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Methods

A Genetic Strategy to Eliminate Self-Activator Baits Prior to High-Throughput Yeast Two-Hybrid Screens

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Large-scale sequencing projects have predicted high numbers of gene products for which no functional information is yet available. Hence, large-scale projects, such as gene knockouts, gene expression profiles, and protein-interaction mapping, are currently under way to initiate the understanding of the function of these gene products. The high-throughput strategies that are currently being developed to generate protein-interaction maps include automated versions of the yeast two-hybrid system. These strategies rely on the large-scale construction of DNA-binding domain/protein-of-interest hybrid constructs (DB-X baits). An inherent problem of large-scale two-hybrid systems is that a high percentage of cloned sequences encode polypeptides that, when fused to DB, can activate transcription in the absence of any two-hybrid-interacting partner protein. Here, we describe and validate a genetic strategy that efficiently eliminates such self-activator baits prior to screening procedures. The strategy is based on a negative-growth selection and is compatible with high-throughput settings.

Complete genome sequences are available for several model organisms [*Escherichia coli* (Blattner et al. 1997), *Saccharomyces cerevisiae* (Goffeau et al. 1997), and *Caenorhabditis elegans* (*C. elegans* sequencing consortium 1998)] and pathogens (e.g., see Stephens et al. 1998). These genome sequences have revealed the need for large-scale projects to convert genomic information into functional information (Guyer and Collins 1995; Lander 1996; Fields 1997). For example, in >30 years, conventional biology has assigned a function to ~500 *C. elegans* genes of the 19,099 predicted ORFs (e.g., see Walhout et al. 1998). Thus, >97% of the *C. elegans* genome remains to be characterized functionally.

Several laboratories have initiated the development of genome-wide functional analysis projects to help address the function of genes identified by large-scale sequencing efforts. Such projects, collectively referred to as functional genomics, are expected to predict gene functions and lead to hypotheses at a drastically increased rate. Functional genomics projects include genome-wide expression analysis (Schena et al. 1995), large-scale gene knockouts (e.g., see Smith et al. 1996), and protein-interaction maps (Evangelista et al. 1997). Protein-interaction maps can be envisioned as publicly available databases containing large numbers of pairs of potentially interacting proteins (Walhout et al. 1998). Their usefulness stems from the fact that many biological processes rely heavily on protein-protein interactions, from the formation of large enzymatic protein complexes (e.g., see Koleske and Young

1994) to the regulatory roles of signal-transduction pathways (e.g., see Choi et al. 1994). So far, most strategies proposed to generate comprehensive protein-interaction maps have relied on high-throughput versions of the yeast two-hybrid system (Bartel et al. 1996; Fromont-Racine et al. 1997; Flores et al. 1999; A.J.M. Walhout, R. Sordella, X. Lu, J. Hartley, G. Temple, M. Brasch, N. Thierry-Mieg, and M. Vidal, in prep.).

The yeast two-hybrid system is a powerful genetic method to identify potential protein-protein interactions. This system circumvents tedious and time-consuming biochemical methods and is thus amenable to automated high-throughput settings. The two-hybrid system makes use of the observation that transcription factors are generally composed of two separable domains, a sequence-specific DNA-binding domain (DB) and a transactivation domain (AD). When a protein X is fused to DB (DB-X) and a protein Y to AD (AD-Y), an interaction between X and Y can be detected by the reconstitution of a functional transcription factor (DB-X/AD-Y) (Fields and Song 1989). In the most common application, X is a single protein, usually referred to as the bait. To identify potential interaction partners of X, the DB-X bait is screened against a complex genomic or cDNA AD-Y library.

There is a major limitation inherent to the two-hybrid system. DB-X fusions that can activate transcription independently of an interaction with an AD-Y protein (self activators) cannot be used in conventional forward two-hybrid screens. Self activators include proteins that act as transcriptional activators in their respective organisms and maintain this ability in yeast (e.g., see Du et al. 1996). They also include proteins that normally act in other processes but ex-

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hibit transcriptional activity when tethered to a promoter in yeast cells (e.g., see Hu et al. 1997). In high-throughput settings, large numbers of baits need to be generated and screened against AD-Y libraries. This can be accomplished by use of either one of two different strategies. In one strategy, referred to here as the random strategy, a complex library of randomly generated DB-X genomic or cDNA clones is screened against an AD-Y library by yeast-mating procedures (Bartel et al. 1996; Vidal 1997). This approach has been used previously to generate an interaction map for the 55 proteins encoded by the bacteriophage T7 genome (Bartel et al. 1996). A more directed strategy has also been proposed in which large numbers of ORFs are PCR amplified and individually cloned into DB plasmids (Hudson et al. 1997; Walkout et al. 1998). Here again, mating can be used to screen a set of predefined DB-X fusions against an AD-Y library (Finley and Brent 1994; Fromont-Racine et al. 1997).

In addition to the two classes of self activators described above, both high-throughput strategies are complicated by the occurrence of spurious DB-X self activators originating from cloning artifacts. These can include genomic or cDNA fragments cloned out-of-frame in the random strategy. For example, ~1% of randomly generated DNA sequences from *E. coli* encode polypeptides that can activate transcription in yeast when fused to DB (Ma and Ptashne 1987). In the directed strategy, spurious self activators originating from PCR-induced mutations can also be a problem if the bait-encoding plasmids are not systematically verified by sequencing (see below).

The percentage of spurious self activators in particular pools of DB-X baits can be as high as 5% ($\sim 5 \times 10^{-2}$) (see below), whereas the frequency of potential interactors for a single bait is usually $<50/10^6$ ($\sim 5 \times 10^{-5}$) in non-normalized cDNA libraries. Because the frequency of self activators can be higher than the frequency of genuine interactors by several orders of magnitude, it is crucial for the quality of protein-interaction maps to develop a convenient method to efficiently eliminate self activators.

Here, we describe such a method, which is applicable to both random and directed strategies prior to high-throughput two-hybrid screens.

RESULTS AND DISCUSSION

A Genetic Selection to Eliminate Self-Activator Baits

Most versions of the yeast two-hybrid system rely on genetic selections on the basis of the transcriptional activation of reporter genes required for growth (e.g., see Vidal and Legrain 1999). When activated by DB-X/AD-Y interactions, these reporter genes allow cell growth under specific conditions (positive selections). Under such conditions, cells expressing DB-X self acti-

vators are equally able to grow and form colonies in the absence of an AD-Y interaction partner. Such a positive growth phenotype can be used to identify and manually remove self activators from a pool of yeast colonies (Bartel et al. 1996). However, this requires time-consuming manipulations and is therefore not applicable to large-scale projects.

Recently, we have developed a reverse two-hybrid system, in which the transcriptional activation of a counterselectable reporter gene is disadvantageous for growth (Vidal et al. 1996a). When activated by DB-X/AD-Y interactions, this reporter gene prevents growth under particular conditions (negative selection). The reverse two-hybrid system is most commonly used for the selection of interaction-defective alleles or dissociator peptides or compounds (Vidal et al. 1996b; Vidal 1997; Vidal and Endoh 1999). These reagents can then be used to validate potential interactions obtained by forward two-hybrid selections. Here, we make use of such a negative selection to eliminate self activators from large pools of yeast cells. Because it is based on a genetic selection, this approach does not require time-consuming manipulations and is thus applicable to large-scale projects.

Our version of the two-hybrid system utilizes two selectable markers that can be transcriptionally activated by two-hybrid interactions (Vidal 1997). The marker used for positive selection is the *GAL1::HIS3* reporter gene (Durfee et al. 1993). The *HIS3* gene encodes an enzyme involved in histidine biosynthesis that can be specifically inactivated by the competitive inhibitor 3-aminotriazole (3AT). Hence, it is possible to titrate the level of 3AT so that growth of yeast cells that express only basal levels of *HIS3* would be prevented. Under these conditions, even moderate increases of *HIS3* expression confer a growth advantage.

The counter-selectable reporter gene used for negative selection is *SPAL10::URA3* (Vidal et al. 1996a). The *URA3* gene encodes an enzyme required for uracil biosynthesis and can be used for positive growth selections on medium lacking uracil. However, this enzyme can also convert the nontoxic 5-fluorouracil (5FOA) into a toxic byproduct (Boeke et al. 1984). Thus, on medium containing both uracil and 5FOA, expression of *URA3* is toxic.

To examine whether negative selection with 5FOA can be used for the elimination of self activators, we asked the following two questions. Can self activators be efficiently eliminated with 5FOA? And, how successful are subsequent two-hybrid selections? We addressed these questions in the context of both the random and the directed strategies described above.

General Scheme

The rationale behind our experiments is analogous to any preclearing step of experiments in which back-

ground activity needs to be reduced or removed prior to performing the relevant functional test(s). In our scheme (Fig. 1), haploid yeast cells of one mating type are first transformed with a pool of DB-X bait plasmids. As described above, these might include a subpopulation of molecules encoding natural or spurious self activators. Transformants are selected on plates lacking leucine (Sc-Leu) because, in our system, the DB plasmid contains the *LEU2* selectable marker. The Leu⁺ transformants are then transferred to the 5FOA negative-selection plates by replica plating. As described above, self-activator-expressing colonies should not be able to grow under these conditions. After a two-day incubation, the 5FOA resistant colonies are transferred to fresh Sc-Leu plates for recovery. Subsequently, the non-self-activator colonies can be mated with cells of the opposite mating type containing an AD-Y library in a *TRP1* vector. Diploids are selected on plates lacking both leucine and tryptophan (Sc-Leu-Trp) and can be submitted to the 3AT-positive selection for the identification of DB-X/AD-Y interactions.

To control for the efficiency of the preclearing step, the haploid colonies expressing DB-X baits are also transferred to 3AT-positive selection plates, both prior (plate 3AT#1) and subsequent (plate 3AT#2) to the preclearing step (Fig. 1). When colonies are still able to grow on the 3AT#2 plate, additional preclearing steps can be performed (Fig. 1, dotted arrow).

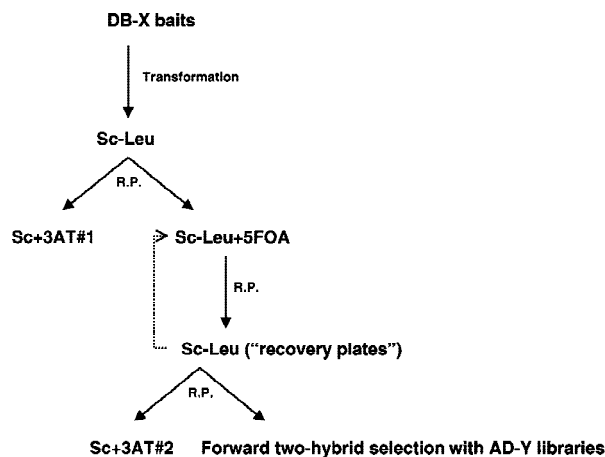


Figure 1 Flowchart of the self-activator preclearing step followed by forward two-hybrid screens. DB-X bait constructs are transformed and selected on synthetic plates lacking leucine (Sc-Leu). These plates are replica-plated (R.P.) onto two plates. One plate contains 20 mM 3AT (Sc+3AT#1) and is used to monitor the number of self activators prior to the preclearing step; the other contains 5FOA (0.2%) (Sc-Leu+5FOA) and is used to eliminate self activators. The Sc-Leu+5FOA plates are then replica plated onto Sc-Leu plates for recovery. The recovery plates are transferred to a second Sc+3AT plate (20 mM) (Sc+3AT#2) to assess the efficiency of self-activator elimination. If necessary, the whole procedure can be repeated. After efficient elimination of self activators, the plates can be used for forward two-hybrid screens. Representatives of the three plates indicated in red in this scheme are shown in the next two figures.

Elimination of Self Activators from a DB-X cDNA Library

To test the scheme described above in the context of the random strategy, we used two non-normalized mouse 13.5-day embryonic cDNA libraries, one fused to DB (DB-cDNA) and the other to AD (AD-cDNA). Different versions of both libraries have already been used to identify a large number of valid interactors (Chevray and Nathans 1992; Hu et al. 1997). We reasoned that the high complexity of these mammalian cDNA libraries would provide an ideal test to evaluate the efficiency of the self-activator preclearing step. In addition, we reasoned that non-normalized libraries provide a good test for the efficiency of recovering protein-protein interactions in subsequent forward two-hybrid selections because several cDNAs that are known to be highly represented in embryonic libraries encode proteins that physically interact (Soares et al. 1994; see below).

The DB-cDNA library was transformed into *MAT α* cells to obtain ~50,000 transformants. These were replica plated onto both 3AT and 5FOA plates. As seen in Figure 2A, ~2500 colonies were resistant to 3AT and are thus presumed to express self-activator fusions. This number corresponds to ~5% of the mouse cDNAs fused to DB and is consistent with the data reported previously (Ma and Ptashne 1987).

To estimate the efficiency of the preclearing step, the recovery plates were also replica plated onto 3AT plates (Figs. 1 and 2A). The preclearing of the DB-cDNA library was compared with that of a reference that consists of a well-described self-activator construct [DB-DCC (Hu et al. 1997)] diluted 100-fold in a solution of empty DB plasmid. Preclearing conditions have already been optimized to allow efficient elimination of this DB-DCC control self activator (Vidal 1997). In the experiment shown here, a few hundred colonies had survived the first negative selection and thus the 5FOA-negative selection was repeated (not shown). As seen in Figure 2A (right, bottom), no 3AT resistant colonies could be detected after two rounds of 5FOA selections, indicating that self activators had been eliminated efficiently.

Simultaneously, the AD-cDNA library was transformed into *MAT α* cells to obtain ~50,000 transformants. No growing colonies were obtained when the corresponding Trp⁺ transformants were replica plated onto 3AT plates, indicating that the frequency of AD-Y self activators is much lower than that of DB-X self activators (<1/50,000) (data not shown). This result was expected, because in the absence of a sequence-specific DNA-binding domain, such as DB, the fusion protein is not tethered to the reporter genes and therefore does not activate transcription.

To demonstrate that forward two-hybrid selections can be applied after self-activator preclearing,

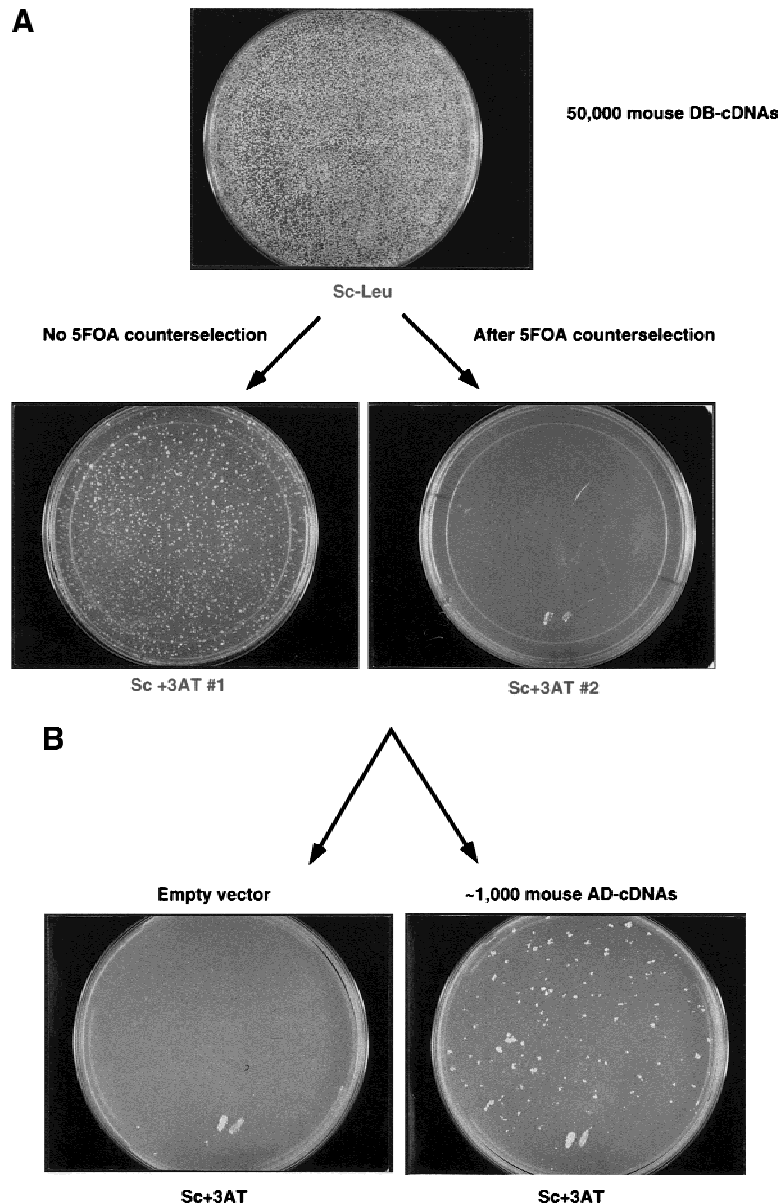


Figure 2 Preclearing of self-activator baits from random DB-cDNA libraries. (A) The mouse 13.5-day embryonic DB-cDNA library cloned in a *LEU2* plasmid was transformed into MaV103 cells. The ~50,000 resulting *Leu*⁺ transformants were treated as described in the legend to Fig. 1. The plates shown here correspond to the Sc-Leu, Sc+3AT#1, and Sc+3AT#2 plates indicated in red in Fig. 1. The Sc-3AT plate at *right* contains two control patches. (B) A mouse 13.5-day embryonic AD-cDNA library cloned in a *TRP1* plasmid was transformed into MaV203 cells and the resulting transformants were mated with the precleared DB-cDNA transformants shown in A. The plate at *right* shows the resulting 3AT-growing colonies indicative of potential two-hybrid interactions. As a negative control, MaV203 was transformed with a *TRP1* empty vector and mated with the precleared DB-cDNA transformants (*left*). Both plates contain two control patches at the bottom.

~1000 *MAT* α AD-Y transformants were pooled, plated, and subsequently replica plated onto ~50,000 precleared *MAT* α DB-X colonies to allow mating (Fig. 2A). The resulting diploids were selected on Sc-Leu-Trp. As a negative control, *MAT* α cells transformed with the

empty AD plasmid alone were used. As expected from the efficiency of preclearing (Fig. 2A), diploids originating from this mating experiment produced only a few 3AT-resistant colonies (Fig. 2B, left). In contrast, when AD-Y and precleared DB-X cells were mated, a substantial number (~200) of 3AT-resistant colonies was observed (Fig. 2B, right). These colonies also exhibited uracil prototrophy and β -galactosidase activity (Vidal 1997), indicating that they represent genuine two-hybrid interactors (data not shown).

Next, the identity of the two potential interacting proteins from a dozen 3AT-resistant colonies was determined by sequencing DB-X and AD-Y PCR products amplified directly from yeast colonies (Wang et al. 1996). The majority of interactor pairs consisted of α - and β -globin, either in the DB- α -globin/AD- β -globin or in the DB- β -globin/AD- α -globin orientation. To evaluate the relative presence of the α -globin/ β -globin interaction in a larger number of 3AT-growing colonies, a yeast-colony hybridization experiment with α -globin or β -globin radiolabeled DNA probes was performed. Approximately 95% of the 3AT-growing colonies represented the globin-interaction pair, which can be explained by the relative abundance of α - and β -globin cDNAs in both the DB-X and the AD-Y libraries (~3%) (data not shown).

The recovery of the α -globin/ β -globin interaction in this experiment demonstrates that relevant interactions can be identified after the elimination of self-activator baits by use of 5FOA. In addition, it indicates that the application of random two-hybrid screens in high-throughput settings requires the use of normalized libraries (Soares et al. 1994).

Elimination of Mutant Self-Activators Generated by PCR

Although potentially powerful, the random strategy for generating protein-interaction maps described above is limited by the fact that it is not directly connected to any particular biological question.

Therefore, several laboratories have recently embarked in a more directed approach involving large numbers of defined DB-X bait constructs rather than random DB-X cDNA libraries. Usually, the baits are selected on the basis of their known or suspected involvement in a biological process of interest.

For example, a protein-interaction map of yeast proteins involved in RNA splicing has been described recently (Fromont-Racine et al. 1997). In the directed strategy, sets of predefined ORFs must be inserted one-by-one into the DB plasmid prior to performing two-hybrid screens.

Conventional cloning methods can be time consuming and are thus not compatible with high-throughput settings. Therefore, they are usually replaced by the PCR-Gap repair transformation method (e.g., see Vidal 1997). This method allows the rapid cloning of PCR products into vectors such as the DB plasmid by direct recombination in yeast. ORFs are amplified by PCR with primers containing, at their 3' end, the gene-specific sequences needed for amplification, and at their 5' end, a tail sequence identical to the polylinker sequence of the DB plasmid. The tail sequences are used to promote recombination of the PCR products into the DB plasmids *in vivo*. The Gap-repair method can easily be automated, because each step of the protocol can be performed in 96-well plates.

Our laboratory is involved in a *C. elegans* protein-interaction mapping project with the directed strategy (Walhout et al. 1998; A.J.M. Walhout, R. Sordella, X. Lu, J. Hartley, G. Temple, M. Brasch, N. Thierry-Mieg, and M. Vidal, in prep). In general, large numbers of worm ORFs are amplified by PCR and inserted into the DB plasmid by Gap repair. In the course of these experiments, we noticed the occurrence of an unexpected class of self activators that could greatly complicate the outcome of the directed strategy, even when applied to other genomes. For several baits tested in our laboratory so far, a small, but significant, fraction of the transformants (~5%) exhibited growth on 3-AT plates. According to our preliminary observations, the number of baits belonging to this class might be as high as 15%. These self activators are derived from PCR-induced mutations as shown below and thus we refer to them as spurious self-activators. They represent false-positive clones and thus need to be removed prior to applying two-hybrid selections.

The *C. elegans lin-5* gene (M. Lorson, H.R. Horvitz, and S. van den Heuvel, in prep.) is used here to exemplify the preclearing procedure for such spurious self-activator baits (Fig. 3). The *lin-5* ORF was amplified by PCR with a CMV-*lin-5* plasmid as template DNA and primers that contain both *lin-5*- and vector-specific sequences (see Vidal 1997). The *lin-5* PCR fragment was transformed into MaV103 yeast cells along with linearized DB vector and several hundreds of transformants were selected on Sc-Leu plates. Approximately 5% of the Leu⁺ transformants could confer a 3AT-resistant

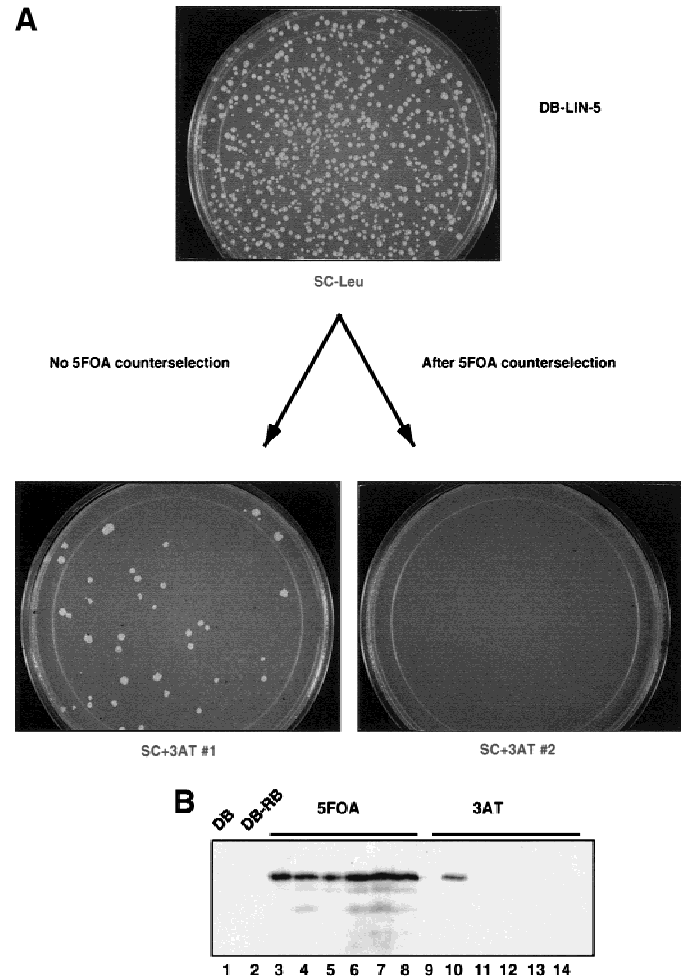


Figure 3 Preclearing of PCR-generated self-activator baits. (A) A PCR-generated *lin-5* sequence was transformed by Gap repair into MaV103 cells. The Leu⁺ transformants were treated as described in the legend to Fig. 1. The plates shown here correspond to the Sc-Leu, Sc+3AT#1, and Sc+3AT#2 plates indicated in red in Fig. 1. (B) Western-blot analysis of extracts from yeast cells expressing self-activator (lanes 9–14) and non-self-activator (lanes 3–8) DB-LIN-5 baits. A mix of three anti-LIN-5 monoclonal antibodies that recognize epitopes in the LIN-5 carboxyl terminus were used. (Lanes 1, 2) Extracts from negative control cells expressing DB alone and DB-RB, respectively (RB corresponds to the pocket domain of the human retinoblastoma protein); (lanes 3, 9) extracts from pools of colonies; (lanes 4–8, 10–14) extracts from individual colonies.

phenotype, suggesting that a subpopulation of DB-LIN-5 behaves as spurious self-activator baits (Fig. 3A, left, bottom).

The genetic selections used here to preclear the self-activator baits were identical to those described above except that only one round of 5FOA selection was sufficient to eliminate all self-activator-expressing colonies (not shown). The preclearing ratio compared with our control was satisfactory, and this is exemplified by the fact that no 3AT-growing colonies could be observed after the recovery on Sc-Leu plates (Fig. 3A, right, bottom).

To address the efficiency of two-hybrid selections subsequent to the preclearing step, a screen was performed with the precleared DB-LIN-5 bait and the data obtained were compared with those from ~100 screens performed without any preclearing step. The LIN-5 interactors identified are still being characterized and will be described elsewhere (M. Lorson, A.J.M. Walhout, M. Vidal, and S. van den Heuvel, in prep.). However, for the purpose of this experiment, the number of different potential interactors found at least twice can be a good indicator of the quality of a screen. Five potential interactors were found for LIN-5, which is in good agreement with the average number of interactors found in the 100 screens mentioned above (M. Vidal, in prep.). Thus preclearing can be applied to DB-X baits that give rise to spurious self activators without significantly affecting the read out of two-hybrid screens.

Finally, we were intrigued by the nature of the molecular events that could give rise to spurious self-activator baits after PCR amplification. As recognized previously, PCR amplification can be very mutagenic (Mulhard et al. 1992). At least three classes of mutations can be envisioned, including missense mutations that lead to single amino acid changes and nonsense or frame-shift mutations that result in a truncated protein. Interestingly, it has been observed that truncated versions of several proteins exhibit transcriptional activation, whereas the corresponding full length does not. Different folding properties of truncated and full-length proteins are sometimes argued to explain this phenomenon. Thus, we decided to test whether the PCR-induced DB-LIN-5-mutated self activators correspond to truncated versions of the fusion protein. An immunoblot was performed on yeast extracts prepared from both 5FOA-resistant (non-self activators) and 3AT-resistant (self activators) colonies, with a mixture of three anti-LIN-5 monoclonal antibodies that recognize the LIN-5 carboxyl terminus (M. Lorson, H. R. Horvitz, and S. van den Heuvel, in prep.) (Fig. 3B). As expected, five of five 5FOA-resistant colonies expressed full-length DB-LIN-5 protein. However, four of five 3AT-resistant clones did not. The absence of a LIN-5-specific signal in most extracts derived from 3AT-resistant clones is consistent with the idea that PCR-induced mutations can create stop codons or frame-shift mutations leading to truncated products. It is possible that spurious activation domains might be accessible to the yeast transcriptional machinery only in the context of truncated rather than full-length proteins.

Conclusions

Self-activator baits cannot be used in conventional forward two-hybrid selections because they do not depend on any interaction to elevate the expression of the reporter genes. The percentage of such sequences

in random cDNA (or genomic) DB-X libraries or in PCR-generated DB-X baits can be higher than the frequency of genuine interactors by several orders of magnitude. Thus, an efficient method is needed to eliminate them prior to performing a forward two-hybrid selection for potential AD-Y interactors.

We have shown here that negative-selection-growth phenotypes can be used to eliminate such self activators from pools of DB-X baits, either present naturally in cDNA libraries, or generated by PCR.

We note that relevant proteins that exhibit self-activation properties in this assay, and thus would be eliminated as DB-X bait fusions, will be tolerated when expressed as AD-Y fusions. Thus, they will not necessarily be precluded from large-scale interaction screens. In theory however, one might imagine situations in which both interacting proteins exhibit self-activation properties. For these cases, an alternative configuration of the two-hybrid system that makes use of Pol III transcriptional regulation could be used (Marsolier et al. 1997).

We envision that the feasibility and quality of protein-interaction mapping projects will be greatly improved with the genetic selection against self activators described here.

METHODS

Yeast Manipulations and DB- and AD-cDNA Libraries

The yeast strains MaV103 and MaV203 have been described previously (Vidal et al. 1996a). All information concerning yeast manipulations such as medium, incubation time, and replica plating can be found in Vidal (1997). The 13.5 mouse embryonic AD-cDNA library used in Figure 2 was described in Hu et al. (1997). The 13.5 mouse embryonic DB-cDNA library used in Figure 2 is a kind gift from M. Heymans and R. Bernards (N.K.I., Amsterdam, The Netherlands). It was generated by cloning into pPC97 (Vidal 1997) the inserts from a pre-existing AD-cDNA library described in Chevray and Nathans (1992) (details will be published later). The worm AD-cDNA library used in Figure 3 will be described in detail elsewhere. PCR reactions directly from yeast cells were performed essentially as described (Wang et al. 1996). Automated sequencing was performed as described in the Perkin Elmer ABI protocol.

PCR/Gap Repair

PCR reactions (50 μ l) were assembled on ice and contained 60 mM Tris-SO₄ at pH 9.1, 18 mM (NH₄)₂SO₄, 2 mM MgSO₄, 50 μ g of BSA, 200 μ M dNTP, 0.5 μ M of each primer, 100 ng of CMV-*lin-5* (M. Lorson and S. van den Heuvel, in prep.), and 0.25 units of Elongase *Taq* polymerase (Life Technologies Inc.). The *lin-5* forward primer was 5'-**TAGTAACAAAGGTCAAAGACAGGTTGACTGTATCGTCGAGG**AGCGTGAGACATCAGTTGTG-3' (the second codon of the *lin-5* ORF is underlined). The *lin-5* reverse primer was 5'-**GCCGTTACTTACTTAGAGCTCGACGCTTACTTACTTAGCT**ACTGCTTTTGCTCGAAAA-3' (the *lin-5* stop-codon is underlined). Sequences identical to the pPC97 polylinker sequences and required for Gap repair are indicated in bold. The *lin-5* sequence was amplified in a reac-

tion consisting of 4 cycles with an annealing temperature of 56°C and 11 cycles with a temperature of 66°C. For Gap repair, MaV103 cells were transformed with 25 ng of pPC97 digested with *Sall* and *Bgl*III, along with 5 µl of PCR product. Colonies were grown on plates SC-Leu to select for pPC97-derived plasmids. Controls included no DNA, linearized vector alone, PCR product alone, and circular pPC97. An increase in the number of colonies of at least 20-fold was observed between linearized vector alone and vector + PCR product.

Western Blot Analysis

The Western blot analysis was performed as described (Vidal 1997). The anti-LIN-5 monoclonal antibodies were raised against a carboxy-terminal domain of the protein (M. Lorson, H. R. Horvitz, and S. van den Heuvel, in prep.).

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