineering suitable deletions in conjunction with mutational analyses reveal domains essential for the interaction.

A number of different vectors have been constructed. Figure 1A shows a common version of BD vector utilizing the product of the *GAL4* gene. The *GAL4* gene product is an endogenously expressed transcription activator protein of 881 aminoacids (aa) and contains a DNA binding domain (aa 1-147) and an activation domain (aa 771-881) (18). Other commonly used BD vector uses *lexA,* a repressor protein from *E. coli* (19). Activation domain vectors from *GAL4* (8,17) and herpes simplex virus's VP16 protein (20) are available. These are shuttle vectors: they can replicate in two different organisms due to the presence of different origins of

replication. Bacterial origin of replication allows maintenance and selection in *E. coli* and yeast auxotrophy marker provides selection of the plasmid in the yeast strain.

## PHARMACEUTICALLY SPEAKING: USING THE REVERSE GEAR OF THE YEAST-TWO HYBRID IN DRUG DEVELOPMENT

The first step in drug development is the identification of biological targets. Among the biological targets, proteins constitute one of the major sites of drug action. Because the characteristics of a protein are a function of its biological interactions, targeting protein-protein interactions is a fundamental issue in drug development. Indeed, some existing drugs, such as immunosupressants and HIV-protease inhibitors, exert their actions by disrupting certain protein-protein interactions, although they were not necessarily selected for this purpose (5). For example, the macrolides FK506 and rifamycin were shown to disrupt the interaction between the type I receptors of transforming growth factor-beta (TGF-β) and the immunophilin, FKBP12 (21). Progress in molecular biology and functional genomics helped a better identification of potential target proteins and protein-interaction-mapping for some organisms, such as phage T7 (22) and *S. cerevisiae* (23) are already reported.

The second step of drug development is lead discovery. A range of small organic molecules that inhibit the chosen target is identified from very large compound libraries during this step. A screening process identifies candidate structures that are effective on the target. Through a focused chemical synthesis effort coupled to computational design, these active compounds can serve as the basis for structure-activity relationships to create specific compounds of high potency (24–26). Although the process is time-consuming and costly, the computational approaches greatly aided by nuclear magnetic resonance (NMR) and X-ray crystallograhy make this process feasible.

The yeast-two hybrid system has a significant impact on pharmaceutical research; once the interaction is demonstrated via Y2H, then the interacting protein partners can be screened against a vast number of compounds to dissociate undesirable interactions by reversing the original system. Traditionally, identification of such dissociator compounds has relied on the biochemical methods including *in vitro* affinity chromatography and radiolabeled ligand-binding reactions (2). However, readout for dissociator molecules using these techniques is such that only a high level of activities can be detected.

Reverse modification of Y2H uses counterselection by employing a reporter gene, whose product is either toxic or cytostatic to growing cells such that only the cells that do not express it can grow (27). Figure 2 shows how the system works. The gene *URA3,* incorporated into the yeast genome under the control of *GAL4* promoter encodes for the enzyme, orotidine-5-phosphate decarboxylase, which is involved in uracil biosynthesis (28). The product of *URA3* also catalyzes the transformation of pro-toxin 5-flouroorotic acid (5-FOA) into a toxic compound that causes cell death. Therefore, *URA3* marker allows both positive- and countergrowth selection on medium lacking 5-FOA or on medium lacking uracil, respectively. The positive selection step of the assay identifies the interacting proteins X and Y among the growing cells using the original system (Fig. 2A). If a test compound does not interfere, the interaction between X and Y proteins will allow *URA3* expression and, subsequentially, the transformed yeast strain will die in 5-FOA-containing media (Fig. 2B). On the other hand, if the test compound interferes with the same interaction, expression of *URA3* is diminished due to dissociation of AD from the promoter allowing the cell to survive in the presence of 5-FOA (Fig. 2C). A parallel line carrying two hybrid proteins that are unrelated to the target proteins with similar reporter gene expression can also be treated with each compound as a control.

Reverse modification of the Y2H was initially used in identifying mutations that cause the dissociation of protein-protein interactions. Li and Fields screened the mutations in the tumour suppressor protein, p53, to disrupt binding with the simian virus 40 (SV40) large T antigen (27). Later reports have employed reverse Y2H with several modifications (29–33). Leanna and Hannink used *CYH2* for counterselection to investigate the interaction between cREL and p40 proteins (29). The *CYH2* gene product provides sensitivity to cycloheximide in a phenotypic reversal from that observed with the *HIS3* reporter gene (34). Therefore, a productive protein-protein interaction drives the expression of *CYH2* gene and makes the yeast strain sensitive to cycloheximide. Another modified basic system employed *E. coli tet*-repressor and operator to search for CREB mutations that disrupt its association with CREB-binding protein (30). Vidal *et al* reported important mutational determinants of E2F family transcription factors using reverse Y2H (31,32). A *lexA*-based screen verified FK506 as the inhibitor of the interaction between the receptor R1 of transforming growth factor-β and FKBP12 (33).

A recent report from Franco's laboratory showed the identification of a novel lead compound using this approach (35). These authors designed a reverse Y2H to screen compounds for their interfere with the associations of the human α1B and β3 subunits of N-type calcium channel involved in neurotransmitter release in the central and peripheral nervous system. A large chemical library comprising over 156,000 diverse and random small synthetic compounds was screened with the plasmids constructed by fusing the entire β3 sequence and α1BI/II intracellular loop to BD and AD, respectively, in a *GAL4*-based assay. An investigational lead compound, WAY141520, was found to reversibly-inhibit the N-type current with therapeutic potential (35). The compound was active in secondary electrophysiology screen using the recording of calcium currents in isolated rat superior cervical ganglion (SCG) neuron.

Targeting proteins to disrupt their physiological interactions during infection is a typical viral strategy. Therefore dissociating the protein-protein interactions, i.e., the interaction between the pathogen and host proteins can halt the infection process. An interesting example is lymphocyte choriomeningitis virus (LCMV) infection, an arenavirus. Some arenaviruses like Lassa cause haemorrhagic fever (36). Recently, we have shown that arenaviral Z protein and promyelocytic leukemia protein (PML) interact with proline-rich homeodomain protein (PRH) (37). The Z protein, encoded by LCMV appears to mimic PML so that it can commandeer the host cell components it requires for replication and survival (38). We are currently working on screening of compounds for their interference with the interactions of viral protein to overcome infection.

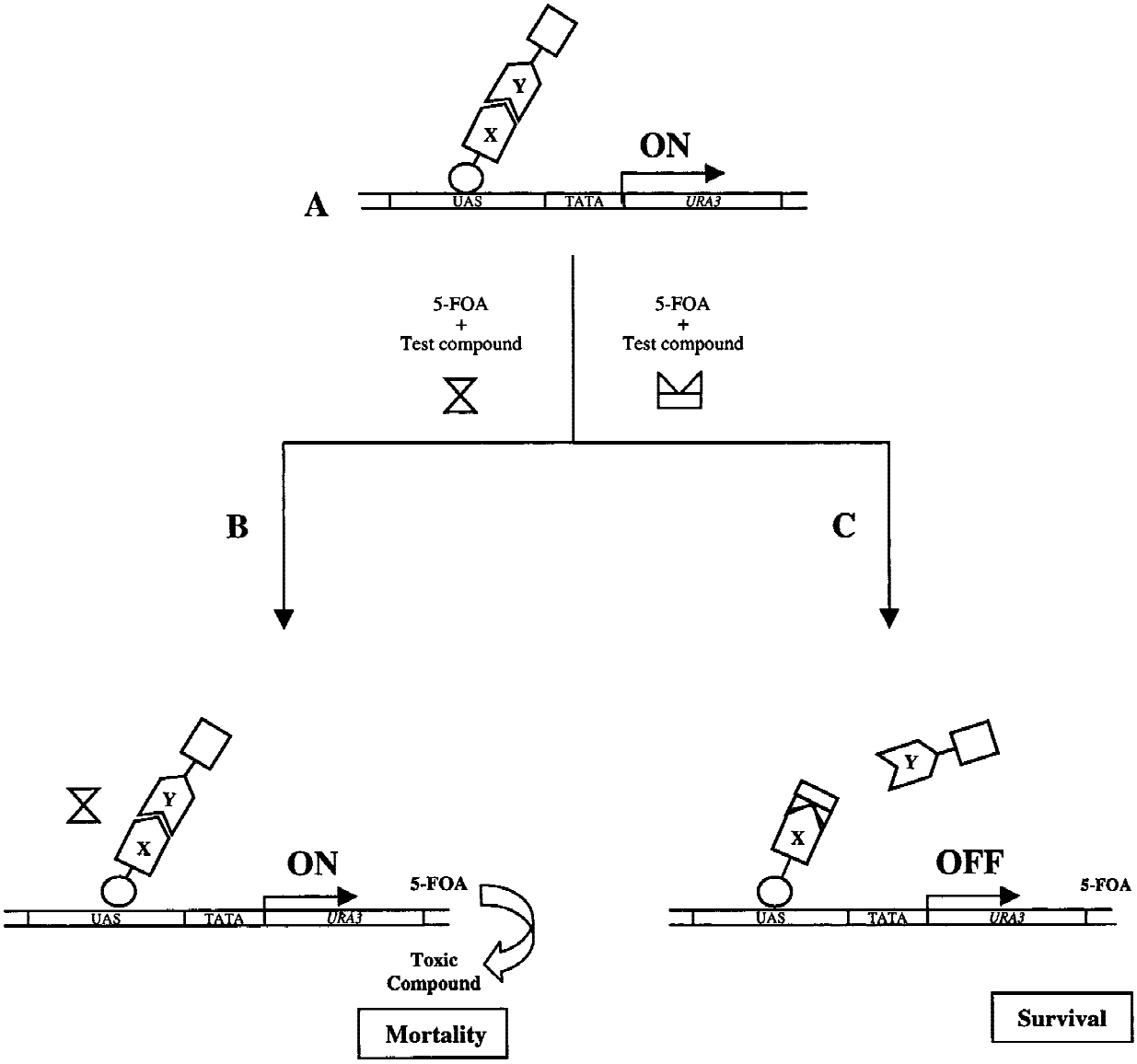


**Fig. 2.** Schematic representation of using yeast two-hybrid assay in drug screening. Panel A shows an interaction detected between the proteins X and Y using the yeast two-hybrid system (see Fig. 1 legend). The yeast *URA3* gene product is essential for uracil biosynthesis and can also catalyze the transformation of 5-FOA into a toxic compound. Expression of *URA3* is controlled by the promoter containing *GAL4* binding sites. If a test compound does not interfere, the interaction between proteins X and Y will allow the *URA3* expression and subsequentally the transformed yeast strain will die in 5-FOA-containing media (panel B). If a test compound disrupts the interaction, AD dissociates from the promoter and transcription of *URA3* is diminished allowing the cell to survive (Panel C).

## VARIATIONS OF THE YEAST TWO-HYBRID SYSTEM

Other pharmaceutically important modifications of the two-hybrid system have been developed to address a growing list of molecular interactions: the yeast one-hybrid (Y1H) (39) and three-hybrid (Y3H) (40,41) systems are employed in investigating protein-DNA and multiprotein interactions, respectively.

### The Yeast One-Hybrid System

The study of protein-DNA interactions is one of the most rapidly growing areas in molecular biology. Such interactions play essential roles in several cellular activities including restriction and modification of DNA, chromatin assembly, replication, transcription, and recombination. The biochemical methods to determine protein-DNA interactions include gel mobility shift, DNase I and Exonuclease III footprintings, filter-binding assay, and crosslinking (42). A simplified version of the basic Y2H framework offers a valuable technique for studying *in vivo* protein-DNA interaction (39). Mainly, binding domain plasmid is eliminated and a hybrid expression library is constructed by fusion of a transcriptional AD to a cDNA library such that expression of a reporter gene is induced when the hybrid protein recognizes the binding site. DNA molecules with different sequences serve as a control to confirm the specificity of binding.

As in the two-hybrid system, Vidal *et al.* reversed Y1H strategy such that the expression or presence of a mutation in the DNA-binding protein results in a decreased expression of reporter gene (31). Their study identified p53 mutations, which abolished the binding of the protein to its consensus nucleotide sequences. This protein-nucleic acid interaction is associated with tumorigenesis. Rudakoff *et al.* tested several compounds for their receptor mediated-progestogenic and antiprogestogenic actions in a one-hybrid reporter composed of the human progesterone receptor fused to the DNA-binding domain of the yeast transcriptional activator *GAL4* (43).

The yeast one-hybrid system offers suitable opportunities for the proteins binding to DNA in a sequence specific manner. Using this system, a library of random protein segments expressed as AD fusion can be screened for affinity to a given nucleotide sequence and a protein of interest can be tested for sequence dependence with a series of nucleotides using site-directed or random mutagenesis of a given DNA sequence. Taking advantage of high sequence specificity of transcription factors, the activity of a transcription factor can be analysed by reporter genes that are fused to a yeast minimal promoter containing the DNA-recognition elements of the respective transcription factor (9).

The mutational analyses of proteins for their affinity to certain DNA sequences can be extended to search for compounds that interfere with protein-DNA interactions. Among the DNA-binding proteins DNA-topoisomerases I and II, the family of DNA-processing enzymes are pharmacologically significant as they are shown to be the cellular targets of a number of clinically useful anticancer drugs (44). Topoisomerases prefer certain nucleotide sequences at their reaction sites (45,46). Drugs targeting these enzymes essentially act by trapping and reversing topoisomerase-DNA cleavable complex, an intermediate in the enzyme's catalytic cycle. Therefore, designing or screening the molecules that interfere with topoisomerase-DNA interactions with a reverse one-hybrid strategy can provide fresh approaches to anticancer drug development.

### The Yeast Three-Hybrid System

A limitation of the currently used form of Y2H is its restriction to bipartite interactions. However, in most cases, the interaction is more complex accomodating multipartite proteins, i.e., signalling pathways often require a third molecule to mediate association. The three-hybrid strategy allows the study of such ternary complexes. The strategy of Y3H is schematized in Fig. 3. DNA-binding domain fused to the receptor for one ligand X, and activation domain fused to the receptor for a second ligand Y cannot activate reporter gene expression (Fig. 3A). Proteins X and Y are brought into close proximity by the third component allowing reporter gene expression (Fig. 3B).

This system can be applied to identifying cDNAs encoding receptors of a ligand of interest and to screen for new ligands that bind to a specific receptor. In one example, Zhang and Lautar used Y3H to verify that, after epidermal growth factor (EGF) stimulation, EGF receptor, and C-terminal region of Sos, a guanine-nucleotide exchange factor for Ras proteins need an adaptor protein, Grb2, for interaction (41). In addition to BD and AD plasmids of the original

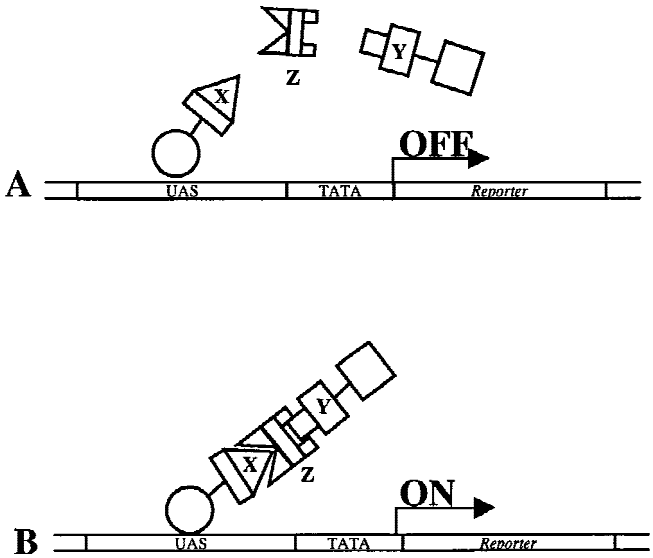


**Fig. 3.** The principle of the yeast three-hybrid system. Binding domain fused to the receptor for one ligand X and the AD fused to the receptor for a second ligand Y can not activate reporter gene expression (panel A). Proteins X and Y are brought into close proximity by the third component allowing reporter gene expression (panel B).

system, the authors expressed a third plasmid as the mediator in the form of a fusion protein with a SV40 T-antigen nuclear localization signal (NLS). The third plasmid also contained a *URA3* gene so that triple transformants were selected in a medium lacking Ura/Trp/Leu.

The mediator can be a synthetic compound as well. Licitra and Liu used this approach in searching the targets against FK506 (40). These authors fused rat glucocorticoid receptor (GR) gene to a *lexA*-based vector and screened a cDNA library from human leukemia Jurkat cells for proteins to induce reporter gene expression in the presence of a synthetic heterodimeric compound, dexamethasone-FK506. Clones expressing a FK506-binding protein (FKBP12) was identified as a mediating partner of the interaction (40).

Once a receptor is identified in the pathogenesis of a disease, application of Y3H can be extended for searching dissociator compounds in receptor-ligand interactions. Small molecules mimicking or blocking natural ligands of the biological target can be screened from compound libraries by modifying this system in a manner similar to Y2H.

## NOBODY IS PERFECT: REQUIREMENTS AND LIMITATIONS OF THE YEAST TWO-HYBRID SYSTEM IN DRUG DEVELOPMENT

The yeast two-hybrid system requires that the genes encoding the target protein are available. The system also requires stable expression of hybrid proteins and correct protein folding in yeast.

Verification of detected interaction is an essential issue in library screening. A reliable method for verification is to test the ability of hybrid proteins to associate when the target protein is switched to the AD and the library-derived protein is fused to the BD. Oberved interaction between reciprocal hybrids is a strong indication of true interaction. However, once the candidate interacting protein has been confirmed within the two-hybrid system, supporting evidences are nec-

essary to show that the interaction is real by using biochemical or immunohistochemical techniques. Cotransfection of mammalian cell lines with cDNAs encoding the two proteins of interest to achieve high levels of expression followed by coimmunoprecipitation, and/or immunolocalization are employed to verify Y2H interaction.

Proteins that are known to interact within mammalian cells do not necessarily display interaction in Y2H. Conversly, positive interactions detected via Y2H may not have physiological significance. The partners can be expressed at different stages and/or subcellular locations. Biological significance of the observed interaction is not established by Y2H, and this issue has to be addressed by using independent strategies.

All necessary posttranslational modifications need to be carried out for determination of protein interactions by YTH screen. Interactions that are dependent on posttranslational modifications, such as glycolization and disulfide bond formation, and the proteins that cannot fold correctly in the cytoplasm or that require other modifications for association such as phosphorylation or acetylation by non-yeast proteins may not be suitable for use in a YTH screen. Although yeast cells are known to carry out some of the mammalian posttranslational modifications, several plasmids were designed to allow conditional expression of a transacting partner to mediate posttranslational modifications, referred to as tribrid systems (47). Adaptation of Y2H to use in cultured mammalian cell lines has also been reported (48).

The interaction between fusion protein constructs must take place in the yeast cell nucleus to be detected, as Y2H relies on the assembly of a BD with AD upon the promoter of the reporter gene. Therefore, bait and prey plasmids must be able to enter the nucleus. Both BD and AD plasmids provide NLS for the fusion proteins (4,7). However, proper localization of YTH vectors with NLS can represent difficulty when working with membrane-anchored proteins containing hydrophobic domains such as integral membrane receptors. This problem can be partially circumvented by using the intracellular hydrophilic region(s) of membrane-bound proteins, as successfull results have been achieved using bait fusion proteins ranging from only a few amino acids in tandem repeats to entire large-protein domains (6).

Permeability of yeast cells to potential compounds examined in a reverse version of the method is an important limitation. Besides the presence of cell wall, yeast plasma membrane has a different composition than that of mammalian cells. Particular genetic modifications can be used to make yeast strains more permeable to a wide range of compounds, including hydrophilic or charged molecules. One way to approach this problem is to incubate with lytic enzymes to dissolve the cell wall and to enhance the permeability of the yeast by using chemicals such as polymixin B (49). Alternatively, using mutants with altered permeability to various compounds have been reported (50,51). For example, the *tum* mutants exhibit an altered uptake of thymidilate (50). Another mutant strain is *erg6,* which affects ergosterol sysnthesis and increases the cell's sensitivity to several compounds such as cycloheximide (51). For the compounds that can penetrate a yeast cell, there should not be any problem with entering a human cell.

The presence of several yeast proteins involved in drug export, collectively known as members of the PDR (pleiotropic drug resistance) is another drawback of using yeast as a drug screening factory (52). Attempts were made to reverse PDR to enhance the intracellular maintenance of drugs (53).

Finally, activating or inhibiting a particular protein-protein interaction in cultured cells by a given lead does not necessarily provide the same effect in treating a disease in a whole organism. Several parameters need to be considered to optimize the interfering compound to make it useful as either therapeutic or a scientific tool. A candidate compound that interferes with a molecular interaction in a yeast screen may not match the required bioavailability and stability characteristics. It must show a high degree of binding specificity. The candidate compound should be analysed for known criteria in drug design such as pharmacokinetics, side effects, specificity, discomfort, and cost.

## FUTURE ASPECTS

The yeast two-hybrid system is a powerful methodology in determination of protein-protein interactions, and screening small molecule ligands for their interference with these interactions is a pharmaceutically significant modification of the method. Compounds of potential therapeutic value can be identified using reverse modification of the yeast two-hybrid system as well as its one- and three-hybrid versions. However, using potential protein targets for an effective drug screening also requires the development of high-throughput assays and several types of technologies for rapid testing or development of pharmaceutical lead compounds (54). Highly sensitive monitoring with specialized reporter systems introduced by high-throughput screening, and advances in combinatorial chemistry will undoubtedly expand the potential applications the yeast two-hybrid system in pharmaceutical research. Besides the human therapeutics, this methodology can also be applied in designing pesticides, herbicides, and veterinarian therapeutics.

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