A Two-hybrid Dual Bait System to Discriminate Specificity of Protein Interactions*

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Biological regulatory systems require the specific organization of proteins into multicomponent complexes. Two hybrid systems have been used to identify novel components of signaling networks based on interactions with defined partner proteins. An important issue in the use of two-hybrid systems has been the degree to which interacting proteins distinguish their biological partner from evolutionarily conserved related proteins and the degree to which observed interactions are specific. We adapted the basic two-hybrid strategy to create a novel dual bait system designed to allow single-step screening of libraries for proteins that interact with protein 1 of interest, fused to DNA binding domain A (LexA), but do not interact with protein 2, fused to DNA binding domain B (cl). Using the selective interactions of Ras and Krev-1(Rap1A) with Raf, RafGDS, and Krit1 as a model, we systematically compared LexA- and cl-fused baits and reporters. The LexA and cl bait reporter systems are well matched for level of bait expression and sensitivity range for interaction detection and allow effective isolation of specifically interacting protein pairs against a nonspecific background. These reagents should prove useful to refine the selectivity of library screens, to reduce the isolation of false positives in such screens, and to perform directed analyses of sequence elements governing the interaction of a single protein with multiple partners.

To understand and manipulate the function of a particular protein of biological interest, it is generally useful to identify other proteins with which it associates. Although identification of protein interactions initially proceeded almost solely by technologically difficult biochemical methods, in recent years yeast two-hybrid systems (1) have developed as a powerful genetic tool to rapidly select previously uncharacterized proteins that specifically interact with a target protein of interest from a suitable library (2–5). In this schema, a protein of interest is synthesized in yeast as a fusion to a DNA binding domain (DBD), which is typically the bacterial repressor protein LexA or the amino-terminal end of the yeast transcription factor GAL4. Interaction of this DBD protein fusion (a “bait”) with a transcriptional activation domain-fused partner protein (either a defined partner or a novel protein screened from a library) allows the activation of reporter genes (lacZ, HIS3, LEU2) responsive to the cognate DBD. More recently, interest has focused on expanding the utility of two-hybrid systems to enable the detection of interactions between proteins and RNA (6, 7), proteins and nonprotein ligands (8), proteins and peptides (9, 10), and proteins and multiple partners (11, 12). A second thrust has been to enable whole-genome applications (13–15), leading to the generation of maps of protein interaction networks with the potential to complement the vast resource of sequence information now being developed as part of the Genome Project. Finally, there has been interest in developing two-hybrid systems as tools in high throughput drug discovery screening strategies to identify agents regulating the activity of biologically important target proteins.

As two-hybrid technologies have evolved to more complex applications, a question of mounting importance has been the degree to which library screens performed in these systems yield partners specific for the utilized bait, as opposed to proteins of broad interaction capability (“false positives”). Although the large number of published two-hybrid papers indicates that many specific partners are obtained, a recent survey has suggested that the majority of library screens isolate at least some cDNAs that are nonspecific. As a related issue, it is clear that many biologically important proteins are organized into families of evolutionarily related members that conserve substantial sequence similarity (e.g. Refs. 17–19). Thus, the degree to which two-hybrid systems isolate proteins partners absolutely specific for individual baits, rather than those that interact generally with a class of protein (“familial positives”), is also an issue. Although existing two-hybrid systems allow discrimination of uniquely specific interactors from false positives or familial positives through use of various methods of specificity testing performed subsequent to a screen (20), these methods are frequently laborious, particularly when many possible interactors must be tested. For this reason, it has been of considerable interest to devise a method to eliminate such clones before selection.

In this report, we describe a novel adaptation of the two-hybrid system designated the dual bait system. This system incorporates controls for false positive or nonspecific interactions in a single step and allows the simultaneous assay of a protein interaction with two related or unrelated partners in a single cell, which should also be useful for a variety of high throughput and genome-oriented studies. We demonstrated that these reagents are effective at selectively identifying two
discrete sets of interacting proteins against an extensive background population of nonspecifically interacting proteins, supporting the idea of reagent appropriateness for large scale genomic applications.

**EXPERIMENTAL PROCEDURES**

Molecular Biology and Genetic Techniques—DH5α Escherichia coli was grown on LB medium (21) where appropriate, and antibiotics were added at the concentrations recommended by the suppliers. Standard DNA manipulation techniques were as described in (21). Yeast were grown on yeast-peptone-glucose or minimal medium and manipulated using standard techniques (22). Two-hybrid experiments and β-galactosidase assays were performed as described (23), with six independent colonies assayed for each value presented; for sensitive plate-based X-Gal and X-Gluc assays, the procedure of Duttweiler (24) was used.

**Dual Bait System Reagents**—Relevant properties of all strains and plasmids are described in the text. The bacteriophage λ repressor protein cI (25) was used as the basis of reagent development, as its size, structure, and DNA binding properties suggested it might behave comparably as a DNA binding domain to the pre-existing two-hybrid system DDBL LexA (26–28).

**cI-responsive LacZ Reporters**—A 68-base pair fragment of the β bacteriophage genome (LAMCG, nucleotides 37230–37940) containing 3 naturally occurring cI operators, and Xho I codons added by PCR. The resulting product was digested with Xho I and inserted into the Xho I site of the plasmid pLR1Δ1 (parent of all currently utilized interaction trap lexAop-lacZ reporters (20)) in either orientation upstream of a basal GAL1 promoter directing expression of the lacZ gene. The resulting plasmids pclep-LacZa and pclep-LacZb have a 2-μm origin of replication and use a URA3 marker for selection in yeast; they differ only in the orientation of the cI operator cassette.

**cI-responsive LYS2 Yeast Strains**—An EcoRI fragment containing a minimal GAL1 promoter, cI operator cassette, and the translational start of the GAL1 gene was excised from clog-lacZa and inserted into pRFILYS8 (a gift of R. Finley) to generate pCIL-1. In this construct, the GAL1 promoter-cI operator cassette directs the expression of a fusion protein in which the first 31 amino acids of the GAL1D cassette (containing three naturally occurring cI operators), and the GAL1 minimal promoter and cI operator cassette, and the translational start of the GAL1 gene was excised from clog-lacZa and recloned immediately upstream of the lacZ gene in the plasmid pLexZeo (Invitrogen), which had been digested with Hpa I-EcoRI. The resulting plasmid, pGK6, used the ADH1 promoter to express a cI fusion. It had a 2-μm origin of replication and used ZEO1 (Invitrogen) resistance for selection in yeast and bacteria. Expression of proteins was assayed by standard lysozome cells expressing appropriate constructs (20) followed by SDS-polyacrylamide gel electrophoresis and Western analysis with antibodies to Krev-1 (Transduction Labs, Inc.), LexA, or cI repressor (a gift of G. Kalmar).

**cI Fusion Bait/clop-gusa Reporter Dual Purpose Vectors**—To allow introduction of all system components into a single yeast strain, a combined cI bait expression/clop-gusa reporter with a single selectable marker (ZeoR) was constructed. To create this plasmid, pGKS8, the Xho I-digested pGKS3, pGKS6, pGKS8, and pEG202, as noted in the text. Nonactivating bait fusions were constructed by cloning the full-length Krev-1 gene (30) into EcoRI-XhoI-digested pGKS3, pGK6, pGKS8, and pEG202, as noted in the text. Nonactivating bait fusions were constructed by cloning the full-length Krev-1 gene (31) into the EcoRI-XhoI site of pGKS8 or into EcoRI-SacI site of pGKS8 and by cloning the Ras gene into the EcoRI-XhoI site of pGKS8. Activation domain fusion plasmids were obtained by cloning Krev1 (full-length) and Raf1 (3 amino acids 1–56) into EcoRI-XhoI sites of the plasmid pEG202 (4) and RalGDS (amino acids 767–848) (a gift of A. Vojtek) into BamHI-EcoRI sites of pYesTrp2 (Invitrogen).

**Assaying Reporter Activation**—Activation of LacZ reporters was assessed qualitatively by on-plate overlay assays (24) using the substrates X-Gal or Magenta-Gal (Diagnostic Chemicals Ltd) and quantitatively using β-galactosidase assays as described in (21). Activation of gusa reporters was assessed qualitatively using the same overlay procedure as for LacZ but with X-Gluc (Diagnostic Chemicals Ltd) as a substrate. A quantitative assay was performed as for a β-galactosidase assay but using 4-nitrophenyl-β-D-glucuronic acid instead of 2-nitrophenyl-β-D-galactopyranoside as the substrate. For both assays, determination was made in a plate reader; in a standard procedure, 100 μl of exponential phase yeast cultures were harvested by centrifugation in a microtiter plate. Yeast pellets were resuspended in 50 μl of Z-buffer, and the A590 was determined in a plate reader, after which the plate was frozen at −70 °C. After thawing, 50 μl of 4-nitrophenyl-β-D-glucuronic acid (2 mg/ml) or 2-nitrophenyl-β-D-galactopyranoside (2 mg/ml) in Z-buffer, as appropriate, was added to each well. Immediately, the A590 was measured, and the plate was then placed in a 30°C incubator. Additional A590 readings were taken between 5 and 45 min, depending on the intensity of the reaction. To calculate β-glucuronidase or β-galactosidase activity, the following formula was used:

\[
\frac{(A_{590} - A_{590})_{0}}{A_{590} t (0)} \times 1000
\]

(Eq 1)

where \(v\) is volume in ml, \(t\) is time, and \((A_{590} - A_{590})_{0}\) is a net increase of optical density at 405 nm between zero time and \(t\), and \(A_{590}\) is optical density at 590 nm.
activation domain-fused prey. Qualitatively identical patterns of protein interaction are obtained with both media; however, because of minor nonspecific growth inhibitory effects of zeocin, growth is faster (~24 h to point of large colonies) without zeocin omitted.

For mixing experiments, yeast were pregrown in medium selective for plasmid retention and inducing for activation domain fusion expression (ura-his-trp-galactose/raffinose + zeocin), diluted as described in results, and plated to selective medium; colony outgrowth was monitored over 4 days.

RESULTS

Outline of Strategy—The general approach taken with a dual bait selection strategy is outlined in Fig. 1. In the interaction trap two-hybrid system (Fig. 1A (4)), a LexA-fused bait (expressed from plasmid pEG202 or a derivative) interacts with a galactose-inducible B42 “acid blob” activation domain-fused partner (expressed from plasmid pGJ4–5) to induce the expression of two reporter genes under transcriptional control of lexA operator (op) sites. These are (lexAop)_n-lacZ (borne on plasmid pSH18–34 (n = 8), pJK103 (n = 2), or pRB1840 (n = 1)) and an integrated (lexAop)_n-LEU2 (in yeast strain EGY48 (n = 6) or EGY191 (n = 2)).

In the dual bait system described here, three further components are added (Fig. 1B). The first of these is a cl-fused alternate bait, expressed from the novel Zeo^{R^2}, 2-μm plasmid, pGKS8. The second is a (clop)_{n}-gusA (β-glucuronidase) (n = 3) reporter gene cassette, additionally borne on the plasmid GKS8. The third is an additional integrated reporter system in which cl operators direct the expression of the LYS2 gene; (clop)_{n}-LYS2 (n = 3) in the yeast strains SKY48 or SKY191 (derivatives of EGY48 and EGY191, respectively). These reagents can be utilized in multiple ways to enhance measurement of protein interactions over currently existing approaches.

As a first example, in a library screen, if an activation domain-fused interacting protein associates uniquely with a LexA-fused primary bait but not with a cl-fused alternate bait, SKY48 or SKY191 yeast containing the appropriate bait and reporter constructs would turn blue on medium containing X-Gal but not on medium containing X-Gluc, and grow on medium lacking leucine, but fail to grow on medium lacking lysine; in contrast, promiscuously interacting clones would be revealed by their growth on medium lacking both leucine and lysine and blue color with both X-Gal and X-Gluc. False positives would be eliminated simultaneously with isolation of true positive clones. As a second example, in targeted examination of the interaction of a single activation domain-fused protein with two defined partners (for example, interaction of activation domain-fused cyclin D with LexA-fused CDK4 and cl-fused CDK6), a randomly mutagenized pool of activation domain-fused partners could be screened to identify mutations that disrupt interaction with either one or both of the partner proteins. As a third example, one source of interest in two-hybrid systems is their use in drug screening approaches to identify compounds that disrupt interactions between discrete pairs of interacting proteins (8, 32, 33); dual bait reagents would apply a simultaneous control to the specificity of such interactions.

Parallel Performance of LexA and cl Expression and Reporter Systems—Given that assessment of protein interactions in two-hybrid systems is a factor of bait expression levels (34) and stringency of reporter systems (23), for these hypothetical uses to be practicable, the two-bait-reporter combinations utilized in the dual bait system must express respective baits to similar levels and possess similar sensitivities to transcriptional activation. Therefore, an initial step was to carefully measure these parameters. To this end, we constructed equivalent pEG202 (LexA) and pGKS3 (cl) fusions to the protein Krev-1/Krap1A (31), a human Ras-family GTPase. These and parent vectors were transformed in parallel into EGY48 yeast, and expression of the synthesized proteins was assayed by Western analysis using antibodies to Krev-1, LexA, or cl (Fig. 2). Expression of the two Krev-1 fusion constructs was found to be comparable in 4 randomly chosen colonies, with slightly higher levels (∼2–3-fold) in the cl constructs. Furthermore, expression of the fusion protein was in each case similar to the matching unfused DNA binding domain, indicating that cl tolerated attachment of a fusion domain without loss of stability. Finally, essentially identical expression levels were observed using pGKS6-Krev1, a Zeo^{R^2} instead of HIS3 version of pGKS3 (not shown), indicating the selectable marker could be exchanged without gross alteration of plasmid copy number.

We next determined the degree to which activation through cl operators was comparable with activation through lexA operators. As a conservative first step, we constructed analogous fusions of pGKS3 and pEG202 to Krit1 (a Krev-1-interacting protein (30) that fortuitously functions as a transcriptional...
activator of moderate strength) and assayed activation of the closely related cIop- and lexAop-lacZ reporters. Parallel transformations were performed with pGKS3-Krit1 (cI-Krit1) plus cIop-lacZA and cIop-lacZB, which contained the three naturally occurring cI operators C1-R3 of A phage in either orientation (25). In addition, for comparison, yeast were transformed with pEG202-Krit1 (LexA-Krit1) plus pRB1840, pJK103, or pSH18–34 (1, 2, or 8 lexAop-lacZ) (23), and as a negative control, with pEG202-Krit1 plus cIop-lacZA. β-Galactosidase assays were used to measure activation of the lacZ reporters (Fig. 3). In these tests, the cIop-Krev-1 fusion protein activated the two cIop-lacZ constructs to equivalent levels, which were closely comparable with that obtained using the combination of LexA-Krev-1 and pJK103. As a negative control, the LexA-Krev-1 construct was also shown not to activate the cIop-lacZ reporters, as expected. Based on this result, we next used the cIop/minimal promoter cassette to develop a cIop-gusA reporter, pRG2, to be used in conjunction with the standard lexAop-lacZ reporters. Yeast were transformed with the reporter and an activating cI-Krev-1 fusion protein or LexA-fused Ras (which does not activate transcription) as a negative control, and gusA transcription was assessed with a quantitative assay, analogous to a β-galactosidase assay (see “Experimental Procedures”); as with the cIop-lacZ reporters, a high degree of specific activation was observed against a much lower background (6300 β-glucuronidase units for cI-Krit1 versus 85 for the negative control, a 75-fold difference), although overall the gusA reporter was more sensitive than lacZ, as reflected in the higher units (700 β-glucuronidase units for cI-Krit1). In plate overlay assays, positive and negative were similarly clearly distinct, with cI-Krit1 medium to dark blue, whereas the negative LexA-fused control was pale blue or white (not shown; also, see below).

Finally, we compared direct activation of the LEU2 versus LYS2 auxotrophy reporters, again using analogous LexA- and cI-fused Krit1(Fig. 4). Using SKY48 and SKY191 as hosts, we determined that LexA-Krev-1 activates the LEU2 reporter of these strains, whereas cI-Krit1 does not (Fig. 4, second panel from top). Conversely, cI-Krit1 is capable of activating the LYS2 reporter of SKY strains, whereas LexA-Krit1 is not (Fig. 4, third panel from top). Finally, each fusion activated the appropriate lacZ reporter to comparable degrees, independent of growth properties on Leu or Lys medium (Fig. 4, bottom panel, middle two rows). Notably, positive growth dependent on activation of the LEU2 and LYS2 reporters could be assessed in similar time frame, with results detectable at 24–48 h after plating yeast on selective media. Based on visual estimation of growth rate, the sensitivity of the cIop-lys2 reporter in these strains appears to be intermediate between that of the LEU2 reporters in EGY48 and EGY191.

Cumulatively, these results indicated that the cI- and LexA-based expression and reporter constructs yielded results in a similar sensitivity range, making them suitable for comparative purposes. One point remaining was the development of reagents suitable for expressing all baits and reporters in the same strain, to allow simultaneous assay. SKY strains (MATα trp1 ura3 his3 lexAop-LEU2 cIop-lys2) utilize the LEU2 and LYS2 markers for reporter genes. The activation domain fusion expression plasmid (pJG4–5) uses a TRP1 marker; the lexAop-LacZ reporter (pJK103) uses a URA3 marker; and the LexA fusion bait plasmid (pEG202) uses a HIS3 marker; the cI fusion bait plasmid (pGKS6) utilizes a ZeoR marker. To introduce the cIop-gusA reporter, we took advantage of the fact that the cIop-gusA cassette is only 2.6 kilobases, whereas the plasmid backbone for the cI fusion plasmid pGKS3 is unusually small, as the ZeoR marker is used for both bacterial and yeast selection. The cIop-gusA cassette was introduced into pGKS6, resulting in a new plasmid, pGKS8, which encompassed both cI-bait and cIop-gusA reporter. Control experiments similar to those outlined above demonstrated that this bait-reporter-combined plasmid yielded results similar to those obtained with the combination of pGKS3 and pRG2 (not shown). This construct was used for the experiments described in the following sections.

Specificity of the Dual Bait System in Controlled Two-hybrid Assay—The major criterion for effective use of a dual bait system is that it should effectively discriminate interactions of a partner with related but distinct proteins. Ras and Krev-1 possess 56% amino acid identity and are known to interact with an overlapping set of protein partners (35–37). In experiments described elsewhere, we determined that Raf preferentially interacts with Ras by a two-hybrid system assay, whereas Krit1 preferentially interacts with Krev-1 (30). The Raf guanine nucleotide dissociation stimulator RalGDS interacts with both Krev-1 and Ras (36). Neither Ras nor Krev-1 activates transcription when expressed as a DNA binding domain fusion. The strain SKY191 with the plasmid pSH18–34 was used as a host for transformation by pEG202-Ras (LexA-Ras) and pGKS8-Krev-1 (cI-Krev-1). We then super-transformed the SKY191/pEG202-Ras/pGKS8-Krev-1 combination in parallel with each of the galactose-inducible expression plasmids pJG4–5-Raf (AD-Raf), pJG4–5-Krit1 (AD-Krit1), or pYesTrp2-RalGDS (AD-RalGDS) or with empty AD vector and assayed for reporter activation/growth properties on selective medium. As noted above, activation through a LexA fusion permits growth on Leu medium and production of LacZ (cleaves X-Gal, Manganese-Gal, etc. to produce colored products); activation through a cI fusion permits growth on Lys medium and production of β-glucuronidase (cleaves X-β-Gluc, etc., to produce colored products).

All yeast grew on nonselective plates (ura – his – trp –, glucose, or galactose, Fig. 5, panel A). No strains grew on either Leu or Lys plates when glucose was present as the carbohydrate source. However, under galactose induction, strains containing pJG4–5-Raf were able to grow preferentially on Leu medium (Fig. 5, panel F) but only minimally on Lys medium, based on the association between Raf and LexA-Ras; conversely, strains containing pJG4–5-Krit1 grew well on Lys medium but only weakly on Leu medium, based on the interaction between Krit1 and cI-Krev-1 (Fig. 5, panel E). Strains
The results of X-Gal and X-Gluc assay on the plates are in good correspondence with the auxotrophic selection assay, with Raf-Ras positive with X-Gal (Fig. 5, panel C), Krev1-Krit1 positive with X-Gluc (Fig. 5, panel B), and Ral-GDS positive with both (Fig. 5, panel B and C). Note: using a complementary set of color-producing substrates (Magenta-Gal + X-Gluc), both LacZ and GusA activities can also be assayed on a single plate (Fig. 5, panel D). These results paralleled those previously obtained using a conventional two-hybrid selection (30) and confirmed that the dual bait system can be used to distinguish interactions between two closely related potential partner proteins. We note that although the α-aminoacidipate counterselection works well in the controlled situation shown above, over time background colonies arose on the counterselected plates, suggesting this particular application may be more useful in targeted disruption of known interactions than in conjunction with library screens.

Selection of Specifically Interacting Protein Pairs from a Nonspecific Pool—The previous results demonstrated that direct streaking of uniform populations of yeast containing predetermined combinations of baits, activation domain fusions, and reporters yields expected results. A more rigorous test of the power of the ability of these reagents to discriminate specific interactions was performed using a mixing experiment, as outlined in Fig. 6. Four populations of SKY191 yeast were generated. Each expressed LexA-Ras and cl-Krev-1 and contained lexAop-lacZ and clop-gusA reporters and an activation domain fused to either 1) Raf, 2) RalGDS, 3) Krit1, or 4) nonspecific (a fragment of hsp90). 10–100 colony forming units (30–300 cells), each of populations 1–3, were mixed together with 2 × 10^6 cells containing the nonspecific control and parallel samples of the pooled cells plated to media selective for the lexAop-LEU2 (ura-his-trp-leu-Zeo) or clop-LYS2 (ura-his-trp-lys-) reporters.

Approximately 50 colonies arose on each of these plates, in good accord with the number anticipated based on the seed. Of these, 24 were chosen from each of the Leu and Lys plates and transferred to a master plate, then retested for growth on both Leu- and Lys- medium, as well as activation of lacZ or gusA reporters. 43 of the 48 analyzed colonies were specific for growth on Leu or Lys medium, whereas 5 of the 48 total colonies analyzed grew on both Leu- and Lys- medium. In tests with the colorogenic reporters, 45 of the 48 displayed expected patterns for lacZ and gusA; LEU+ colonies were blue with X-Gal, LYS+ colonies were blue with X-Gluc, LEU+LYS+ colonies were blue with both; the remaining 3 colonies were white with both substrates. Finally, 5 colonies for each group (LEU+ Lacs+ LYS-gusa-, LEU+ Lacs+ LYS+ gusa-, LEU-Lacs-LYS+ gusa+) were selected at random and used for PCR using primers containing sequences complementary to library vector sequence-flanking inserts to identify inserts
**DISCUSSION**

In this report, we describe the development and characterization of novel dual bait reagents that can be used to study the interaction of a protein with two distinct partners in a single yeast cell. The cI repressor/cI operator system utilized in the SKY yeast strains and cIop-LacZ plasmids is demonstrated to function with a sensitivity range closely comparable with the pre-existing LexA repressor/lexA operator system in the interaction trap, facilitating their combined use. In a model system assaying the interaction of the related GTPases Ras and Krev-1 with their preferred partners Raf and Krit1 and their shared partner RalGDS, the dual bait system clearly differentiates higher affinity versus lower affinity interactions. In addition to effectiveness in discriminating interactions in grids of yeast streaked to selective plates, the discrimination observed is sufficiently robust to allow the isolation of yeast containing specifically interacting protein pairs against a vast excess of noninteracting pairs. These properties support the idea that these reagents will be useful in library screening and genome-scale applications. The reagents described here offer the option of performing two independent, simultaneous screens in a single yeast, with one set of positives registering through lexAop-LEU2 and cIop-LYS2 activation of LEU2 and lacZ and a second set through cIop activation of LYS2 and gusA, both negatively controlled against each other. Together, these developments have the potential to greatly expand two-hybrid system contributions to studies of biological interactions.

Several groups have recently described the use of two simultaneously expressed baits to identify mutations that selectively affect interactions of an activation domain-fused protein with one of two partners (38–40). In each case, introduction of a second bait-reporter system was obtained by eliminating one of the two reporters used for the primary bait, greatly reducing the screening power available to the system. However, in these novel reagents, both baits retain two distinct reporters, greatly facilitating the screening process. The value of having two separate reporters is evident even in the mixing experiment performed here (Fig. 6), as their use allowed the immediate discrimination of the noninteracting background clones from specific partners, which activated both reporters. The dual bait reagents described here can be similarly used for mutational analysis and have been recently used to successfully identify mutations in Pak1 kinase, which selectively reduce interaction affinity for either of two partners, the Cdc42 or Rac GTPases.3

Finally, there is preliminary evidence that these reagents possess the power necessary to perform library screens in organisms with complex genomes; as in several recent library screens using the above reagents, cI- and the LexA-fused baits have yielded specific partners.4

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3 M. Reeder, J. Chernoff, E. A. Golemis, and I. Serebriiskii, unpublished data.
4 M. J. Russell, personal communication; V. Khazak, unpublished data; Y. Z. Zhang and E. Golemis, unpublished data.
On a more basic level, the system allows considerable savings in the effort required by individual investigators wishing to perform multiple two-hybrid screens without invoking the dual bait selectivity function. Instead of having to perform two separate library transformations or matings (generally the most laborious step in a screening process) and subsequent selection of positive clones, only one such step is required for any two baits. Furthermore, in the case where only one-half of a dual bait screen of a previously untested cDNA library is positive (e.g. if a LexA bait yields interactors but a cI bait does not), the fact that positive interactors are obtained for at least one bait will provide useful information toward the determination of whether the library utilized was of acceptable quality. Finally, it has previously been noted that some proteins of interest for cDNA library screening perform better with particular fusion domains (i.e. are more usefully utilized as LexA fusions but not as GAL4 fusions; or vice versa (23)). In cases where specific DNA binding domain/fusion domain problems are suspected, an investigator could express a bait of interest both as a LexA and as a cI fusion and screen with the bait in both configurations to maximize chances of obtaining valid positive interacting partners.

The dual bait reagents are built upon the interaction trap form of two-hybrid system (4). cI and LexA are similar in size (237 versus 202 amino acids) and structure (41) and use related amino-terminal helix-turn-helix domains to bind palindromic operator sites with similar \(K_m\) values, ranging from 200 pM to 20 nM for LexA (discussed in Estojak et al. (23)) similar to cI (42). Because of these many similarities, it is clear that the LexA and cI systems are well matched. However, in addition to use in the current interaction trap shell, the cI “add-on” parts of this system have been constructed to potentially supplement any of the currently existing two-hybrid variants. Thus, the reporter system developed in this study purposely uses a DNA binding domain (cI), reporter genes (\(gusA\) and \(LYS2\)), and plasmid marker (zeocin resistance) not in use in any other two-hybrid-based system (2, 3, 5), including the recently described membrane-based Sos recruitment system (16). Thus, these reagents could readily be integrated with any of the other screening systems operating on two-hybrid principles; in the case of the Sos recruitment system, this raises the possibility that with minor modifications of the library vector, a single bait could be simultaneously used to identify interactors using either a membrane-based or a transcriptional activation-based selection strategy, enlarging the potential pool of interacting proteins obtained. An additionally useful feature of the \(gusA\) reporter is that it is assayed using similar protocols even on the same plates as the \(lacZ\) reporter standardly used in two-hybrid systems, again contributing to ease of use. Finally, although the dual bait reagents here described have been optimized for use in conjunction with LexA fusions, parameters have been previously established to test and vary sensitivity levels (23), making merging of two-hybrid systems a relatively simple and certainly useful effort that should contribute to efforts to understand complex protein-protein interactions on the genome scale.

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