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Design of a single plasmid-based modified yeast one-hybrid system for investigation of in vivo protein-protein and protein-DNA interactions

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We have developed a modified yeast one-hybrid system (MY1H) useful for in vivo investigation of protein-protein and protein-DNA interactions. Our single-plasmid expression system is capable of differential protein expression levels; in addition to a GAL4 activation domain (AD) fusion protein, a second protein can be coexpressed at either comparable or higher transcriptional levels from expression vectors pCETT or pCETF, respectively. This second protein can play a structural, modifying, or inhibitory role that restores or blocks reporter gene expression. Our MY1H was validated by use of the well-characterized DNA-binding protein p53 and its inhibitory partners, large T antigen (LTAg) and 53BP2. By coexpressing LTAg or 53BP2 at comparable or higher levels than the GAL4AD-p53 fusion in the MY1H, we show that DNA binding of p53 decreases by different, measurable extents dependent on the expression level of inhibitory partner. As with the traditional Y1H, our system could also be used to investigate proteins that provide coactivational or bridging functions and to identify novel protein- or DNA-binding partners through library screening. Our MY1H provides a system for investigation of simultaneous protein-protein and protein-DNA interactions, and thus is a useful addition to current methods for in vivo investigation of such interactions.

INTRODUCTION

Gene expression is a sophisticated, finely tuned process that involves the regulated interactions of multiple proteins with promoter and enhancer elements. A variety of approaches are currently used in the study of these interactions, including phage display and yeast-based assays, as well as other biophysical and biochemical methods (1). The yeast one-hybrid system (Y1H), a variant of the yeast two-hybrid system (Y2H) (2), is a powerful and commonly used in vivo genetic assay for identification of protein-DNA interactions. The Y1H is useful for isolation of genes encoding proteins that bind to *cis*-acting regulatory elements and for further characterization of known protein-DNA interactions, whereas the Y2H allows detection of protein-protein interactions (2–4).

In many cases, protein-protein and protein-DNA interactions are intertwined in vivo: DNA-binding proteins are often modulated by the recruitment of accessory proteins that cannot bind DNA directly but rather serve to repress or coactivate transcription through the formation of transcriptional complexes (5,6). Most bZIP and bHLH families, such as Jun-Fos (7), Myc-Max (8), and the classic bacteriophage λ repressor and Cro proteins, belong to this class of transcription factors. A number of yeast genetic approaches have been reported for investigation of a protein in complex with a known partner, thereby forming

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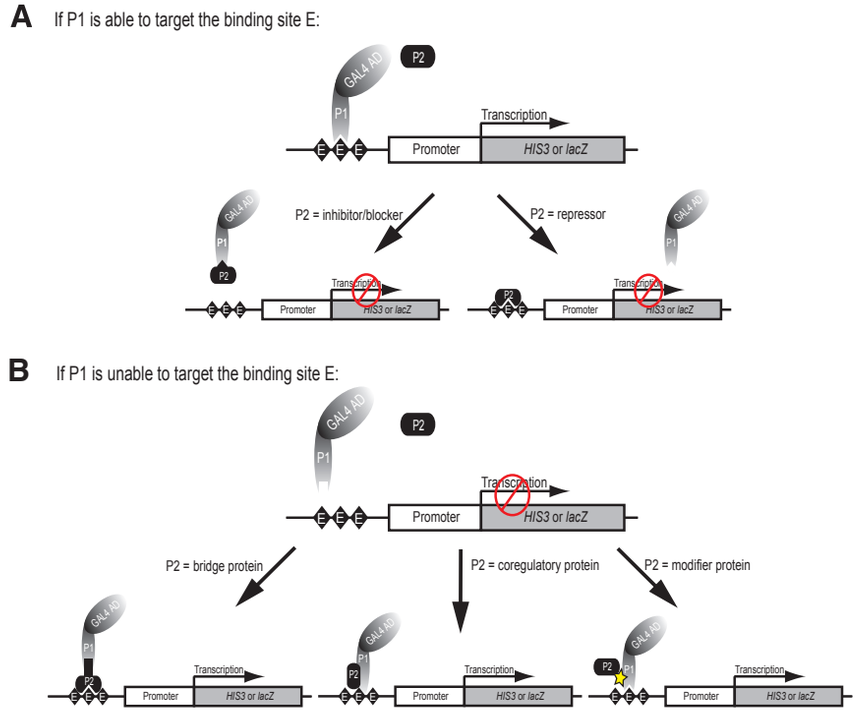

Figure 1. Five different types of interactions between proteins and DNA can be detected with the MY1H system. (A) If P1 is able to target the binding element E, the second protein P2 can be expressed either to block the DNA-binding region of P1 or directly bind to binding element E, thereby inhibiting reporter gene expression. (B) If P1 is unable to target the binding element E, P2 can be expressed as a bridging protein or coregulatory accessory protein, or it can modify the structure of P1 to enable DNA binding, thereby restoring reporter gene expression. As a coregulatory protein, P2 may function with or without making direct contact with P1.

a heterodimeric complex that binds DNA; these complexes may include one partner lacking intrinsic DNA-binding capability enabled by dimerization with an accessory protein (9–14). Traditionally these studies have used two separate plasmids for expression of the two different proteins.

We have developed a single plasmid-based modified Y1H system (MY1H) useful for examination of both protein-protein and protein-DNA interactions in vivo. In addition to an AD fusion protein, a second protein is coexpressed at either comparable or excess levels. The interaction of this second protein with the AD fusion, via cooperative oligomerization, structural modification, or inhibition, can restore or block reporter gene expression (Figure 1). We chose to validate our MY1H using the extensively studied interactions of DNA-binding protein p53 and its inhibitory partners, Simian Virus 40 (SV40) LTAg and 53BP2 (15–19). Both LTAg and 53BP2 inhibit wild-type p53 function through a protein-protein interaction at the DNA-binding domain of p53, thereby preventing p53 from binding to its consensus DNA target site (18–22). The well-characterized p53-LTAg and p53–53BP2 interactions—protein-protein interactions that modulate DNA-binding ability—provide an ideal system for validation of our MY1H.

Our MY1H combines the features of the Y1H and Y2H systems, and also extends their scopes such that simultaneous protein-protein and protein-DNA interactions can be investigated; hence, this MY1H is speculated to have broad utility (Figure 1) and may provide a widely applicable approach for investigation of various types of interactions, including heterodimer-DNA interactions or the effects of different protein modifiers on the DNA-binding capability of a transcription factor.

MATERIALS AND METHODS

Unless otherwise stated, reagents were purchased from BioShop Canada (Burlington, ON, Canada), enzymes were purchased from New England Biolabs (Pickering, ON, Canada), and oligonucleotides were synthesized by Operon Biotechnologies (Huntsville, AL, USA).

Bacterial and Yeast Strains

Escherichia coli DH5α (Stratagene, La Jolla, CA, USA) or dam-/dcm- C2925H (New England Biolabs) was used for standard cloning and for rescue of plasmids from yeast cells. *Saccharomyces cerevisiae* YM4271 [*MATa, ura3–52, his3–200, ade2–101, lys2–801, leu2–3, 112, trp1–901, tyr1–501, gal4-Δ512, gal80-Δ538, ade5::hisG*] was used for plasmid construction via homologous recombination and reporter strain construction. Two yeast reporter strains, YM4271[p53HIS] and YM4271 [p53BLUE], were created according to the Matchmaker One-hybrid System User Manual (Clontech, Palo Alto, CA, USA) for reporter assay analysis in the MY1H. These two strains contain three tandem copies of the consensus p53 binding site upstream of the *HIS3* and *lacZ* reporter genes, respectively.

Transformation, DNA Preparation, and Plasmid Rescue

Recombinant plasmids were transformed into *E. coli* by the standard TSS procedure (23). Plasmids were isolated from bacteria using the Wizard *Plus* SV Miniprep DNA Purification System (Promega, Madison, WI, USA). Yeast transformations were performed using either the standard lithium acetate method (Clontech; Yeast Protocols Handbook: www.clontech.com/images/pt/PT3024-1.pdf) or the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, USA). Transformants were selected by leucine prototrophy. Isolation of yeast

Table 1. Oligonucleotides Used in This Study

No.	Sequence
1	5′-ACTATCTATTCGATGATGAAGATACCCCACCAAACCCAAA-3′
2	5′-GGCGCTCGCCCTATAGTGAGTCGTATTAAAGATCTCTTTTTTTGGGTTTGGTGGGGTATC-3′
3	5′-CTCACTATAGGGCGAGCGCCGCCATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACC-3′
4	5′-GCGCGCACCTTGTCGACCGCGGCCTCCATGGCCATATGCAGGTCCTCCTCTGAGATCAGC-3′
5	5′-GCGGTCGACAAGGTGCGCGCTCTAGATGATCATGAATCGTAGATACTGAAAAACCCCGCA-3′
6	5′-ATGCACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCT-3′
7	5′-AGAAAGGTCGAATTGGGTACCGCCGCCAATAAAGAGATCTTTAAT-3′
8	5′-CTCGCCCTATAGTGAGTCGTATTAAAGATCTCTTTATTGGCGGCG-3′
9	5′-AAA**GACGTC**GCATGCAACTTCTTTTCTTT-3′
10	5′-ATT**GACGTC**AAGCTTGCATGCCGGTAGAGGT-3′
11	5′-AAA**GACGTC**CCTGCAGGTCGAGATCCGGGA-3′
12	5′-AAAA**GTCGAC**CCTGTCACCGAGACCCCTGG-3′
13	5′-ACGC**TCTAGA**TCAGTCTGAGTCAGGCCCCA-3′
14	5′-AAA**GAATTC**GGAACTGATGAATGGGAGCAG-3′
15	5′-AAA**GGATCC**TTATGTTTCAGGTTCAGGGGGAG-3′
16	5′-AAA**GAATTC**CCGCCTGAAATCACCGGGCAG-3′
17	5′-AAA**GGATCC**TCAGGCCAAGCTCCTTTGTCTT-3′

Restriction sites used for cloning are in bold.

plasmids was performed using the Zymoprep II Yeast Plasmid Miniprep kit (Zymo Research). PCR reactions were performed using Phusion high-fidelity DNA polymerase (New England Biolabs). PCR products and DNA fragments for cloning were purified using the QIAquick Spin kits or MinElute kits (Qiagen, Mississauga, ON, Canada).

Plasmid Construction

All new constructs were confirmed by dideoxynucleotide DNA sequencing on an ABI (Applied Biosystems, Foster City, CA, USA) 3730XL 96-capillary sequencer at the DNA Sequencing Facility in the Centre for Applied Genomics, Hospital for Sick Children (Toronto, ON, Canada).

pGAD424-MCS I and pGAD-424-MCS II. pGAD424-MCS I and pGAD424-MCS II were constructed by homologous recombination (24) in YM4271 to replace the original multiple cloning site (MCS) in pGAD424 (25) using the 6.6 kb *Eco*RI/*Pst*I pGAD424 fragment along with the CE4MCS fragment and 679 bp *Bst*Z17I/*Mlu*I pGADT7 (Clontech) fragment, respectively. The CE4MCS fragment was assembled by self-priming PCR (26) using oligonucleotides 1–6 (Table 1); the fragment contains a T7 promoter, a c-Myc epitope tag, and a multiple cloning site (MCS I) with recognition sequences for five restriction enzymes (*Sac*II, *Sal*I, *Bss*HII, *Xba*I, and *Bcl*I). Similarly, the *Bst*Z17I/*Mlu*I pGADT7 fragment contains a T7 promoter, a HA epitope tag, and a multiple cloning site (MCS II) with recognition sequences for six restriction enzymes (*Eco*RI, *Sma*I, *BamH*I, *Sac*I, *Xho*I, and *Pst*I).

pGAD424-MCS IIΔAD and pGADT7ΔAD. In pGAD424-MCS IIΔAD and pGADT7ΔAD, the GAL4AD was deleted while the open reading frame was maintained. To create these two recombinant plasmids, the FINALREC fragment was assembled by mutually primed synthesis (27) using oligonucleotides 7 and 8. The 5′ and 3′ ends of the FINALREC fragment contain 30 and 35 bp homology, respectively, to both *Bgl*II-linearized pGAD424-MCS II and *Bgl*II-linearized pGADT7. YM4271 was cotransformed with either *Bgl*II-linearized pGAD424-MCS II or *Bgl*II-linearized pGADT7 and the FINALREC fragment to give rise to pGAD424-MCS IIΔAD and pGADT7ΔAD, respectively.

pCETT and pCETF. The T2 fragment was amplified with oligonucleotides 9 and 10 from pGAD-424-MCS IIΔAD. The F2 fragment was amplified with oligonucleotides 10 and 11 from pGADT7ΔAD. The amplified fragments T2 and F2 were digested with *Aat*II, treated with alkaline phosphatase, and then ligated into the *Aat*II site of pGAD424-MCS I to generate pCETT and pCETF (Figure 2; see Supplementary Figure S1 in the Supplementary Material available online at www.BioTechniques.com), respectively.

pCETT/53 and pCETF/53. The sequence encoding amino acids 72–390 of the murine p53 gene was amplified from pGAD53m (Clontech)

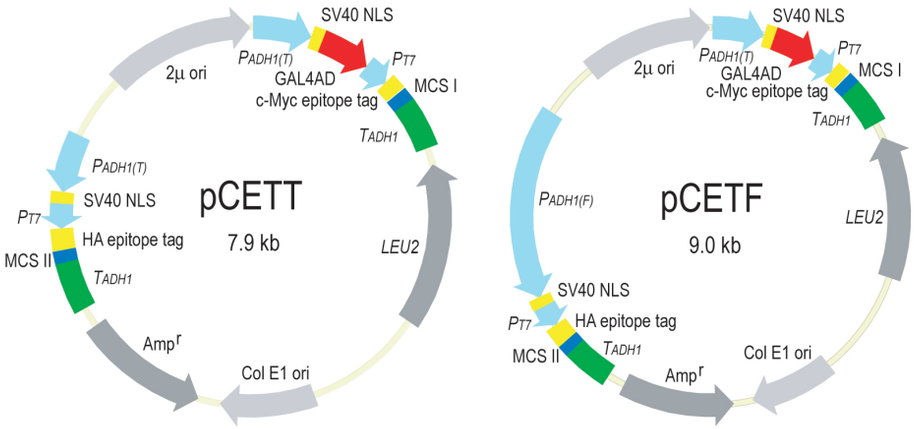

Figure 2. Plasmids pCETT and pCETF were constructed for coexpression of two proteins in a yeast model system. The two vectors have unique restriction sites located in both the MCS I and MCS II regions. MCS I is at the 3′-end of the open reading frame for the GAL4AD sequence allowing a fusion protein combining amino acids 768–881 of the GAL4AD and the cloned protein of interest to be expressed at low levels from a truncated constitutive *ADH1* promoter. The expression of genes inserted into MCS II is controlled by either the truncated *ADH1* promoter (pCETT) or full-length *ADH1* promoter (pCETF). Therefore, a second protein can be coexpressed at either low levels (pCETT) or high levels (pCETF). Both vectors also contain a T7 promoter at both MCS regions, a c-Myc epitope tag at MCS I, and an HA epitope tag at MCS II.

using oligonucleotides 12 and 13. This fragment was inserted between the *Sal*I and *Xba*I sites of pCETT and pCETF to construct pCETT/53 and pCETF/53, respectively.

pCETT/53/T and pCETF/53/T. The fragment encoding amino acids 87–708 of SV40 LTAg was amplified from pGADT7/T (Clontech) with oligonucleotides 14 and 15. This fragment was inserted into the *EcoR*I and *BamH*I sites of pCETT/53 and pCETF/53 to construct pCETT/53/T and pCETF/53/T, respectively.

pCETT/53/BP2 and pCETF/53/BP2. The cDNA plasmid pGBT9, which contains the human 53BP2 sequence, was kindly given to us by Kuniyoshi Iwabuchi (Kanazawa Medical University, Kahoku-gun, Ishikawa, Japan) (19). The sequence encoding amino acids 768–1005 of 53BP2 was amplified with oligonucleotides 16 and 17 and inserted between the *EcoR*I and *BamH*I sites of pCETT/53 and pCETF/53 to generate pCETT/53/BP2 and pCETF/53/BP2, respectively.

3-AT Titration Analysis

HIS3 gene expression was measured by growing transformed yeast cells on selective media lacking leucine, uracil, and histidine. Activity of the *HIS3* reporter was quantified as survival rates of yeast transformants on plates containing increasing amounts of 3-aminotriazole (3-AT), a competitive inhibitor of the His3 protein.

Transformed yeast cells were initially grown at 30°C with shaking in SD/-L media for 2 days or until OD_{600} >1.5 was reached, and then used to inoculate a fresh culture of SD/-L media. This secondary culture was grown overnight until OD_{600} 1.0–1.3 was reached. An aliquot of the secondary culture was resuspended in yeast peptone dextrose adenine (YPDA) to give a starting OD_{600} ~0.2. The YPDA culture was then grown for 3–5 h until OD_{600} 0.60–0.65 was reached. The culture was then diluted by a factor of 4000 and 100 µl of the diluent was plated on SD/-H/-L/-U plates containing increasing 3-AT concentrations ranging from 0 to 80 mM. Individual colonies were counted after 5 days growth at 30°C. The survival rate of a specific transformant was calculated as the number of colonies on the SD/-H/-L/-U plate containing a specific 3-AT concentration divided by the number of the colonies on the control SD/-L/-U plate. This assay was performed in triplicate and independently repeated at least three times in order to ensure reproducibility.

X-gal Colony-lift Filter Assay and ONPG Liquid Assay

The X-gal colony-lift filter assay and *ortho*-nitrophenyl-galactoside (ONPG) liquid assay were performed according to the protocols provided in the Yeast Protocols Handbook (www.clontech.com/images/pt/PT3024-1.pdf) with the following modifications: in the X-gal assay, the cells were subjected to two cycles of freeze-thaw in order to lyse the cells. In the ONPG assay, yeast cells were grown as described above for the 3-AT titration assay before harvesting for lysis. Results are presented as mean values ± SEM of 3–4 independent experiments, each performed in triplicate.

RESULTS

Design of Protein Expression Vectors

In order to examine protein-protein and protein-DNA interactions simultaneously in a single yeast genetic system, two GAL4AD fusion vectors, pCETT and pCETF, were constructed based on pGAD424, the protein expression plasmid provided in the Matchmaker One-Hybrid System. In both pCETT and pCETF, the gene encoding the AD fusion protein is inserted into MCS I where transcription is under the control of a truncated *ADH1* promoter, leading to low protein expression levels (Clontech; Yeast Protocols Handbook: www.clontech.com/images/pt/PT3024-1.pdf) (28). A second gene inserted into the second multiple cloning site, MCS II, can also be expressed from the same plasmid under the control of the truncated *ADH1* promoter identical to that in MCS I. In pCETF, MCS II is under the control of the full-length *ADH1* promoter, leading to higher transcription levels (Clontech; Yeast Protocols Handbook: www.clontech.com/images/pt/PT3024-1.pdf) (29).

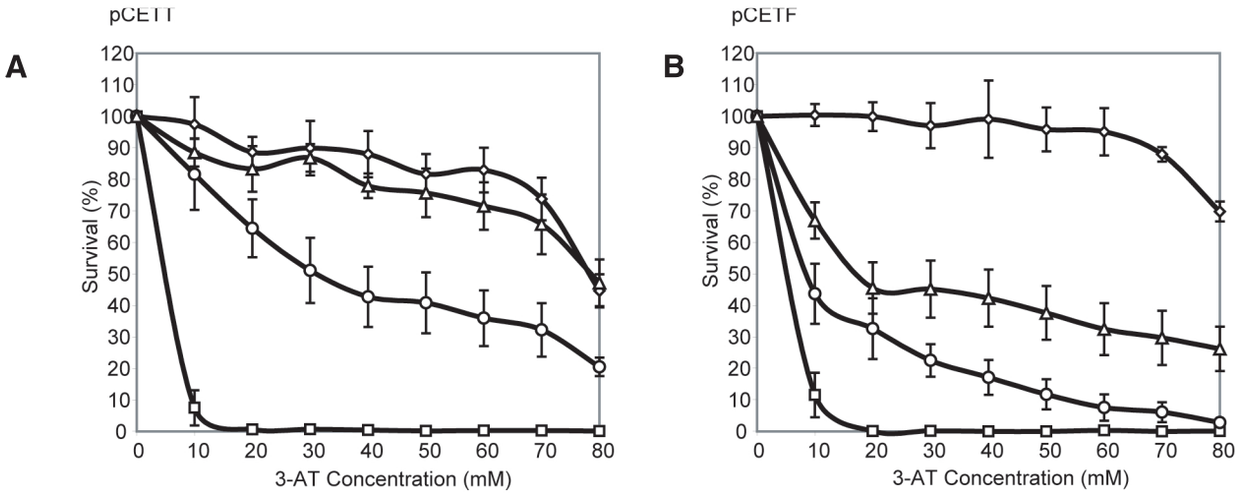

Figure 3. 3-AT titrations reveal that the survival rates of transformants decrease when the inhibitory proteins are expressed. YM4271[p53HIS] cells were transformed with (A) pCETT (□), pCETT/53 (◊), pCETT/53/T (○) or pCETT/53/BP2 (Δ) or (B) pCETF (□), pCETF/53 (◊), pCETF/53/T (○) or pCETF/53/BP2 (Δ). Key for labels as follows: pCETT and pCETF, negative controls, no protein expression; pCETT/53 and pCETF/53, positive controls, expression of GAL4AD-p53 from MCS I only; pCETT/53/T and pCETF/53/T, low-level expression of GAL4AD-p53 from MCS I, low- or high-level expression of LTAg from MCS II; pCETT/53/BP2 and pCETF/53/BP2, low-level expression of GAL4AD-p53 from MCS I, low- or high-level expression of 53BP2 from MCS II. Cells were grown to exponential phase in YPDA media and plated at equal densities on selective media containing increasing concentrations of 3-AT. Individual colonies were counted after 5 days growth at 30°C. The survival rate of a specific transformant was calculated as the number of colonies on the SD/-H/-L/-U plate containing a specific 3-AT concentration divided by the number of the colonies on the control SD/-L/-U plate. The assay was conducted in triplicate and independently repeated at least three times. Data are presented as average values ± SEM.

Our plasmid design provides the option of allowing the second protein to be expressed at a comparable level with the AD fusion protein (pCETT; truncated *ADH1* promoters in MCS I and II) or in excess (pCETF; truncated *ADH1* promoter in MCS I, full-length *ADH1* in MCS II). In addition, these plasmids were designed with different epitope tag-coding sequences upstream from each multiple cloning site and with a T7 promoter upstream of both multiple cloning sites to allow in vitro transcription and translation.

Coexpression of LTAg or 53BP2 Decreases the Transactivation Potential of GAL4AD-p53 in the MY1H

In our MY1H, we used three assays to measure the inhibitory strengths of LTAg and 53BP2 on binding of the p53 DNA consensus site by GAL4AD-p53, including titration on inhibitory 3-AT-containing media (*HIS3* assay) and two colorimetric *lacZ* reporter-based assays: the qualitative X-gal colony-lift filter assay and the quantitative ONPG liquid assay. All three assays showed that the ability of GAL4AD-p53 to activate transcription of reporter genes was adversely affected when coexpressed with LTAg or 53BP2 in the MY1H.

We titrated the yeast transformants on SD/-H/-L plates containing 0 to 80 mM 3-AT and plotted the survival rates of each transformant at increasing 3-AT concentrations (Figure 3). Survival rates in the presence of 3-AT correlate with transcriptional activity (30). Therefore, lower survival of transformants on 3-AT-containing plates indicates stronger inhibitory strengths of LTAg or 53BP2 on the ability of GAL4AD-p53 to bind the p53 consensus DNA site. As expected, the survival rates of yeast cells transformed with pCETT or pCETF decrease sharply as 3-AT concentration increases and reach zero at 20 mM 3-AT, while cells transformed with pCETT/p53 or pCETF/p53 only begin to show decreased survival at 3-AT concentrations over 70 mM. In contrast, when LTAg or 53BP2 is coexpressed with GAL4AD-p53, an immediate decrease in cell survival is observed at 10–20 mM 3-AT and continues to decline at higher 3-AT concentrations, consistent with the inhibitory role both LTAg and 53BP2 play in the DNA-binding ability of p53.

The sensitive X-gal colony-lift filter assay corroborates the 3-AT titration results in the *HIS3* assay (Figure 4). In the colony-lift assay, reporter gene activation is visualized by the blue chromophore released by the action of β-galactosidase encoded by the *lacZ* gene. Transformants expressing only GAL4AD-p53 become blue very quickly (within 10 min, Figure 4, B and F), and continue to increase in intensity, becoming vivid blue after 45 min. When LTAg or 53BP2 is coexpressed at low expression levels from pCETT (Figure 4, C and D), blue color only begins to appear after 15 min and is considerably less intense after 45 min, indicating discernible inhibition of reporter gene expression. Higher expression of either LTAg or 53BP2 from pCETF (Figure 4, G and H) inhibits *lacZ* transcription to a much greater extent, as only a faint blue color is achieved after 45 min.

The quantitative ONPG assay further corroborates the qualitative results obtained from both the 3-AT titration and the colony-lift assays. Expression of GAL4AD-p53 from either pCETT

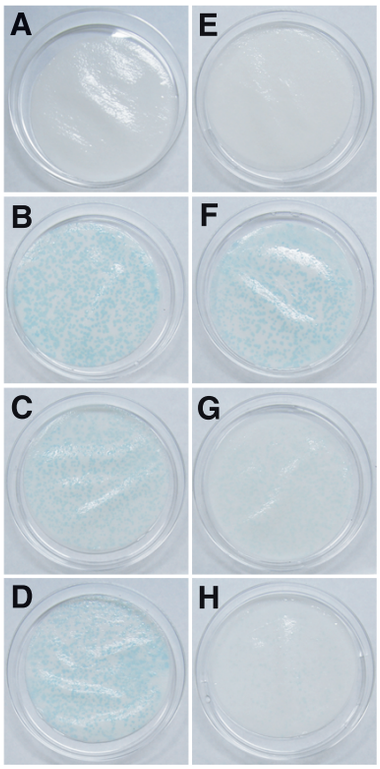

Figure 4. Colony-lift filter assay indicates LTAg and 53BP2 inhibit DNA binding of p53 to different extents. YM4271[p53BLUE] cells were transformed with (A) pCETT; (B) pCETT/53; (C) pCETT/53/T; (D) pCETT/53/BP2; (E) pCETF; (F) pCETF/53; (G) pCETF/53/T; or (H) pCETF/53/BP2 and plated on SD/-L/-U plates. The X-gal colony-lift assay was performed after 4 days growth at 30°C. Photos were taken after 45 min incubation at 30°C. See caption for Figure 3 for the key to labels.

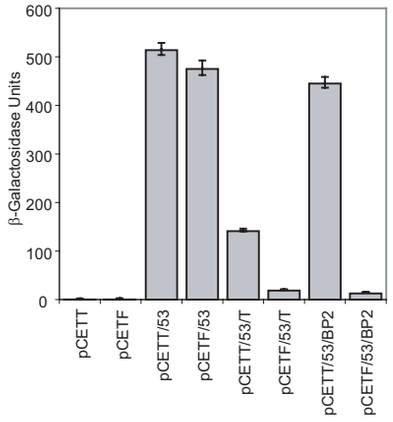

Figure 5. Histogram comparing the effects of different expression levels of inhibitory proteins LTAg and 53BP2 on DNA binding by p53. Quantitative assessment of β-galactosidase activity of the GAL4AD-p53 fusion in yeast strain YM4271[p53BLUE] in the presence/absence of high/low expression of 53BP2 or LTAg. Vertical axis indicates the mean values of β-galactosidase units. Error bars represent standard error from at least three independent trials conducted in triplicate. See caption for Figure 3 for the key to histogram labels.

or pCETF leads to comparable β-galactosidase activities (Figure 5), demonstrating that expression from MCS I is not affected by the different *ADH1* promoters (truncated versus full length) that control the transcription of genes cloned into MCS II. When no gene is cloned into MCS II, a nonsense protein (76 residues) from the cloning vector is expressed. We further tested the effect of this nonsense protein by assay of β-galactosidase activity and compared these results to those obtained when using singly transformed yeast expressing GAL4AD-p53 from the original pGAD53m plasmid supplied by Clontech. The results of this assay are not statistically different, suggesting that this nonsense protein expressed from pCETT or pCETF, regardless of its high or low expression levels, does not affect GAL4AD-p53 in either an enhancing or inhibitory fashion, nor does its expression adversely affect normal yeast growth (data not shown).

Low-level coexpression from pCETT of GAL4AD-p53 and LTAg (pCETT/53/T, Figure 5) gives a ~3.5-fold decrease in β-galactosidase activity compared with the same cells expressing GAL4AD-p53 alone (pCETT/53, Figure 5), compared with a ~26-fold decrease when LTAg is expressed from the full-length *ADH1* promoter in pCETF (pCETF/53/T, Figure 5). Similar results were observed for the coexpression of GAL4AD-p53 and 53BP2: a minimal decrease in β-galactosidase activity was observed when 53BP2 was expressed from pCETT (pCETT/53/BP2, Figure 5), while a 36-fold decrease was observed when using pCETF (pCETF/53/BP2, Figure 5). As expected, expression of either LTAg or 53BP2 alone does not result in reporter gene activation (data not shown).

DISCUSSION

The DNA-binding Activity of p53 and Its Interaction with LTAg or 53BP2: Comparison of MY1H Observations with Earlier Studies

In our MY1H system, the decrease in positive signal from transactivation of GAL4AD-p53 requires the interaction of LTAg or 53BP2 with GAL4AD-p53: LTAg and 53BP2 prevent the DNA binding of p53 in vivo, thereby leading to decreased activation potential. This conclusion is supported both by our experimental data and by evidence gained from previously published studies. First, as discussed above, previous studies have already proven that LTAg and 53BP2 inhibit DNA binding of p53 by binding to and masking p53's DNA-binding domain (18–22). Second, we observed by the *HIS3* reporter assay that neither LTAg nor 53BP2 interacts with the p53 consensus DNA site (also known as the p53 *cis*-acting DNA target element, data not shown), which excludes the possibility that LTAg or 53BP2 inhibits transcription of GAL4AD-p53 through occupying the p53 DNA consensus target. Third, there is no evidence that LTAg or 53BP2 can interact with GAL4AD, indicating the unlikelihood of LTAg or 53BP2 interference with GAL4AD function. Fourth, no abnormal growth of yeast upon expression of either LTAg or 53BP2 was observed in our experiments, and none was reported by other labs in their experiments on p53 interactions with either LTAg or 53BP2 (19,20,31). In our MY1H, all three assays provide convincing in vivo evidence that either LTAg or 53BP2 excludes p53 from binding to its DNA consensus sequence.

Interestingly, all three assays show that at low expression levels, the inhibitory activity of 53BP2 is not vastly different from the positive control expressing only GAL4AD-p53, whereas LTAg shows strong inhibition of reporter gene activation (Figures 3–5). At high expression levels, both 53BP2 and LTAg efficiently inhibit transcription potential of the reporter gene, leading to comparable minimal

levels of reporter gene activity; the 3-AT titration assay shows, however, that LTAg still appears to be a more effective inhibitor at low and high expression levels (Figure 3). Although these observations suggest that LTAg may be a more capable inhibitor of transcription potency than 53BP2, they do not necessarily prove that LTAg inhibits the binding of p53 to its consensus site more efficiently than does 53BP2. The reasons lie in the complexity of the in vivo system and the inherent differences between LTAg and 53BP2, including size, ability to permeate the yeast nucleus, and potential differences in cellular concentrations. Given such differences between dissimilar proteins in the in vivo environment and that truly quantitative comparisons of data obtained from in vivo genetic systems is impractical (32), a more appropriate and reliable use of our MY1H would be, for example, the in vivo comparison of the effect of several mutant versions of a targeted protein on the DNA-binding ability of a transcription factor, with potential for correlation of in vivo data with in vitro measurements.

Different Types of Interactions between Two Proteins and a DNA Target Can Be Examined in Our MY1H System

By expressing a second protein in our modified system, different interactions can be investigated (Figure 1). If P1, which is expressed as a fusion to GAL4AD, is able to target DNA element E, the second protein P2 can interact with the DNA-binding domain of P1 (protein-protein interaction) or directly bind to element E (protein-DNA interaction), or P2 can recruit repressors to P1: either of these scenarios can result in blockage or decrease of reporter gene activation. If P1 itself is unable to target element E, the second protein can serve to rescue or restore reporter gene expression by serving as a bridge between P1 and element E, by dimerizing with P1 to enable binding at element E, or by modifying the structure of P1 to allow DNA binding.

As demonstrated here, our MY1H system might be particularly useful for testing the effects of a new protein, or mutant versions of a protein, on the DNA-binding activity of a transcription factor. As we study the effects of a particular protein on the DNA-binding activity of a transcription factor, this protein can be transcribed at low or high levels from MCS II, which is a considerable advantage during the study of inhibitors of a DNA-binding protein or of DNA-binding competition assays. The activities of strong inhibitors can be evaluated with pCETT, while the activities of weaker inhibitors can be distinguished with pCETF. Such a combined use of the two plasmids allows reliable qualitative assessment of the strength of the protein-DNA interaction of interest. For example, we have successfully applied this MY1H system to examine two other protein-DNA systems (unpublished results). In the first case, the MY1H was used to test the repression of Max-DNA interactions with several mutants of Max such that the competitive binding of two proteins vying for the same DNA target was examined: hence, a protein-DNA interaction was assayed, as shown in Figure 1 where P2 serves as a repressor. In the second case, we used the MY1H for examination of mutants of AhR and Arnt that must heterodimerize in order to bind to a specific DNA target site: hence, a protein heterodimer-DNA interaction was assayed, as shown in Figure 1, where P2 acts as a coregulatory protein. In the p53/LTAg/53BP2 system presented here, a protein-protein interaction was assayed, and hence, P2 serves as a blocker.

Moreover, investigation of cooperative heterodimer-DNA interactions benefits from expression of both proteins from the same plasmid. The dimeric complex comprises two different monomers at equimolar ratio, and such expression is more controllable when plasmid copy number is not a complicating factor. In the MY1H system, the two genes inserted into pCETT are transcribed at the same level, resulting in comparable protein concentrations within the cell. (We note that although both proteins encoded in each plasmid should be expressed comparably, their actual concentrations in the cell can depend on other factors including protein size, stability, and posttranslational processing.) In contrast, previously published yeast genetic approaches for investigation of multiprotein-DNA interactions use two separate plasmids to express two different proteins, commonly controlled by different promoters (9–14). These two-plasmid systems inherently lack the ability to control the expression of both proteins at comparable levels, as fluctuations in plasmid copy numbers and differential promoter strengths can lead to variable and unpredictable expression levels of the two different proteins. Our one-plasmid system, therefore, eliminates the issue of variable protein expression levels stemming from differential copy numbers between plasmids within the same cell.

Although not shown in this report, our system could also be used to examine proteins that contribute coactivational or bridging functions to the protein under investigation (Figure 1). Furthermore, with some prior knowledge of possible target proteins, this system could potentially be extended toward library screening for identification of novel accessory proteins that rescue DNA-binding capability of a target protein that is otherwise incapable of specific DNA binding.

In summary, we have developed a modified Y1H system that can be used to detect both protein-protein and protein-DNA interactions in vivo. The system was validated by use of DNA-binding protein p53 and inhibitors LTAg and 53BP2. This MY1H system should be particularly useful in the investigation of the effects of a regulatory protein on the target transcription factor-DNA interaction, and should complement current methods available for identifying and investigating novel protein-protein or protein-DNA interactions in yeast *S. cerevisiae*.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Sambrook, J. and D.W. Russell.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. CSH Press, Cold Spring Harbor, NY.
2. **Fields, S. and O.-K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature *340*:245-246.
3. **Wang, M.M. and R.R. Reed.** 1993. Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature *364*:121-126.
4. **Luo, Y., S. Vijaychander, J. Stile, and L. Zhu.** 1996. Cloning and analysis of DNA-binding proteins by yeast one-hybrid and two-hybrid systems. BioTechniques *20*:564-568.
5. **Ptashne, M. and A. Gann.** 1997. Transcriptional activation by recruitment. Nature *386*:569-577.
6. **Wolberger, C.** 1998. Combinatorial transcription factors. Curr. Opin. Genet. Dev. *8*:552-559.
7. **Ransone, L.J. and I.M. Verma.** 1990. Nuclear proto-oncogenes Fos and Jun. Annu. Rev. Cell Biol. *6*:539-557.
8. **Atchley, W.R. and W.M. Fitch.** 1997. A natural classification of the basic helix-loop-helix class of transcription factors. Proc. Natl. Acad. Sci. USA *94*:5172-5176.
9. **Dalton, S. and R. Treisman.** 1992. Characterization of Sap-1, a protein recruited by serum response factor to the c-fos serum response element. Cell *68*:597-612.
10. **Naya, F.J., C.M.M. Stellrecht, and M.J. Tsai.** 1995. Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev. *9*:1009-1019.
11. **Peterson, B.R., L.J. Sun, and G.L. Verdine.** 1996. A critical arginine residue mediates cooperativity in the contact interface between transcription factors NFAT and AP-1. Proc. Natl. Acad. Sci. USA *93*:13671-13676.
12. **Sun, L.J., B.R. Peterson, and G.L. Verdine.** 1997. Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1. Proc. Natl. Acad. Sci. USA *94*:4919-4924.
13. **Yu, Y., M. Yussa, J.B. Song, J. Hirsch, and L. Pick.** 1999. A double interaction screen identifies positive and negative *ftz* gene regulators and Ftz-interacting proteins. Mech. Dev. *83*:95-105.
14. **Koszewski, N.J., K.W. Henry, E.J. Lubert, H. Gravatte, and D.J. Noonan.** 2003. Use of a modified yeast one-hybrid screen to identify BAF60a interactions with the Vitamin D receptor heterodimer. J. Steroid Biochem. Mol. Biol. *87*:223-231.
15. **Wang, Y., J.F. Schwedes, D. Parks, K. Mann, and P. Tegtmeyer.** 1995. Interaction of p53 with its consensus DNA-binding site. Mol. Cell. Biol. *15*:2157-2165.
16. **Zhao, K., X. Chai, K. Johnston, A. Clements, and R. Marmorstein.** 2001. Crystal structure of the mouse p53 core DNA-binding domain at 2.7 Å resolution. J. Biol. Chem. *276*:12120-12127.
17. **Farmer, G., J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives.** 1992. Wild-type p53 activates transcription in vitro. Nature *358*:83-86.
18. **Lilyestrom, W., M.G. Klein, R.G. Zhang, A. Joachimiak, and X.J.S. Chen.** 2006. Crystal structure of SV40 large T-antigen bound to p53: interplay between a viral oncoprotein and a cellular tumor suppressor. Genes Dev. *20*:2373-2382.
19. **Iwabuchi, K., P.L. Bartel, B. Li, R. Marraccino, and S. Fields.** 1994. Two cellular proteins that bind to wild-type but not mutant p53. Proc. Natl. Acad. Sci. USA *91*:6098-6102.
20. **Bargonetti, J., I. Reynisdottir, P.N. Friedman, and C. Prives.** 1992. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. Genes Dev. *6*:1886-1898.
21. **Segawa, K., A. Minowa, K. Sugasawa, T. Takano, and F. Hanaoka.** 1993. Abrogation of p53-mediated transactivation by SV40 large T antigen. Oncogene *8*:543-548.
22. **Gorina, S. and N.P. Pavletich.** 1996. Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science *274*:1001-1005.
23. **Chung, C.T., S.L. Niemela, and R.H. Miller.** 1989. One-step preparation of competent *Escherichia coli*—transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA *86*:2172-2175.
24. **Orr-Weaver, T.L., J.W. Szostak, and R.J. Rothstein.** 1981. Yeast transformation: A model system for the study of recombination. Proc. Natl. Acad. Sci. USA *78*:6354-6358.
25. **Bartel, P.L., C.-T. Chien, R. Sternglanz, and S. Fields.** 1993. Using the two-hybrid system to detect protein-protein interactions, p. 153-179. *In* D.A. Hartley (Ed.), Cellular Interactions in Development: A Practical Approach. Oxford University Press, Oxford, England.
26. **Dillon, P.J. and C.A. Rosen.** 1990. A rapid method for the construction of synthetic genes using the polymerase chain reaction. BioTechniques *9*:298-300.
27. **Oliphant, A.R., A.L. Nussbaum, and K. Struhl.** 1986. Cloning of random-sequence oligodeoxynucleotides. Gene *44*:177-183.
28. **Tornow, J. and G.M. Santangelo.** 1990. Efficient expression of the *Saccharomyces cerevisiae* glycolytic gene *ADH1* is dependent upon a *cis*-acting regulatory element (UAS_{RPG}) found initially in genes encoding ribosomal proteins. Gene *90*:79-85.
29. **Ammerer, G.** 1983. Expression of genes in yeast using the ADCI promoter. Methods Enzymol. *101*:192-201.
30. **Titz, B., S. Thomas, S.V. Rajagopala, T. Chiba, T. Ito, and P. Uetz.** 2006. Transcriptional activators in yeast. Nucleic Acids Res. *34*:955-967.
31. **Thukral, S.K., G.C. Blain, K. Chang, and S. Fields.** 1994. Distinct residues of human p53 implicated in binding to DNA, simian virus 40 large T antigen, 53BP1, and 53BP2. Mol. Cell. Biol. *14*:8315-8321.
32. **Estojak, J., R. Brent, and E.A. Golemis.** 1995. Correlation of two-hybrid affinity data with in vitro measurements. Mol. Cell. Biol. *15*:5820-5829.

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