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Identifying and Modifying Protein–DNA and Protein–Protein Interactions Using a Bacterial Two-Hybrid Selection System

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Abstract A bacterial two-hybrid system based on transcriptional activation in *E. coli* has recently been described. A variety of different protein–DNA and protein–protein interactions from bacteria, yeast, and humans have been studied using this bacterial-based system. The method, because it is based in bacteria, offers significant advantages relative to its yeast counterpart including the ability to analyze complex libraries $> 10^8$ in size, ease of use, and speed. The ability to easily and rapidly process very large libraries make this system a powerful tool for identifying, modifying, or optimizing protein–DNA and protein–protein interactions. J. Cell. Biochem. Suppl. 37: 53–57, 2001. © 2002 Wiley-Liss, Inc.

Key words: two-hybrid selections; protein-DNA; protein-protein; interaction trap

In the post-genomic era, the need for general methods to study protein–protein and protein–DNA interactions that mediate biological processes continues to grow. Many different selection-based technologies (e.g., the yeast two-hybrid system and phage display) have been developed in recent years, and these methods have provided powerful tools for identifying and studying the details of important macromolecular interactions [for recent reviews, see Sidhu, 2000; Toby and Golemis, 2001].

Recently, a bacterial-based genetic selection method similar to the yeast two-hybrid system has been described [Joung et al., 2000; Shaywitz et al., 2000]. In this bacterial two-hybrid system, as in its yeast counterpart, a selectable reporter gene is expressed only if a desired protein–protein or protein–DNA interaction occurs within a test cell. A significant advantage of this *E. coli*-based method relative to the yeast-based system is the ability to assess very large libraries of proteins ($> 10^8$ in size for the bacterial system compared with 10^6–10^7 for the yeast method). This capability results from the extremely high transformation efficiency possible only in bacteria and is particularly useful for the analysis of complex randomized and cDNA libraries. In addition, the method provides an alternative for studying proteins that can not be used in the yeast two-hybrid system (e.g., due to toxicity or poor expression). Finally, the system offers advantages of speed and ease of use since all manipulations occur within rapidly growing *E. coli*.

This review will provide an introduction to the technology that underlies the bacterial two-hybrid. It will also discuss successful applications of the system for identifying both protein–DNA and protein–protein interactions. Protocols and descriptions of strains and plasmids for using the bacterial two-hybrid system have recently been published for those interested in additional practical detail [Serebriiskii and Joung, 2001].

BACTERIAL TWO-HYBRID SYSTEM BASED ON TRANSCRIPTIONAL ACTIVATION IN *E. Coli*

The bacterial two-hybrid system described in this review is based on the original observation by Hochschild et al. that an arbitrary pair of interacting proteins (X and Y in Fig. 1) can mediate transcriptional activation of a weak synthetic promoter in *E. coli* [Dove et al., 1997; Dove and Hochschild, 1998]. In order for activation to

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occur, one protein (X) must be tethered near the promoter via fusion to a DNA binding domain (X-DBD hybrid) while the other protein (Y) must be fused to a subunit of the *E. coli* RNA polymerase (Y-RNAP hybrid). In this configuration, the interaction between X and Y permits the X-DBD hybrid (bound to its specific DNA binding site positioned near the promoter) to recruit an RNAP complex harboring the Y-RNAP hybrid to the weak promoter. The resulting transcriptional activation can be measured as an increase in the expression of a selectable reporter gene appropriately positioned downstream.

A number of experiments have demonstrated that various protein–protein and protein–DNA interactions can mediate transcriptional activation in this engineered system. Different interacting proteins X and Y from bacteria [Dove et al., 1997, Dove and Hochschild, 2001], yeast [Dove and Hochschild, 1998; Joung et al., 2000], and mammals [Shaywitz et al., 2000; Joung and Hochschild, unpublished communications] can all function to increase expression of the reporter gene. Various monomeric and dimeric DBDs from prokaryotes [Dove et al., 1997] and eukaryotes [Joung et al., 2000; Joung and Pabo, unpublished communications] have been successfully used to tether Protein X to the DNA. Finally, the magnitude of activation observed appears to correlate with the relative affinity between proteins X and Y [Dove et al., 1997] and with the affinity between the DBD and its specific DNA site (Joung et al. unpublished communications).

These findings suggested that this system could be used as a method to identify candidates

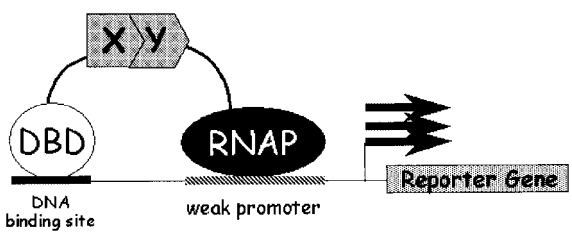

Fig. 1. Arbitrary protein–protein and protein–DNA interactions can mediate transcriptional activation in *E. coli*. X and Y are proteins that interact with each other. DBD is a DNA-binding domain that binds specifically to its DNA binding site positioned near a weak synthetic promoter. Because of the interaction between X and Y, a protein X/DBD hybrid protein can bind to its DNA site and recruit an RNA polymerase (RNAP) complex harboring protein Y to the promoter. The resulting transcriptional activation leads to increased expression of a reporter gene positioned downstream of the promoter.

(from libraries) that interact with a specific DNA or protein target. That is (in the parlance of the yeast two-hybrid system), the bacterial system could be used to identify specific "preys" that interact with a DNA or protein "bait" of interest. For example, to identify proteins that bind to a specific target DNA site (bait), one would position the bait sequence near a weak promoter that controls expression of a selectable reporter gene (Fig. 2a). In addition, one would create a library of potential DBD preys fused to a constant protein X which in turn interacts with a fixed protein Y fused to a RNAP subunit (Fig. 2a). To identify prey DBDs that interact with the target site bait, one would introduce the library into the reporter cells and then identify candidates that confer the ability to grow under selective conditions. As shown in Figure 2b, cells that receive a DBD-X hybrid that does not bind well to the target DNA site will fail to grow under selective conditions. By contrast, cells that receive a DBD-X hybrid that can bind to the target DNA site will activate expression of the selectable reporter and will, therefore, form colonies on selective medium (Fig. 2b). The DNA encoding the desired DBD-X can then be isolated from the cells and sequenced to determine its identity. A similar strategy, in which protein X would serve as the bait and protein Y would be the prey (and in which the DBD and its target site would remain constant), could be used to find preys from a library that interact with a specific protein target.

Using these selection strategies, one should be able to identify novel or altered interaction partners for a wide variety of targets by selecting from randomized, mutagenized, or cDNA libraries. To date, the bacterial two-hybrid system has been used successfully to isolate desired candidates from randomized and mutagenized libraries (reviewed below). Although selections have yet to be performed using cDNA libraries, the method is sufficiently robust, so that extending it to this type of experiment is not expected to be problematic.

SUCCESSFUL APPLICATIONS OF THE BACTERIAL TWO-HYBRID SYSTEM

Selection of Variant Zinc Finger Proteins With Altered DNA Binding Specificities

To test whether the bacterial two-hybrid could be used as a selection method to study protein–DNA interactions, Joung et al. [2000]

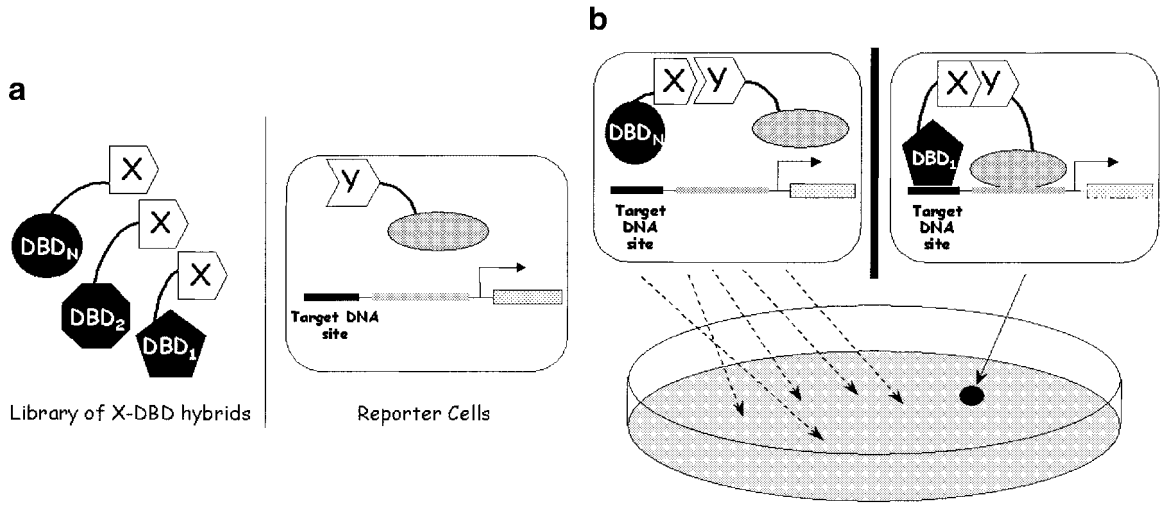

Fig. 2. **a**: Components of a bacterial two-hybrid selection experiment. The left side of the figure depicts a library of hybrid proteins, each bearing a different DBD fused to a constant protein X. The right side of the figure depicts a reporter cell harboring the target DNA site (bait) positioned near a weak promoter (grey line), which in turn controls expression of a selectable reporter gene (stippled box). A fixed protein Y is fused to a subunit of RNA polymerase (RNAP) resulting in its display on the RNAP complex (grey oval). **b**: Performing a bacterial two-hybrid selection experiment. Reporter cells receiving a protein X-DBD hybrid protein that does not bind to the target DNA site (left) do not activate expression of the selectable gene, and thus do not form colonies when plated on selective medium. However, in reporter cells receiving a protein X-DBD hybrid protein that binds to the target DNA site (right), transcriptional activation of the selectable reporter gene occurs and the cell can form a colony (black circle).

used the system to identify Cys_2His_2 zinc finger domains (hereafter referred to as simply zinc fingers) with altered DNA binding specificities from a large randomized library. Zinc fingers bind to specific DNA sequences utilizing an alpha helix (the "recognition helix") that inserts into the major groove of DNA [for recent review, see Wolfe et al., 2000]. The mammalian Zif268 protein contains three tandem zinc fingers that bind to a specific 10 bp DNA sequence. Each zinc finger in Zif268 recognizes and binds to a 3–4 bp "subsite" within the larger DNA recognition site [reviewed in Wolfe et al., 2000]. Using the method of phage display, many laboratories had previously identified Zif268 variants in which the DNA binding specificity of one of the three fingers had been altered [reviewed in Pabo et al., 2001]. To accomplish this, investigators created libraries of Zif268 variants in which as many as six amino acid positions within and adjacent to the recognition helix of one of the three fingers were randomized. Phage display was then used successfully to identify candidates from the library that could bind to modified Zif268 DNA binding sites in which one of the three subsites was altered.

Joung et al. [2000] performed experiments to determine whether the same zinc finger variants found by phage display could also be identified using the bacterial two-hybrid selection. One major motivation for applying the bacterial two-hybrid to this particular problem was to determine whether desired zinc finger variants could be selected in a single step (compared with phage display which requires multiple rounds of enrichment and amplification). To test this possibility, Joung et al. [2000] constructed a library of Zif268 variants in which the six recognition residues in one of the fingers were randomized. The theoretical size of this library is nearly 2×10^8 and the actual library constructed by Joung et al. [2000] contained 10^9 independent transformants. The bacterial two-hybrid system was then used to identify zinc fingers from this library that could bind to each of three different target DNA subsites. The three specific DNA subsites tested (AAA, TGT, and TCA) had been used in previous zinc finger phage display experiments [Greisman and Pabo, 1997; Wolfe et al., 1999], and thus the results from the two approaches could be easily compared. A summary of the results obtained by the two methods is shown in Figure 3.

The results summarized in Figure 3 demonstrate that the bacterial two-hybrid system is at least as effective as phage display in identifying desired candidates from the randomized zinc finger library. Figure 3 shows the consensus

	Consensus sequence(s) from Phage Display	Consensus sequence(s) from Bacterial 2-Hybrid
DNA site	-1 1 2 3 5 6	-1 1 2 3 5 6
AAA	Q + _ N _ _ N S G A _ N	Q + _ N _ _ N S G A θ N
TGT	H _ H H _ _	H _ H H _ _ Q + W L N R W _ S S _ V E R W R _ R
TCA	T _ T N _ S	N S G S W K

Fig. 3. Recognition helix sequences of variant zinc fingers selected by phage display and by the bacterial two-hybrid system. Shown are consensus sequences of positions within the recognition helix derived from variant fingers selected by phage display [Greisman and Pabo, 1997; Wolfe et al., 1999] and the bacterial two-hybrid system [Joung et al., 2000]. The numbering given indicates the position of residues relative to the start of the zinc finger recognition helix (+1). + indicates a positively charged residue, θ an aromatic residue, and _ no discernible consensus. DNA target sites shown are displayed 5′–3′.

sequence(s) that can be derived from each selection experiment for the six positions randomized in the variant zinc finger. For the AAA subsite selection, the two consensus sequences derived from bacterial two-hybrid selection experiments match those obtained using phage display. For the TGT subsite selection, four distinct consensus sequences were obtained using the bacterial two-hybrid system; one of these four matches the single consensus previously obtained using phage display which again validates the bacterial two-hybrid approach. The remaining three TGT consensus sequences all contain an aromatic residue (Phe, Tyr, Trp) that was not permitted by the randomization strategy used to make the phage display library and thus could not have been isolated in those experiments. For the TCA subsite selection, the consensus from the bacterial two-hybrid results was completely different from the weak consensus obtained from the phage display experiments (see Fig. 3). In fact, a number of different observations [see Joung et al., 2000] suggest that the fingers isolated using the bacterial two-hybrid system might actually bind more tightly (and perhaps more specifically) to the target TCA subsite than the fingers isolated by phage display. Taken together, the results of these three selections demonstrate that the bacterial two-hybrid works at least as well as phage display for identifying variant zinc fingers with altered DNA binding specificities, and in certain cases, may actually isolate fingers with greater affinity and specificity.

Perhaps more impressively, the results of the zinc finger selections also show that the bacterial two-hybrid system can be successfully used to isolate rare desired candidates from very large libraries $> 10^8$ in size. The ability to successfully interrogate such large libraries depends critically not only upon the higher transformation efficiency currently possible only with bacteria, but also upon a low background breakthrough frequency of the selectable reporter gene used. For example, if one used a selectable marker with a breakthrough frequency of 10^{-6}, there would be 100 false positives attributable just to breakthrough when looking at 10^8 different candidates; if the desired candidates in the library were relatively rare, then one could quickly spend an enormous amount of time examining false positives. The selectable HIS3 reporter gene, as used by Joung et al. [2000] has an extremely low frequency of breakthrough ($< 3 \times 10^{-8}$), and hence, is ideal for assessing libraries $> 10^8$ in size. In addition, varying the concentration of 3-aminotriazole (a competitive inhibitor of the HIS3 enzyme) in the growth medium provides a simple way to vary the stringency of the selection [Joung et al., 2000].

Selection of a Mutant CREB-Binding Protein With Enhanced Affinity for CREB

To test whether the bacterial two-hybrid could be used as a selection method for studying protein–protein interactions, Hochschild et al. used the system to study the phosphorylation-dependent interaction between the mammalian transcription factors cyclic AMP response element binding protein (CREB) and the CREB-binding protein (CBP) [Shaywitz et al., 2000]. They demonstrated that fragments of CREB and CBP did not associate with each other when tested in the bacterial two-hybrid system, but that an interaction did occur when a mammalian protein kinase (known to phosphorylate CREB) was also co-expressed in the bacterial cell. Thus, just as they do in their native mammalian cell context, these proteins specifically associate in the bacterial two-hybrid system in a phosphorylation-dependent manner.

These investigators then used the bacterial two-hybrid system to select a mutant of CBP with enhanced affinity for CREB. A mutagenized library of CBP fragments was constructed and 5×10^6 transformants were assessed for their ability to activate a selectable reporter gene (the beta-lactamase gene conferring

resistance to the antibiotic ampicillin). A mutant CBP was identified that exhibits enhanced affinity for CREB as judged in the bacterial two-hybrid system as well as within mammalian cells in culture [Shaywitz et al., 2000].

SUMMARY AND FUTURE PROSPECTS

The results reviewed in this article demonstrate the power of the bacterial two-hybrid system for studying protein–DNA and protein–protein interactions from organisms ranging from bacteria to mammals. The system has been successfully used to identify candidates of interest from randomized and mutagenized libraries and should prove useful for analyzing cDNA libraries as well. Perhaps the most significant advantage of this bacterial system (relative to its yeast counterpart) is its ability to easily and rapidly assess very large libraries $> 10^8$ in size. 10^8 candidates can be quickly assessed on a single regular size Petri dish using standard molecular biology techniques and requiring no specialized equipment [for additional details, see Serebriiskii and Joung, 2001]. The ability to handle very large numbers of candidates is critical for experiments that seek to interrogate complex randomized or cDNA libraries. One potential limitation of the system is the inability of *E. coli* to carry out post-translational modifications necessary for the proper functioning of certain eukaryotic proteins. However, even this limitation might be overcome by constructing specialized *E. coli* strains expressing proteins that perform post-translational modifications (as for the CREB–CBP interaction described above). Given its power for handling large libraries and its ease of use, the bacterial two-hybrid system provides a powerful tool for identifying and studying important protein–DNA and protein–protein interactions.

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