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Correlation of Two-Hybrid Affinity Data with In Vitro Measurements

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Since their introduction, the interaction trap and other two-hybrid systems have been used to study protein-protein interactions. Despite their general use, little is known about the extent to which the degree of protein interaction determined by two-hybrid approaches parallels the degree of interaction determined by biochemical techniques. In this study, we used a set of *lexAop-LEU2* and *lexAop-lacZ* reporters to calibrate the interaction trap. For the calibration, we used two sets of proteins, the Myc-Max-Mxi1 helix-loop-helix proteins, and wild-type and dimerization-defective versions of the lambda *c*I repressor. Our results indicate that the strength of interaction as predicted by the two-hybrid approach generally correlates with that determined in vitro, permitting discrimination of high-, intermediate-, and low-affinity interactions, but there was no single reporter for which the amount of gene expression linearly reflected affinity measured in vitro. However, some reporters showed thresholds and only responded to stronger interactions. Finally, some interactions were subject to directionality, and their apparent strength depended on the reporter used. Taken together, our results provide a cautionary framework for interpreting affinities from two-hybrid experiments.

Biological systems depend on interactions between protein components. These interactions affect such diverse processes as the coordination of signal transduction by assembly of multisubunit complexes (57, 58), the regulation of apoptosis by the sequestration of Bax (54), and the control of gene expression through the selective association of transcription factors (19). Efforts to understand the functions of proteins often include identification and characterization of other cellular proteins with which they can interact. While some protein interactions are of high affinity and are easily detectable by physical techniques, a number of biologically important interactions, such as those of many enzymes with their substrates, are often relatively weak or transient and are not easily detectable by these methods.

A number of approaches for studying protein association are in use, including cosedimentation through gradients, coimmunoprecipitation of purified proteins, assay of DNA binding activity for proteins that must dimerize to recognize a DNA site, and assay by two-hybrid systems (20) such as the interaction trap (31). In the last approach, a first protein (P1, or "bait") is fused to a known DNA-binding domain such as LexA (10) or GAL4 (41) and a second protein (P2) is fused to a transcriptional activation domain (AD). Coexpression of the two chimeric proteins in yeast cells in which the cognate binding site for the DNA-binding domain is located upstream of a reporter gene results in transcriptional activation of the reporter by the P2-fused AD if the chimeric proteins associate.

Two-hybrid/interaction trap approaches have gained considerable popularity because they can detect novel interacting proteins that interact with a given bait by substituting an appropriate cDNA library for P2. A recent offshoot of these approaches, interaction mating, can be used to rapidly establish associations between large numbers of known proteins and promises to further expand the applications of two-hybrid technology (22). However, despite the frequent successes associated with the use of such systems, a systematic comparison of two-hybrid system-derived in vivo affinity determinations with in vitro determinations has not yet been undertaken. Such a comparison is critical in order to gauge the strength and significance of interactions observed in two-hybrid experiments.

Here, we tested the extent to which data obtained from a two-hybrid system, the interaction trap, paralleled those obtained from in vitro assays. To this end, we studied associations between two groups of proteins whose oligomerizations have been estimated in vitro. As a first group, we utilized a set of functionally interrelated helix-loop-helix proteins (47): Myc (1), Max (8), and Mxi1 (60). Myc is overexpressed in a large number of cancers (1) and exerts its effects, at least in part, by associating with a second protein, Max, to form heterodimers that bind a CACGTG motif upstream of genes whose products may contribute to carcinogenesis and stimulating their transcription (2). Max associates with high affinity with Mxi1 (60) and Mad (4); these heterodimeric complexes bind the same CACGTG motif but repress transcription through the action of a ternary partner that coassociates with Mad and Mxi1, a mammalian homolog of the yeast SIN3 protein (5, 56). Approximation of the dimerization affinity of these proteins by cosedimentation through gradients (for Myc-Max and Myc-Myc) and by competitive binding to DNA containing the CACGTG motif (for all combinations) suggests that Myc and Max and Max and Mxi1 heterodimerize with similar high affinities (K_d, <5 nM) and are likely to form complexes at physiological concentrations in mammalian cells, while Myc-Myc and Max-Max homodimerize only at much higher protein concentrations (maximum K_d for dimerization, >1 μM) in an interaction probably not physiological for these proteins (4, 43, 60).

As a second group, we utilized the bacteriophage lambda repressor protein *c*I (50) and a series of *c*I mutants (14, 26). The biological activity of lambda repressor protein *c*I depends on its ability to form homodimers (reviewed in reference 50).

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Lambda is a temperate phage. During lysogenic growth, lambda expresses the *c*I protein, which dimerizes to bind and repress operators of genes required for lysis. Upon treatment with UV light or other DNA-damaging agents, cleavage by RecA separates the *c*I amino-terminal DNA-binding domain and carboxy-terminal dimerization domain. The resulting monomeric *c*I DNA-binding domains dissociate from the operators of lysis-specific phage genes, allowing their induction. The full-length wild-type *c*I protein (*c*I-WT) has been rigorously established to dimerize in solution with a K_d of 20 nM (55), while the mutants in our analysis set homodimerized with K_ds ranging from ~200 nM to greater than 1 μM (14, 26).

We constructed a series of LexA-fused proteins (baits) and AD-fused proteins derived from Myc, Max, Mxi1, and *c*I. We assembled a series of LexA-responsive *lacZ* reporter genes that differed in their sensitivities to transcriptional activation because they contained different numbers of operators for LexA upstream of the *lacZ* transcription start, and we constructed a similar set of *lexA*-responsive *LEU2* reporter strains. We then assayed activation of these reporters by combinations of baits and AD-fused proteins within each group. We found that the measured strength of a protein-protein interaction generally correlated with in vitro determinations of dimerization affinity, in that interactions determined in vitro to be of high, intermediate, or low affinity could be similarly discriminated in yeast strains. Moreover, some of the reporters showed thresholds of activation, such that weak interactions (K_d, >1 μM) were generally not detected. However, there was no single reporter gene for which the strength of interaction correlated linearly to affinities reported in vitro, suggesting that it is inappropriate to use differences in reporter transcription as a direct measure of interaction affinity. With the *LEU2* reporter, differences in interaction affinity were reflected in plating efficiency on medium lacking leucine rather than a general decrease in growth rate, suggesting that expression of the LEU^+ phenotype is also subject to a threshold. Finally, in some cases, affinity was affected by whether the fused moiety was attached to LexA or the AD, and in some cases, it appeared to be affected by higher-order oligomerization of the fused moiety. These studies provide a framework for investigators evaluating interaction affinity by using two-hybrid assays.

MATERIALS AND METHODS

Cloning and bacterial strains. *Escherichia coli* DH5αF′ [F′/*endA1 hsdR17*(r_K^- m_K^+)*supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*lac*Δ(*lacZ*) *M15*)] was used as a host for all plasmid constructions. DNA was manipulated by standard methods (3, 52).

Construction of EGY strains. *lexAop-LEU2* reporter strains were made as follows. A *Hin*dIII cassette containing the *URA3* gene was inserted into the plasmid pHR33 (a gift of R. Rothstein) to create the yeast integrating plasmid pXLEU2, which contained the 5′ end of the *LEU2* coding region, a unique *Bgl*II site located upstream of the *LEU2* minimal promoter, and Ty element sequences further upstream. The *Bgl*II cloning site is located 220 bp upstream of the *LEU2* transcription start site. One, two, or three copies of a *Bam*HI-ended double-stranded 42-mer that contained the overlapping double *lexA* operator found upstream of the colicin E1 gene (18, 36), with the plus-strand sequence 5′GATC CTGCTGTATATAAAACCAGTGGTTATATGTACAGTACG3′, were inserted into the *Bgl*I site to generate plasmids p1LexLEU2, p2LexLEU2, and p3LexLEU2. These were linearized by digestion with *Cla*I and transformed into *Saccharomyces cerevisiae* U457 (*MAT***a** *SUP53-a ade2-1 can1-100 ura3-1 trp1-1* [*phi*$^+$]), also a gift of R. Rothstein, and URA^+ colonies were selected. The starting chromosomal array of genes in U457 is Ty1 element-*SUP53-LEU2*: the *SUP53-a* allele suppresses the *trp1-1* gene, so U457 is TRP^+ for growth. *ura3* mutant revertants were selected by their ability to form colonies on medium that contained 5-fluoroorotic acid. *ura3 trp1 leu2* yeast mutants were identified: these presumably resulted from recombination events that resolve the integrated plasmid by crossover between Ty1 sequences present on pXLEU variants and the chromosomal Ty1 element, resulting in loss of the *SUP53-a* gene and substitution of *lexA* operator sequences for the *LEU2* UAS. Strains containing one (EGY18), two (EGY23), or three (EGY38) *colEI* operators upstream of the single chromosomal *LEU2* gene were isolated. These strains were made *his3*$^-$ by being mated to the strain GG100-14D (*MAT*α *his3 trp1 pho5*) (32), with selection for LEU^+ HIS^+ diploids, sporulation, and selection for random spore products that were *leu2*, *ura3*, *trp1*, *his3*, and *GAL*$^+$. EGY48, EGY195, and EGY191 are derivatives of EGY38, EGY22, and EGY18, respectively.

***lacZ* reporter plasmids.** *lexAop-lacZ* reporters have been previously described. The backbone for all reporters is pLR1del1 (59): oligonucleotides encoding *lexA* operators are inserted at a unique *Xho*I site −167 from the transcription start of *GAL1-lacZ*. p1840 contains a single *lexA* operator derived from the *recA* promoter and binds two *lexA* monomers (12). pJK103 (35) has a single high-affinity overlapping *colE1* operator (which presumably binds two LexA dimers) (18) made by insertion of an oligonucleotide similar to that shown above but with *Xho*I ends. pSH18-34 (a gift of Steve Hanes) contains two tandem insertions of a double-stranded 78-bp oligonucleotide encoding two *colE1* operators (plus-strand sequence, 5′-TCGACTGCTGTATATAAAACCAGTGGTTATATGTA CAGTACTGCTGTATATAAAACCAGTGGTTATATGTACAGTACG-3′), resulting in four *colE1* (overlapping) operators upstream of the *GAL1-lacZ* gene (29).

LexA fusion plasmids. pEG202 (29, 31) or its parent plasmid, pLexA202+PL (51), was used as an expression vector for constructing LexA-fused proteins. pLexA-HEF1 expresses LexA fused to a novel 185-amino-acid open reading frame isolated from an ongoing genetic screen for regulators of cell morphology (42). pLexA-RPB7 expresses LexA fused to the full-length coding sequence of the *S. cerevisiae* RNA polymerase II subunit RPB7 (45).

pLexA-Myc and pLexA-Max have been described previously (60). pLexA-Myc expresses the carboxy-terminal 176 amino acids of human c-Myc, encompassing the complete helix-loop-helix and leucine zipper domain but lacking the N-terminal acidic domain (37). pLexA-Max encodes the full-length (151-amino-acid) Max protein (8).

Plasmids that carried *c*I-WT (49) and the E233K and the P158T (14, 26) mutants were obtained from A. Hochschild, and a clone for the A152T (26) mutant was obtained from J. Hu. PCR was used to prepare fragments of *c*I-WT and mutants corresponding to papain fragment c (amino acid residues 132 to 236) (48) that contained 5′ *Eco*RI sites and 3′ *Xho*I sites. These fragments were treated with Klenow fragment and cloned into the *Sma*I site of pUC119, and inserts in the resulting plasmids were completely sequenced. *c*I-WT, E233K, and P158T were reexcised with *Eco*RI and *Xho*I and cloned into similarly cut pEG202: the final construct proceeded from LexA through the amino acid linker sequence EFAS to the TTKKAS. . . of the *c*I sequence. Sequencing of A152T revealed a single point mutation deleting a C at position 401 of the DNA sequence (amino acid 133). To correct for this, the A152T clone was excised as were the other *c*I clones with *Eco*RI and *Xho*I but was introduced into pEG202ATT, a derivative of pEG202 with the *Eco*RI site in an altered reading frame (27). For this clone, the final amino acid sequence would be LexA, the linker sequence NLGIRKHN, and then KKAS. . . of the *c*I mutant, with the substitution of HN for the TT of the original *c*I sequence.

AD fusion plasmids. pJG4-5 (29, 31) or an altered reading frame derivative, pJG4-5ATT (27), was used as the expression vector for all AD fusions. This plasmid expresses proteins under control of the galactose-inducible *GAL1* promoter as fusions to a nuclear localization sequence, the hemagglutinin epitope tag, and the B42 AD as previously described.

p4-5/Myc and p4-5/Max contain the same fragments of Myc and Max as the LexA fusions described above. p4-5/Mxi1 has been previously described and contains amino acids 46 to 228 of Mxi1 (60), commencing with KPPRR (residues 10 to 220 in reference 60); this corresponds approximately to the mMxi1-WR clone and lacks sequences required for interaction with SIN3 (56).

*Eco*RI-*Xho*I fragments containing *c*I-WT, P158T, E233K, and A152T from the pUC119 clones described above were cloned into either pJG4-5 (*c*I-WT, P158T, E233K) or pJG4-5ATT (A152T).

Interaction trap. EGY48, EGY195, and EGY191 yeast cells were transformed by standard methods (34) with plasmids expressing LexA fusions, AD fusions, or both, together with *lexA* operator-*lacZ* reporters as indicated in Results. For all fusion proteins, synthesis of a fusion protein of the correct length in yeast cells was confirmed by Western blot (immunoblot) assays of yeast extracts (53) by using polyclonal antiserum specific for LexA (11) or for hemagglutinin (Babco, Inc.), as appropriate. The ability of the LexA fusions used in this study to bind operator DNA was confirmed by repression assay as previously described (11). Activation of the *lacZ* reporter was determined in liquid β-galactosidase assays as previously described (12) with EGY48 as the strain background. The numbers shown reflect the average values of assays with at least six independent colonies performed on 2 or 3 different days: deviations in β-galactosidase values between individually selected colonies were present in less than 30% of the total values obtained. Activation of the *LEU2* reporter was determined by observing the growth rate of yeast cells patched on complete minimal medium lacking leucine, because this is the standard method used for evaluating library transformants in interactor screens. At least six independent colonies were analyzed for each pair of constructs. Activation is expressed by the following scale: ++++, growth within 24 to 36 h from a light streak to an essentially saturated patch (i.e., growth on medium lacking leucine equivalent to that on medium containing leucine); +++, growth on leucine-minus medium clearly detectable by eye within 24 to 36 h but with a patch only ~20 to 30% as dense as that on medium containing leucine; ++, growth on leucine-minus medium detectable by eye after 48 h, with

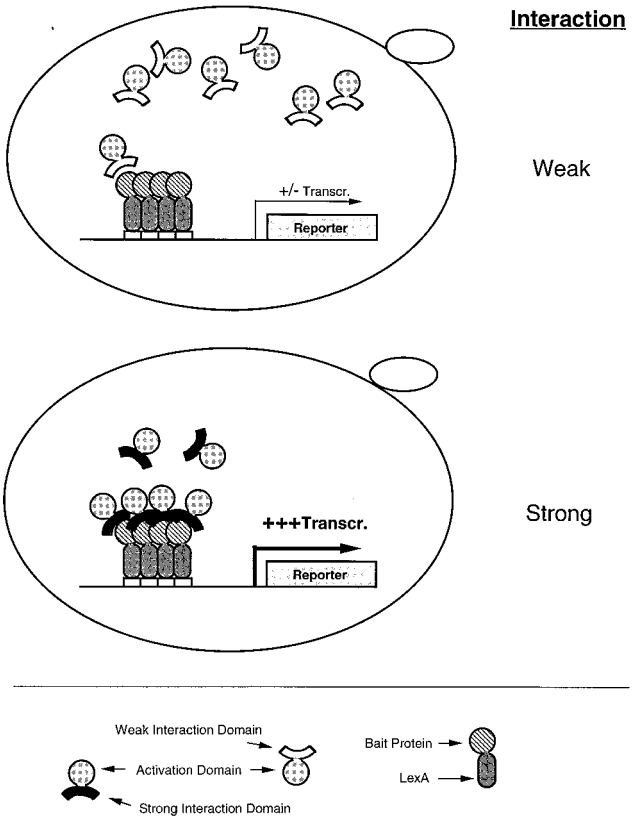

FIG. 1. Schematic of the interaction trap. A LexA-fused protein of interest is expressed in yeast cells modified so that one or multiple *lexA* operators are located upstream of a reporter gene (*lacZ* or *LEU2*). Potential interacting proteins are expressed as fusions to a transcriptional AD provided by the B42 acid blob (31). The LexA-fused protein binds to the *lexA* operators but is unable to activate transcription (Transcr.) of the reporter gene in the absence of interaction with the AD-fused partner. When the interaction is of low affinity, the reporter is turned on moderately or not at all; when the interaction is of high affinity, the reporter is strongly transcribed.

growth saturated at 72 to 96 h after streaking; +, outgrowth of microcolonies detectable by dissecting microscope at 48 h and with visible outgrowth of colonies at ~4 days; and −, no growth discernible even microscopically at 5 days.

RESULTS

Preliminary considerations. In the interaction trap (29, 31) and other two-hybrid systems (6, 13, 17), a first gene is expressed as a protein fusion to a DNA-binding domain to create the bait and a second gene or cDNA library is expressed as a protein fusion to an AD. The bait and the AD fusion are coexpressed in yeast cells in which one or more copies of the binding site for the DNA-binding domain are located upstream of a reporter gene. If the two fused proteins associate, the reporter is transcribed. In screens of cDNA libraries for novel interacting proteins, a dual-reporter system is generally used, with one reporter being *lacZ* and the second being a gene for a nutritional auxotrophy, such as *LEU2* or *HIS3*. In studies of interactions between predefined sets of proteins, often only a single *lacZ* reporter is used.

Assessment of the strength of interaction between sets of proteins in a two-hybrid system presupposes differential activation of reporter genes by strongly versus weakly interacting protein pairs (Fig. 1). The amount of activation (the penetrance of the interaction phenotype) depends on the magnitude of a number of variables, particularly the fraction of the operator sites that are occupied by the DNA-binding fusion protein bait, the fraction of operator-bound bait occupied by the AD-tagged protein, differences in the amount of transcription that might arise from differences in the geometry with which DNA-bound ADs are presented to the transcription apparatus, and the amount of transcription necessary to produce a scorable phenotype for a given reporter. The first two variables depend further on the expression level of baits and AD-tagged proteins in the cell and on any competing interactions between yeast proteins and the bait versus those between the bait and AD-fused protein.

Some of these variables can be estimated, and some cannot. For example, when tested by repression assays (11), occupancy of at least one operator by bait is typically at least 50%; since occupancy cannot exceed 100%, the variation in this parameter is typically low. Similarly, the AD-tagged proteins are generally expressed to comparable levels, typically estimated to be 10^2 to 10^4 (usually $>10^3$) molecules per cell, which should result in intranuclear concentrations of 10^{-7} to 10^{-5} M (28). Other variables have not been quantified. Effects on transcription due to differences in the precise geometry of DNA-associated transcription domains clearly exist but at present cannot be predicted in advance. Similarly, for most reporters, little is known about how many molecules of mRNA are necessary to express the transcription phenotype.

To determine the degree to which the strength of interaction as assayed by a two-hybrid approach parallels biochemically derived estimates of protein affinity, we utilized multiple reporters to systematically measure the interaction phenotypes of closely related pairs of proteins that differed in their oligomerization efficiency, while attempting to hold other variables constant.

Expanding the sensitivity range of the interaction trap. In the interaction trap (29, 31), DNA binding is provided by the *E. coli* LexA protein (11, 12), while the AD is provided by the B42 "acid blob" (44), which is a moderately strong activation domain in yeast cells. To maximize the range of interactions detectable, we assembled two series of *lexAop-lacZ* and *lexAop-LEU2* reporters (Fig. 2). p1840 (*1op-lacZ*) has a single LexA binding site (12), pJK103 (*2op-lacZ*) has a high-affinity, overlapping operator derived from the *colEI* promoter (18), and pSH18-34 (*8op-lacZ*) has four *colE1*-derived operators (29). We also constructed three strains that contained different numbers of *lexA* operators 220 bp upstream of the transcription start site of the *LEU2* gene: EGY191 (*2op-LEU2*) has a single *colE1* operator, EGY195 (*4op-LEU2*) has two *colE1* operators, and EGY48 (*6op-LEU2*) (29, 31) has three *colE1* operators.

We previously used the *lacZ* reporters described above to assist in accurately differentiating the strength of transcriptional activation by different proteins (28). Prior to embarking on large-scale assays of interacting proteins, we performed an initial gauge of the sensitivity of the new *lexAop-LEU2* reporter strains. We made LexA fusions to acid blobs B42 and B6 (44) and to an additional protein, HEF1 (previously been shown by β-galactosidase assay to be medium, fair, and weak activators of transcription, respectively [18a, 28]). We introduced plasmids that expressed LexA-B42, LexA-B6, and LexA-HEF1 and a vector control that expressed LexA into the *2op-LEU2*, *4op-LEU2*, and *6op-LEU2* yeast strains and streaked transformants onto media lacking leucine. LexA-B42 and LexA-B6 stimulated rapid growth of all three strains on leucine-minus medium, whereas native LexA did not (not shown). For LexA-HEF1, at 3 days after streaking, *6op-LEU2* yeast cells grew well on leucine-minus medium, *4op-LEU2* yeast cells grew moderately, and *2op-LEU2* yeast cells did not grow (Fig. 3), although

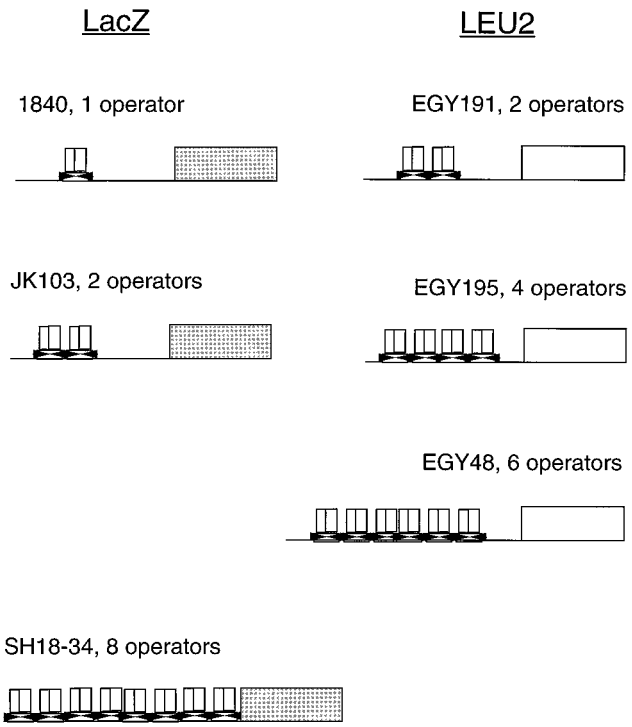

FIG. 2. *lexA* operator-containing strains and reporters. Dark grey boxes represent the *lacZ* reporter gene, while light grey boxes represent the *LEU2* reporter gene. Small rectangles containing palindromic dark arrows represent *lexA* operator motifs, each of which binds two monomers of LexA (white rectangle). See text for further detail.

after 6 days, a small number of colonies (<20 per streaked colony) appeared on leucine-minus plates (not shown).

We plated dilutions of *6op-LEU2*, *4op-LEU2*, and *2op-LEU2* yeast cells containing these LexA fusions onto media with and without leucine and determined the efficiency of plating (29). Sixty-five percent of *6op-LEU2* cells with LexA-HEF1 formed colonies on leucine-minus medium, compared with ~14% of *4op-LEU2* cells and less than 10% of *2op-LEU2* cells (with these last colonies appearing later than those in the other strains). In contrast, all strains containing LexA-B42 and LexA-B6 grew with 100% efficiency on leucine-minus medium. These results show that plating efficiency can vary in a manner that is correlated with the sensitivity of the reporter and indicate that these strains can differentiate weak from moderate transcriptional activation.

Interactions between Myc, Max, and Mxi1. The strength of heterodimerization of the helix-loop-helix (47) proteins Myc (1), Max (8, 9), Mad (4), and Mxi1 (60) has been measured either by directly assaying the association in solution in vitro or by using the K_d for binding to consensus DNA motifs to extrapolate an approximate minimum affinity. Max associates with Myc (43), Mad (4), or Mxi1 (60) to form heterodimers that bind DNA when proteins are present at levels of 1 to 5 nM. Myc has been observed to form homodimers at concentrations of ~20 μM (15, 43). Finally, while bacterially expressed, unphosphorylated Max homodimerizes and binds DNA at concentrations on the order of 10 to 50 nM (5, 7), eukaryotically expressed Max is phosphorylated by casein kinase II and homodimerizes only at a much higher concentration (greater than 20-fold increase in maximum K_d for dimerization, or ~1 μM) (7), suggesting that Max homodimers, like Myc homodimers, are scarce or absent in mammalian cells.

We used the expanded set of reporter strains and plasmids described above to reexamine the interactions of Myc, Max, and Mxi1. We used LexA fusions to Myc and Max and B42-AD fusions to Myc, Max, and Mxi1 to transform yeast cells in conjunction with the *lacZ* and *LEU2* reporters (Fig. 4 and Tables 1 and 2). LexA-fused RPB7 (45) and the B42-AD fusion vector containing no insert were negative controls. We verified that all LexA and B42 fusions were expressed to similar levels with antibody to LexA or to the hemagglutinin epitope tag on the B42 fusion vector (not shown). We have shown previously that the majority of LexA-fused proteins are expressed at intracellular levels of 200 to 800 nM (28), and because of nuclear localization sequences on the fused moiety, intranuclear concentrations are likely to be higher. We measured activation of *lacZ* reporters by β-galactosidase assay and activation of the *LEU2* reporters by observing the growth of cells patched onto leucine-minus medium (Tables 1 and 2).

Pairing of LexA-Myc with AD-Myc or of LexA-Max with AD-Max did not activate transcription of any *lacZ* or *LEU2* reporter. This result agrees with biochemical data that suggest these interactions are of low affinity (maximum affinity in vitro is a K_d of 1 to 20 μM [7, 15, 43]) and implies for this group of proteins that detectable interactions must occur with a K_d of <1 μM.

In contrast, LexA-Max interacted strongly with both AD-Myc and AD-Mxi1. With the *8op-lacZ*, *2op-lacZ*, and *1op-lacZ* reporters, the LexA-Max and AD-Myc pair gave 278, 172, and 61 U of β-galactosidase activity; with the *6op-LEU2* and *2op-LEU2* reporters, it gave ++ and − growth. On the same reporters, the LexA-Max and AD-Mxi1 pair gave 512, 81, and 4 U of β-galactosidase activity, respectively, and showed ++++, and ++ growth (Tables 1 and 2). These results parallel the in vitro findings that these proteins associate with high affinity and demonstrate that interactions that occur in vitro with K_ds of 1 to 5 nM are easily detectable by interaction trap. However, intriguingly, the rank order of these interactions differed between the *lacZ* and *LEU2* reporter genes and also was dependent on the number of *lexA* operator sequences present upstream of the reporter gene. Using the most sensitive reporters, *8op-lacZ* and *6op-LEU2*, Max-Mxi1 appeared to interact more strongly than Max-Myc. However, on less-sensitive reporters, different rankings between *lacZ* and *LEU2* were

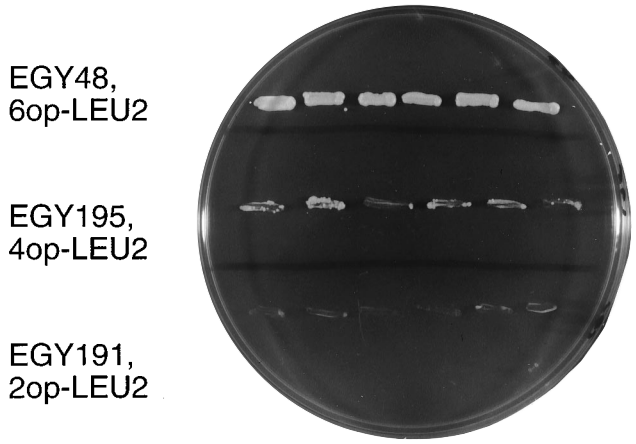

FIG. 3. LexA-HEF1 in strains with different numbers of *lexA* operators upstream of the *LEU2* gene. Colonies were initially grown on histidine-minus glucose medium to select for the presence of the LexA-HEF1 expression plasmid, and then independent transformants were restreaked onto leucine-minus, histidine-minus glucose medium to measure growth. The picture was taken 72 h after the colonies were streaked.

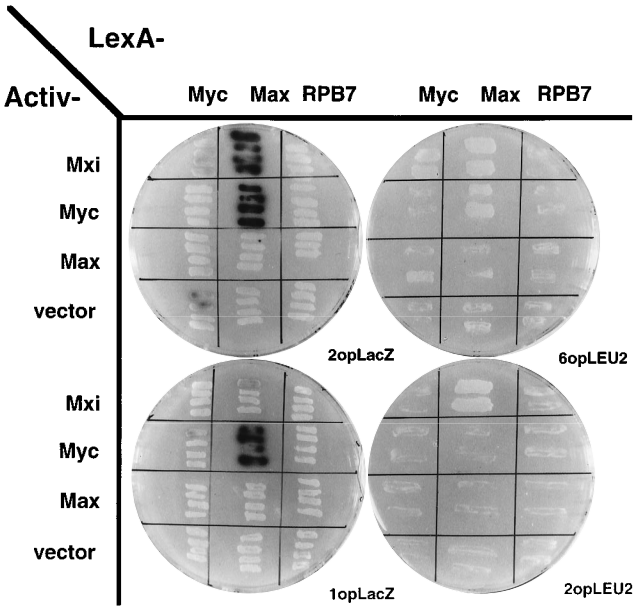

FIG. 4. Interactions between Myc, Max, and Mxi1 as assayed by four distinct reporters. Combinations of plasmids as indicated in the figure were cotransformed into yeast cells and selected on uracil-minus, histidine-minus, tryptophan-minus glucose yeast medium to select for the presence of all plasmids. Independent transformants were restreaked either onto uracil-minus, histidine-minus, tryptophan-minus, leucine-minus galactose medium to score activation of the *LEU2* reporters or onto uracil-minus, histidine-minus, tryptophan-minus galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to score activation of the *lacZ* reporters. The picture was taken 72 h after the colonies were streaked. All interactions were galactose specific (i.e., dependent on expression of the AD-fused protein).

obtained. In *2op-LEU2* cells, Max-Mxi1 appeared to interact more strongly than Myc-Max (+++ versus −). In *2op-lacZ* and *1op-lacZ*, Myc-Max dimers appeared to be preferred over Max-Mxi1 (172 versus 81 U and 61 versus 4 U, respectively, for the two reporters). These differences were quite substantial: whereas *1op-lacZ* suggested a 17-fold preference for LexA-Myc and AD-Max over LexA-Max and AD-Max-Mxi1, *8op-lacZ* suggested a 1.8-fold preference for LexA-Max and AD-Mxi1. In addition, the Myc-Max interaction was sensitive to the direction in which it was tested. While LexA-Max and AD-Myc interacted strongly and detectably with most reporters, the interaction between LexA-Myc and AD-Max was only marginably detectable in *6op-LEU2* yeast strains and was not detectable with any *lexAop-lacZ* reporters.

Finally, LexA-Myc interacted with Mxi1, although significantly less than did Max-Mxi1 and Myc-Max heterodimers. We are unaware of in vitro data demonstrating Myc-Mxi1 interactions, and our data in vivo indicate that this interaction has significantly lower affinity than the interaction between Max and Mxi1; we thus cannot speculate as to its physiological significance.

Interactions between lambda *c*I repressor and repressor mutants. Wild-type repressor (*c*I-WT) dimerizes with a K_d of 20 nM (55). The E233K mutant has a mild defect in dimerization ability, with a K_d for dimerization estimated to be ~50 to 100 nM. A152T and P158T mutants have a K_d for dimer formation estimated to be >1 μM, (a greater than 50-fold reduction relative to the K_d of the wild type). Since the carboxy-terminal region (papain fragment c) of *c*I dimerizes essentially as the full-length wild type but does not form higher-order structures (i.e., tetramers and octamers) (48) that might complicate analysis, we made a series of LexA and AD fusions to residues 132 to 236 (corresponding to this region) of *c*I-WT repressor and the E233K, A152T, and P158T mutants. We introduced plasmids that directed the synthesis of all possible combinations of these, together with controls, into the *6op-LEU2* yeast strain in conjunction with the reporter plasmid *1op-lacZ*, *2op-lacZ*, or *8op-lacZ*. We confirmed expression of comparable levels of correctly sized fusion proteins by Western blots (data not shown) and assayed them for β-galactosidase activity (Table 3).

We first examined the behavior of LexA- and AD-fused proteins with *1op-lacZ*, the least-sensitive reporter. The values for LacZ were 182 β-galactosidase units for *c*I-*c*I, 125 U for E233K-E233K, 1 U for A152T-A152T, and 120 U for P158T-P158T. The rank order of affinity of these homodimeric interactions approximately paralleled that determined in vitro, although that of the P158T homodimer appeared to be unexpectedly strong. It is worth noting, however, that LexA-P158T activated transcription in the absence of any AD-fused partner (see values for the vector control). On this reporter, interactions with K_ds of <50 to 100 nM were clearly detected, while the A152T-A152T interaction, with a K_d of >1 μM, was not detected.

We then examined the interaction of these protein pairs with a more-sensitive reporter, *2op-lacZ*. Here, the strongest single interaction was still that of *c*I with *c*I, followed by that of E233K with E233K, followed by that of A152T with A152T. With this reporter, the A152T-A152T interaction was significantly above the background level, suggesting that this reporter can detect interactions with K_ds of >1 μM. This ranking paralleled that obtained with *1op-lacZ*, but with a compression of affinities in the middle range and with clear detection of weak A152T interactions. On this reporter, LexA-P158T activated transcription of *2op-lacZ* extremely strongly in the absence of any interactive partner, limiting the ability to detect interactions when it was used as a bait.

Finally, we examined interaction of the *c*I domains by using the highest-sensitivity *8op-lacZ* reporter. Here, LexA-P158T activated transcription extremely strongly and LexA-A152T

TABLE 1. Myc, Max, and Mxi1 interaction-dependent activation of *lacZ* reporters containing different numbers of *lexA* operators

AD	β-Galactosidase activity (U)								
	LexA-Myc			LexA-Max			LexA-RPB7		
	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH18-34, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH18-34, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH18-34, *8op-lacZ*
Mxi1	2.2	3.4	26.0	3.7	81.0	511.9	1.3	<1	<1
Myc	3.1	<1	1.3	61.5	172.2	278.3	<1	1.1	1.4
Max	3.2	1.8	1.8	<1	1.2	<1	<1	<1	<1
Vector	1.5	2.0	6.6	1.9	1.5	11.2	1.3	1.4	3.1

TABLE 2. Myc, Max, and Mxi1 interaction-dependent activation of *LEU2* reporters containing different numbers of *lexA* operators

AD	Growth score 72–96 h after streaking[a]					
	LexA-Myc		LexA-Max		LexA-RPB7	
	EGY191, 2op-LEU2	EGY48, 6op-LEU2	EGY191, 2op-LEU2	EGY48, 6op-LEU2	EGY191, 2op-LEU2	EGY48, 6op-LEU2
Mxi1	−−−	++	+++	++++	−−−	−−−
Myc	−−−	−−−	−−−	++	−−−	−−−
Max	−−−	+/−	−−−	−−−	−−−	−−−
Vector	−−−	−−−	−−−	−−−	−−−	−−−

[a] Growth was scored on a scale in which ++++ represents rapid growth within 36 h and −−− represents no detectable growth (see Materials and Methods for details).

activated transcription appreciably. We also note that the negative control protein, LexA-RPB7, showed a moderate level of background activity with all *c*I variant AD fusions, particularly on the *8op-lacZ* reporter. Thus, with this reporter, interactions with K_ds of >1 μM are detected, above very high background levels of activity.

We also examined heterooligomeric interactions between *c*I and the *c*I mutants. On the *1op-lacZ* reporter, heterodimers between LexA-*c*I and AD-E233K and the reciprocal combination LexA-E233K and AD-*c*I interacted with apparent affinity comparable to that of the E233K-E233K interactions (76 and 151 U, respectively). The interactions between LexA-*c*I and AD-A152T and the reciprocal interaction between LexA-A152T and AD-*c*I resulted in somewhat less induction of the *1op-lacZ* reporter (47 and 49 U, respectively). Still weaker were interactions between LexA-*c*I and AD-P158T and LexA-E233K and either AD-P158T or AD-A152T, ranging from 23 to 36 U. Of the lowest affinity, and essentially indistinguishable from the background level of activity, were interactions of the LexA-A152T fusion with AD-E233K, AD-P158T, and AD-A152T. Finally, examination of the LexA-P158T data suggests that this protein can heterodimerize moderately, with the greatest degree of heterodimerization occurring with *c*I and E233K and the least occurring with A152T, although because of the high background level of transcription, this is not directly comparable with the other data.

On the *2op-lacZ* reporter, the strongest heteromeric interaction was that between LexA-*c*I and E233K, followed by those between LexA-*c*I and A152T and P158T, LexA-E233K and AD-P158T or AD-A152T, and LexA-A152T and AD-*c*I. Finally, the LexA-A152T fusion in conjunction with AD-E233K, AD-P158T, or AD-A152T yielded values significantly above background. LexA-P158T activated transcription of *2op-lacZ* extremely strongly in the absence of any interactive partner, limiting its usefulness as a probe. This rank order paralleled that obtained with *1op-lacZ*, but with a compression of differences in the middle range and clear detection of the weaker A152T interactions. In contrast, with the *8op-lacZ* reporter, there were numerous deviations in rank order of affinity of interactions and general compression of differences between different protein pairs.

Taken together, these results indicated that the *1op-lacZ* reporter was able to detect and discriminate between interactions with affinities predicted to range from 20 nM to close to 1 μM, with interactions with affinities of less than 1 μM not detected. The *2op-lacZ* reporter was comparable but allowed more resolution of differences between weaker interactions and detection of interactions in the range of 1 μM. Finally, the *8op-lacZ* reporter allowed clear detection of interactions in the 1 μM range but had high background levels of activity for many of the baits and showed compression of differences between strong and moderate interactions.

We then tested combinations of LexA-*c*I and AD-*c*I proteins with the *LEU2* reporters. In contrast to the results with the Myc-Max-Mxi1 proteins, in which the data from the *LEU2* reporters approximately paralleled those from the *lacZ* reporters, when used with the *c*I proteins, the *LEU2* reporters did not detect interactions. In *2op-LEU2*, the presence of LexA-*c*I, -E233K, or -A152T alone or in conjunction with any AD-fused partner did not permit growth on leucine-minus medium (not shown). In *6op-LEU2*, the presence of LexA-*c*I, -E233K, or -A152T alone resulted in moderate growth of yeast cells on leucine-minus medium that was unaffected by the presence of an AD-fused partner protein. In both *2op-LEU2* and *6op-LEU2*, expression of the LexA-P158T fusion alone or in conjunction with any AD-fused protein resulted in strong activation of the reporter, making it impossible to score any activation resulting from interaction.

DISCUSSION

These experiments have allowed us to draw a number of conclusions about the use of two-hybrid/interaction trap methods to study protein interaction. First, for a number of proteins known to dimerize with in vitro K_ds ranging from 20 μM to 2 nM, it is possible to discriminate high-, intermediate-, and low-affinity interactions on the basis of the relative ability to activate transcription of a reporter. Second, such rankings of interaction strength are facilitated by the use of multiple reporter systems. Third, the measured relative strengths of interactions varied in a manner dependent on which reporter construct was used. Fourth, in scoring related groups of fusion proteins, individual reporters demonstrated thresholds corresponding to the minimum affinity of interaction required to score as positive. Different groups of fusion proteins possessed different thresholds. In general, some reporters only recognized moderate-to-high-affinity interactions (K_d for dimerization, <1 μM), while others were also responsive to weak interactions (K_d for dimerization, >1 μM). Fifth, in no case did differences in activation of a single reporter gene correspond linearly to differences in dimerization affinity reported in vitro. Sixth, in some cases, interaction was detected when one partner was the bait and the other carried an AD but were not observed in the other orientation (directionality). These results have significant implications for two-hybrid/interaction trap studies of protein interactions and for screens of libraries to look for new interacting partners. Finally, the *lexAop-LEU2* reporters we describe should be valuable for future studies of protein interaction.

Comparison of in vivo and in vitro measures of Myc-Max-Mxi1 heterodimerization. In agreement with earlier findings, our data indicate that Myc (15, 43) and Max (7, 38, 43) homodimers are disfavored, while both Myc-Max (8, 9) and Max-Mxi1 (60) can form heterodimers with high affinity. Our data suggest that the affinity of the Mxi1-Max dimerization is comparable to that of Myc-Max. This finding agrees with those of previous experiments in which the dimerization affinities of Myc-Max and Max-Mad were extrapolated from binding affinities to the CACGTG DNA consensus site in vitro (4). Furthermore, this ordering of affinities is compatible with those in recent experiments in which cotransfection of equimolar quantities of Mad or Mxi with Myc in a transformation assay resulted in a greater than 90% reduction in formation of foci (40). However our results emphasize the difficulty of attempting to quantitate differences in affinity from two-hybrid exper-

TABLE 3. *c*I and *c*I mutant interaction-dependent activation of *lacZ* reporters containing different numbers of *lexA* operators[a]

AD	β-Galactosidase activity (U)											
	LexA–*c*I-WT			LexA-E233K			LexA-A152T			LexA-P158T[b]			LexA-RPB7		
	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH1834, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH1834, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH1834, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH1834, *8op-lacZ*	1840, *1op-lacZ*	JK103, *2op-lacZ*	SH1834, *8op-lacZ*
*c*I-WT	182	588	434	151	167	460	50	112	462	187	766	1,726	3	14	30
E233K	76	351	364	125	301	442	6	69	355	130	1,127	1,824	2	8	21
A152T	47	139	188	29	90	45	1	88	843	76	620	1,295	2	8	39
P158T	36	112	144	23	94	214	7	77	236	120	683	848	6	19	85
Vector	2	5	10	3	5	9	2	11	49	27	494	959	2	3	3

[a] Results reflect calibration of λ and dimerization-deficient mutants.
[b] These values are anomalous and are discussed in Results.

iments alone. For example, depending on the *lacZ* reporter used, the LexA-Max–AD-Myc interaction could be assumed to be 18-fold stronger or 2-fold weaker than the LexA-Max–AD-Mxi1 interaction, while the *LEU2* reporters both identify the LexA-Max–AD-Mxi1 interaction as stronger. The point on which all reporters concurred was in repeatedly identifying these two sets of interactions over other presumably nonphysiological interactions. For this group of proteins, our data indicate that strong interactions (K_ds of 1 to 10 nM) are detected strongly by the interaction trap, while weak interactions (K_ds of >1 μM), thought to be nonbiological for this group of proteins, are not. Other considerations affecting the interpretation of these interactions, and in particular the directional nature of the Myc-Max interaction, are discussed further below.

Comparison of in vivo and in vitro measures of *c*I protein and mutant homo- and heterodimerization. Bacteriophage λ repressor is an important model system for the study of DNA-protein and protein-protein interactions, and a wealth of data bearing on both processes exists. E233K (originally isolated as ind^s-1), P158T, and A152T all bind operators with lower affinity than *c*I and are more sensitive to *recA*-dependent cleavage, which targets only monomers (14). Previous estimations of dimerization affinity have suggested that the defect in P158T is most severe (K_d, >1 μM), that in A152T is intermediate (K_d, ~1 μM), and that in E233K is least severe (K_d, ~50 to 100 nM) (14, 26, 33). Our data generally agree with this ranking (Table 3 [results with LexA-*c*I]). However, the A152T dimer data obtained with the three reporters illustrate the difficulty of using transcription to draw simple conclusions about relative interaction affinity. Depending on which reporter is used, A152T-A152T affinity is 200-fold less, 7-fold less, or 2-fold greater than that of *c*I-*c*I homodimers. In this case, the data from the least-sensitive *1op-lacZ* reporter most closely parallel the in vitro data. Notably, the *LEU2* reporters proved completely unable to detect interactions for this set of proteins. While the reason for this failure remains unclear, a number of differences between the reporters are discussed below.

Interactions between different heterodimeric pairs of *c*I mutants have not been previously quantitated. However, it is reasonable to assume that these affinities are likely to fall between a K_d of 20 nM (wild-type *c*I homodimerization and >1 μM (P158T mutant homodimerization). Our data are consistent with this idea, and several different levels of affinity can be discerned. Of the highest affinity are heterodimers between *c*I and E233K. The next highest affinities are those between *c*I and A152T, followed by those between E233K and A152T. Moreover, despite the fact that the LexA-P158T protein activates transcription (for reasons that remain opaque), our data indicate that it interacts with other *c*I proteins with similar affinity to or perhaps slightly lower affinity than A152T. All of these rankings are in accord with predictions based on the homodimerization constants and support the idea that the interaction trap can meaningfully order the affinity of protein variants (29, 31).

Considerations affecting affinity measurements. At the simplest level, the amount of interaction between two protein components in a two-hybrid/interaction trap method is a function of the affinity of the AD-tagged protein for the DNA-bound bait. Our results have for the most part supported the idea that when other variables are held constant, affinity is the predominant determinant of the magnitude of the transcription we measure. However, our results also show that the apparent interaction affinities are affected by other considerations that we do not fully understand.

The most striking departure of our results from those predicted from in vitro affinity is that one of the interactions—that

between Myc and Max—was directional: a LexA-Max bait interacted strongly with AD-Myc, but LexA-Myc interacted only marginally with AD-Max. All fusion proteins were expressed, so the mechanism underlying directionality is unclear, but this phenomenon has been observed for numerous protein pairs in two-hybrid systems, including a number of Cdk and cyclin-related proteins (22, 23); such directionality might be explained by positing that oligomerization of some baits obscures residues on these proteins needed to interact with the AD-tagged protein. In a similar vein, it has also been noted that some proteins such as Rel (35) or Gfi-1 (25), which have important functional domains at their amino termini, possess markedly different phenotypes in transcription-based assays, depending on whether LexA is fused to the amino terminus or the carboxy terminus or is absent (30, 35).

An additional difference between the results expected from in vitro data and those obtained in this study lay in the dependence of the rank order of interactions on the reporter used. These fell into two classes. First, the reporter gene used was sometimes significant. For the *c*I proteins used in these experiments, the *lacZ* reporters produced rankings comparable to those biochemically determined, while the *LEU2* reporters did not yield utilizable data. This is at variance with some cases in which the *LEU2* reporters, particularly the highly sensitive *6op-LEU2*, can detect biologically relevant interactions which the *lacZ* reporters cannot (16, 27). Second, the number of *lexA* operators present on a reporter was significant. We have previously shown that the number of operators upstream of *lacZ* can affect estimation of the strength of LexA-activator proteins (28). In this study we found similar bias occurring: in particular, we observed compression of differences in activation for strongly interacting pairs when reporters with large numbers of *lexA* operators were used.

Several factors might contribute to directionality or promoter bias. First, the *LEU2* and *lacZ* reporter series are quite different. They contain different promoter sequences, which might affect the number of ADs brought to the DNA, and the geometry with which the interacting activation domain is presented to the transcription apparatus; they are present in different numbers of copies intracellularly (1 for the chromosomal *LEU2* reporters, ~20 for the *lacZ* reporters); and, in these experiments, the *lacZ* reporters were carried on plasmids, while the *LEU2* reporters are integrated into the chromosome. Such differences between reporter systems are not unique to the interaction trap or other two-hybrid systems; in bacteria, the choice of reporter gene has been shown to have an impact on measurement of transcriptional activation (24). Second, each LexA fusion and AD fusion is a unique chimeric protein. We have previously shown that different fused moieties can affect the interaction of LexA with operator sequences (28). For any given protein, it is possible that either LexA, the AD, or oligomerization dependent on the fused moiety may obscure residues necessary for interaction or interfere with the conformation of the protein in such a way as to render it unable to interact. Either the LexA-fused or AD-fused protein may associate with extraneous yeast proteins, and these associations could bias all of the considerations presented above. Given these issues, our data suggest that prudent use of a two-hybrid system to study protein interactions would involve an initial assay of a low-sensitivity *lacZ* reporter, a high-sensitivity *lacZ* reporter, and at least one *LEU2* reporter and should test interactions in both orientations.

Finally, particularly in the case of the *c*I repressor mutants studied, we note that the potential dimeric interactions occurring are more complex than the heterodimerizations between the LexA-fused component and the AD-fused component with which this discussion primarily deals. LexA binds its operator sequences as a dimer (10) and possesses dimerization functions in its carboxy-terminal domain. The *c*I proteins were chosen as a test set because they also possessed an intrinsic ability to homodimerize. Thus, we would expect that yeast cells also express some AD-*c*I/AD-*c*I homodimers, some LexA-*c*I/LexA-*c*I homodimers in which dimerization is mediated in part by the *c*I domain, and, inevitably, LexA-*c*I/LexA-*c*I homodimers in which dimerization is mediated by the LexA carboxy terminus, in addition to the LexA-*c*I/AD-*c*I heterodimers whose interaction we are assaying in the final transcription phenotype induced. Because we are able to assay the LexA-P1/AD-P2 interaction, it is clear that the capacity to homodimerize does not preclude the use of a two-hybrid approach. Rather, it is perhaps impressive that the system does as well as our data indicate in ranking heterodimeric interactions of different affinities in the background of possible homodimeric noise.

Implications for library screening. Perhaps the most common current use of two-hybrid–interaction trap technologies is to select new interacting partners from interaction cDNA libraries. Our work here has a number of implications for that application.

First, our results support the idea that through judicious choice of reporters, it should be possible to isolate interacting proteins that are physiologically significant to the chosen bait, whether this describes interactions occurring with affinities of 20 nM or those occurring with affinities of 1 μM.

Second, to date, baits that activate transcription have been unsuitable for use in interactor hunts because of the background of growth they induce in *6op-LEU2* reporter strains. Because of their decreased sensitivity, the *4op-LEU2* and *2op-LEU2* reporter strains are suitable for hunts with some such baits (39, 42, 46).

Third, given the fact that most current two-hybrid/interaction trap library screens depend on dual-phenotype strategies, one intriguing implication of our data is that the pool of positive clones selected may be biased by which of the two reporters is used first. Proteins that appear to interact strongly on the basis of the LEU2 phenotype but which activate even a sensitive *lacZ* reporter only weakly may still be valid (16, 23). Since the reporters used in GAL4-based two-hybrid experiments are different from those used here, it is not unreasonable to imagine that some interactions might be detected differently in these systems (13, 17), such that nonidentical sets of protein interactors are obtained. In addition, the directionality we and others have observed suggests an explanation for interactor hunts that fail to identify known protein partners.

Fourth, our pilot experiments demonstrated that increased activation strength at the *LEU2* reporters resulted in an increase in the proportion of cells in a population able to form colonies on leucine-minus medium rather than a general variance in the growth rate of all cells in a population. This variation in plating efficiency implies both that activation of the *LEU2* reporters varies over a substantial range from cell to cell and that there must exist a threshold of *LEU2* transcription that is necessary for a cell to form a colony. Such a threshold effect has been previously described for transcription of higher eukaryotic genes (21) and may in fact describe the situation of the *lacZ* reporters as well; however, *lacZ* activity is assayed as the average of a population. Heterogeneity of expression may affect the interpretation of the significance of cDNAs isolated from colonies in interactor hunts that arose from a nutritional selection, since colonies are derived from single founder cells (26, 27). In conventional library screening—a labelled probe or an antibody against a filter—the number of hybridizing organisms reflects the frequency with which the clone appears in the

library (3). In an auxotrophy screen with a LexA-*X* bait, if one cDNA, *Y*, is isolated 50 times and a second cDNA, *Z*, is isolated 5 times, this may reflect the abundance of *Y* and *Z*, if the two have equivalent affinity for *X*, but it is equally likely to reflect the fact that *Y* has a higher affinity for *X* than *Z* does.

In conclusion, this study highlights the utility of the interaction trap in identifying and analyzing interactions between proteins. The fact that affinity measurements by two-hybrid/interaction trap systems are subject to variables such as those we have described is not surprising and does not vitiate the fact that these systems represent a powerful tool for rapidly establishing the preliminary strength of interactions, which should aid in the daunting task of assessing the roles of ever-increasing numbers of genes.

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