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pBT, a novel vector for tetracycline-regulated yeast three-hybrid assay

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ABSTRACT

A novel yeast three-hybrid (Y3H) vector pBT was developed, which contains a tetracycline (Tet)-sensitive transactivator (tTA) expression unit and a Tet-responsive element (TRE)-driven 3rd protein expression unit within a single plasmid. To optimize tTA expression levels, several promoters for driving tTA expression were tested, and the weakest human cytomegalovirus (CMV) promoter showed the best induction/background ratio. Culturing yeast cells in different doses of doxycycline (Dox) resulted in a dose-dependent reduction of 3rd protein expression. Screening a cDNA library with pBT successfully identified functional Y3H interactions that could be easily discriminated from Y2H interactions by culturing on Dox-containing plates. At 5.0 μg/ml Dox, Y3H interactions were undetectable by the colony-forming assay under high-stringency selection conditions or by a lacZ colorimetric assay. A low-copy-number version of the pBT vector, pBT(L), completely eliminated the leakage activity of pBT found under low-stringency condition. In conclusion, the pBT system is a useful tool for studying the structures of higher-order protein complexes.

INTRODUCTION

The yeast two-hybrid (Y2H) system is a powerful tool for detecting protein interactions. It has been widely used to identify novel protein interactions and to analyze cell signaling networks (1). The Y2H system is usually used for assessing direct interactions between two partners, despite the fact that most native protein complexes are formed by more than two proteins. Even in the case of two-protein interactions, a 3rd protein is often required to stabilize or facilitate the binding between the two partners. To study this kind of higher-order protein complex, a yeast three-hybrid (Y3H) system was developed. In this Y3H system, a 3rd protein is expressed together with a DNA-binding domain (BD)-bait fusion protein and a transcription activating domain (AD)-prey fusion protein. The 3rd protein is incorporated into the protein interaction between bait and prey through direct binding or protein modifications such as phosphorylation. If the bait and prey—which do not form a complex by themselves—can interact with the addition of a 3rd protein, the reporter genes will be activated.

Several Y3H systems have been developed thus far (2–5). From a practical point of view, the use of an inducible promoter for driving the 3rd protein makes it easy to isolate triplex interactions from duplexes formed only by bait and prey. The Met25 promoter has been used for this purpose (3,5), and several other inducible promoters can be used as well (6–9). However, these promoters utilize inducing reagents that may affect cell metabolism and possibly cause unwanted phenotypes. For example, methionine depletion for the Met25 promoter hampers basal growth of the AH109 strain (unpublished data). To circumvent this problem, a tetracycline (Tet)-regulated expression system was chosen. Originally reported in mammalian cells (10), Tet-regulated gene expression systems can also be used in yeast (11–13). Doxycycline (Dox), an inducing reagent of the Tet-regulation system, has no obvious effect on the phenotype and global gene expression pattern of yeast even at a high dose of 40 μg/ml (14).

In this article, the construction of a novel Y3H vector pBT is reported, which has all the Tet-OFF components within a single plasmid with optimizations to minimize background leakage activity. This pBT vector has been successfully used to isolate functional triplexes from a cDNA library. This simple-to-use pBT Y3H system will facilitate the high-throughput analysis of higher-order protein complexes.

MATERIALS AND METHODS

Suppliers

Yeast strains (AH109, Y187), yeast vectors (pBridge, pACT2), the mouse brain cDNA library in pACT2,

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Tet-OFF system vectors (pTet-OFF, pTRE) and pEGFP-N1 were obtained from Clontech (Palo Alto, CA, USA). pCI-neo was purchased from Promega (Madison, WI, USA), and pTEF1/Zeo was purchased from Invitrogen (Carlsbad, CA, USA). p415CYC1 (15) was from the American Type Culture Collection (ATCC). pCH110 was obtained from Pharmacia Biotech (Uppsala, Sweden). *Escherichia coli* strain XL2-blue was from Stratagene (La Jolla, CA, USA). HRP-conjugated anti-Flag M2 antibody was from Sigma (St. Louis, MO, USA). SuperSignal Femto chemiluminescent substrate was from Pierce (Rockford, IL, USA). All other reagents were from Nacalai Tesque (Kyoto, Japan).

Plasmids

pBridge was used as a backbone for all vectors. pBP constitutive promoter vectors were constructed by replacing the pBridge Met25 promoter with one of the exogenous mammalian or yeast promoters as described below. The CMV promoter (pCI-neo BglII–HindIII fragment), SV40 promoter (pCI-neo KpnI–HindIII fragment), HSV-tk promoter [pMC1neo (16) XhoI–PstI fragment], EF1a promoter [pEF-BOS (17) EcoRI–BamHI fragment] and CYC1 promoter [p415CYC1 (15) SacI–XbaI fragment] were isolated by restriction enzyme digestion. The TEF1 promoter was isolated from pTEF1/Zeo by polymerase chain reaction (PCR) using the following primers: 5′-GT CGCTAGCCAGCCCACACACCATAGCTTC-3′ and 5′-TTCCATATGGCCCATCCGCCCCTTAGATTA-3′. The isolated promoter fragments were digested with NheI/NdeI, and subcloned into pBridge in place of the Met25 promoter. For pBT three-hybrid vectors, the Tet-regulated expression cassette was constructed by fusing the Tet operator (TetO) with a minimal CYC1 promoter (minCYC1) and CYC1 terminator (CYC1Term). Seven TetO repeats (TetO[7]) were amplified from pTRE by PCR using the primers 5′-GCTGAGCTCTGTACGGG CCAGATATACGCG-3′ and 5′-GCTCATATGGACCC GGGTACCGACCTCGAC-3′. The single TetO element (TetO[1]) was created by annealing two oligonucleotides 5′-TATGTTTACCACTCCCTATCAGTGATAGA GAAAAGTGAAAGTCGAG-3′ and 5′-TACTCGACTT TCACTTTTCTCTATCACTGATAGGGAGTGGTAA ACA-3′. minCYC1-CYC1term was isolated as a NdeI–KpnI fragment from p415CYC1(15). The TetO[n]-minCYC1 promoter-CYC1Term cassette was inserted into the PmaCI site of pBP. The Tet transactivator (tTA) was amplified from pTet-OFF using the primers 5′-ACTC GGCCGATGTCTAGATTAGATAAAAG-3′ and 5′-GT CTGGATCCTCGCGCCCCCTACCCACCG-3′ and subcloned into the NotI-BglII site of pBP. pBT(L), a low-copy-number version of pBT, was constructed by replacing the original 2 μm replication origin with a centromeric CEN6/ARSH4 sequence from p415CYC1(15). For reporter assays, full coding regions of EGFP (AA 1–239) and lacZ (AA 1–1085) were isolated from pEGFP-N1 and pCH110, respectively, and N-terminally tagged with Flag tag (MDYKDDDDK-) using PCR. For Y2H and Y3H assays, a C-terminal fragment of mouse epidermal growth factor receptor (EGFR, AA 671–1210) and the full coding region of rat growth factor receptor-bound protein 2 (Grb2, AA 1–217) were isolated using RT-PCR.

Strains

Saccharomyces cerevisiae strains AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1) and Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1UAS-GAL1TATA-lacZ, MEL1) were used to test promoter activity and three-hybrid interaction. Media preparation, growth, transformation and selection of yeast were carried out as described previously (18,19). *Escherichia coli* strain XL2-blue was used as a bacterial host for vector construction and plasmid preparation.

EGFP fluorescence

pBP-transformed AH109 colonies on SD/-Leu/+Dox (0–2 μg/ml) plates were suspended in dH_2O and mounted on a glass slide. EGFP fluorescence was observed under an Axiophot microscope (Carl Zeiss, Germany). Images were captured using a Digital Sight DS-5Mc CCD camera (Nikon, Japan) and NIS-Elements D software (Nikon, Japan). Image-capture conditions were kept constant throughout the experiments. Captured images were trimmed and composed using Photoshop software (Adobe, USA). No level/contrast enhancement was applied.

β-Galactosidase assay

β-Galactosidase activity was quantified using an ONPG (*O*-nitrophenyl-β-D-galactopyranoside) liquid assay. Yeast cells in log phase liquid culture were collected by centrifugation, incubated at 28°C in 500 μl of Z buffer [100 mM NaPi (pH 7.2), 10 mM KCl, 1 mM $MgSO_4$, 0.36% β-mercaptoethanol], and permeabilized with 20 μl of chloroform and 35 μl of 0.1% SDS. Enzyme reaction was started by the addition of 100 μl of Z buffer containing 4 mg/ml ONPG and terminated by the addition of 250 μl of 1 M Na_2CO_3. β-Galactosidase activity (Miller unit) was calculated using the equation $1000 \times (OD_{420}/OD_{600} \times V \times t)$, where V is the culture volume and t is the reaction time. Activation of the endogenous lacZ reporter by three-hybrid reaction was also monitored by an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) agarose overlay assay. One percent low-melting point agarose dissolved in Z buffer was cooled down to 40°C, and dimethylformamide, SDS and X-gal were added to final concentrations of 6%, 0.1%,and 0.25 mg/ml, respectively, and poured onto the yeast colonies. After hardening, plates were incubated at 30°C overnight.

Western blotting

Protein extraction from yeast cells was performed as described previously (20). Extracted proteins were separated by SDS–polyacrylamide gel electrophoresis, blotted

onto nitrocelulose membranes, and probed with HRP-conjugated anti-Flag M2 antibody (1 : 10000 dilution). SuperSignal Femto HRP substrate was used for chemiluminescent detection.

Three-hybrid screening

AH109 cells transformed with pBT-EGFRc-TetO[1]-Grb2-CMV-tTA were mated with Y187 cells pretransformed with a mouse brain cDNA library. Mated cells were selected on the SD/-Trp/-Leu/-His/-Ade plates for 2 weeks at 30°C. A total of 1.8×10^8 colonies were screened and 350 colonies were isolated. Colonies were duplicated onto SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade plates with or without 5.0 μg/ml Dox. Reporter activation was assessed by colony formation and the X-gal overlay assay. The prey plasmids were purified, amplified in XL2-blue and subjected to DNA sequencing. The isolated plasmids were used to retransform AH109 with the bait or control plasmids to verify the three-hybrid interaction.

RESULTS

Comparison of promoter activity in *S. cerevisiae*

One of the key parameters for establishing an efficient Tet-OFF system is the tTA protein expression level. An excess of tTA expression will cause significant leakage activity under the OFF condition (in the presence of Dox). To simultaneously achieve sufficient inducibility and decreased leakage, it is critical to maintain an adequate but not an excessive tTA expression level. To identify the best promoter for tTA expression, the activities of several constitutive mammalian, viral and yeast promoters were compared, with the expectation that these mammalian and viral promoters may also be active in yeast, as was found in the case of the CMV promoter (11). TEF1 (21) and CYC1 (15) promoters from yeast, the EF1a (17) promoter from humans and the CMV (22), SV40 (23) and HSV-tk (16) promoters from mammalian viruses were chosen. These promoters were inserted into the pBP vector and FlagEGFP or the lacZ gene was used as a reporter (Figure 1A).

EGFP fluorescence and β-galactosidase activity measurements showed similar trends (Figure 1B and C). Quantitative measurements showed that the TEF1 and CYC1 yeast promoters were more than 10 times as active as the mammalian promoters (Figure 1C). Among the mammalian promoters, the SV40 promoter was the most potent, and the weakest CMV promoter had only one-tenth the activity of the SV40 promoter. The activity of HSV-tk and EF1a promoters was between those of the CMV and SV40 promoters (Figure 1C). The CYC1 (strong), SV40 (medium) and CMV (weak) promoters were chosen for further evaluation.

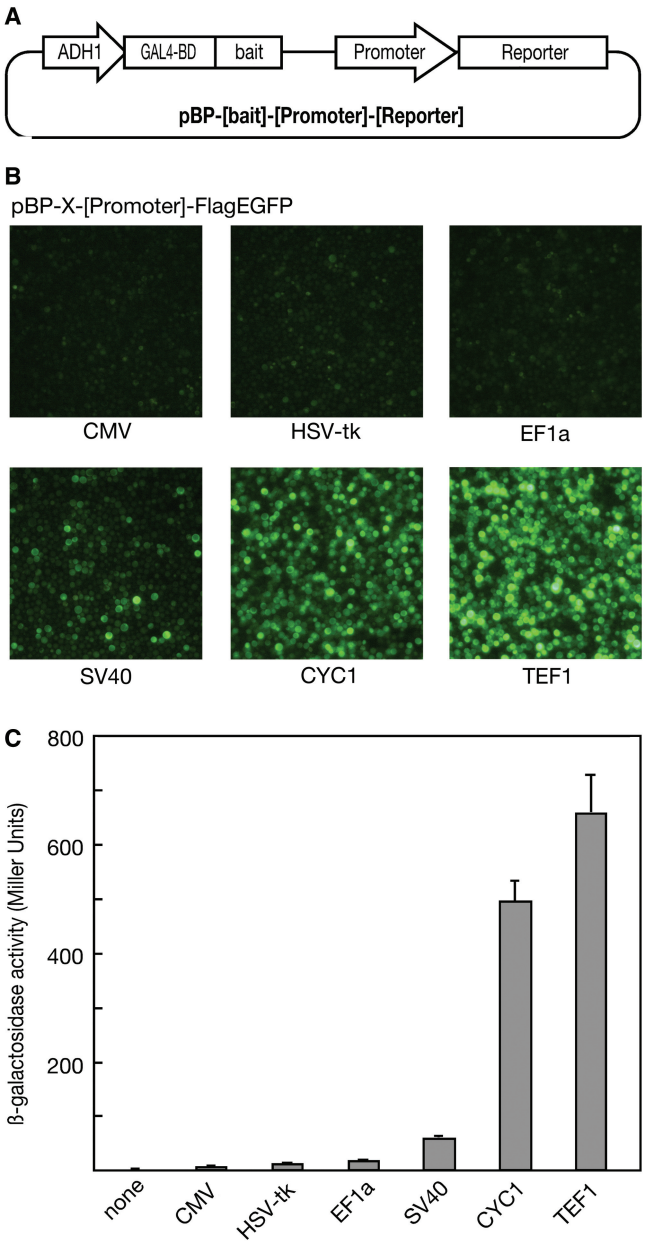

Figure 1. Comparison of promoter activities in *S. cerevisiae*. (**A**) A map of the pBP vector for assaying promoter activity. Several mammalian, viral or yeast constitutive promoters were used to drive reporter gene expression. (**B**) Semi-quantitative comparisons of promoter activity. A Flag-tagged EGFP gene was inserted downstream of the indicated promoter. Images of EGFP-expressing yeast cells were captured under similar conditions. (**C**) Quantitative comparisons of promoter activity using the lacZ gene as a reporter. β-Galactosidase enzyme activity was measured by the ONPG assay. Each bar represents at least three independent samples (mean ± SEM).

Tet-regulated gene expression in *S. cerevisiae*

To establish the Tet-mediated regulation system, the pBT vector was constructed by inserting the tTA gene downstream of the CYC1, SV40 or CMV promoter, respectively, and added a TRE-driven expression unit between the GAL4-BD and tTA expression units (Figure 2A). The Tet-responsive promoter was composed of 0, 1 or 7 repeats of the Tet operator (TetO) motif fused to a minimal CYC1 (minCYC) promoter (Figure 2A). To characterize the pBT vector system, FlagEGFP or a lacZ reporter gene was inserted downstream of the

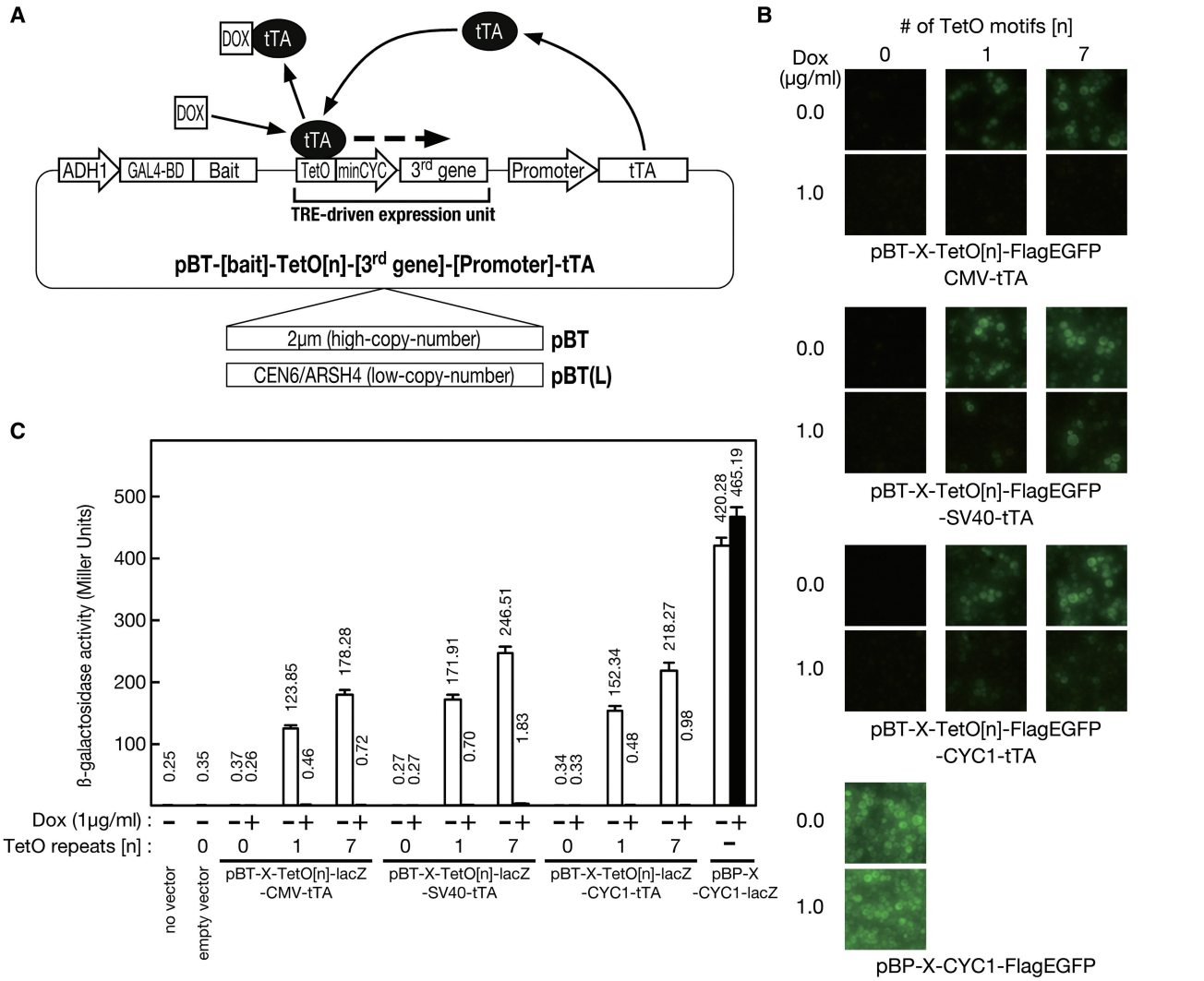

Figure 2. Tet-mediated regulation in *S. cerevisiae*. (**A**) A plasmid map and a schematic diagram of the Tet-controlled Y3H vector pBT. GAL4BD-bait fusion protein was expressed using the ADH1 promoter. tTA (Tet transactivator) expression was driven by one of the mammalian,viral or yeast constitutive promoters. 3rd gene was under the control of the TetO (Tet operator)-minimal CYC1 fusion promoter. 0, 1 or 7 repeats of the TetO motif were tested. tTA binds the TetO motif(s) and activates transcription from a downstream minCYC promoter. Dox detaches tTA from the TetO sequence and consequently inhibits 3rd gene transcription in a dose-dependent manner. High-copy-number (2 μm) and low-copy-number (CEN6/ARSH4) replication origins were used in pBT and pBT(L), respectively. (**B** and **C**) Testing Tet-mediated gene regulation. Flag-EGFP (B) or a lacZ (C) reporter was inserted as the 3rd gene downstream of the TetO[n]-minCYC promoter. [n] Indicates number of TetO motifs (0, 1 or 7). Vectors with CMV, SV40 or CYC1 promoter-driven tTA were tested. pBP-X-CYC1 vector (Figure 1) was used as a control vector without a Tet regulation system. (B) Semi-quantitative monitoring of Tet-mediated gene regulation. Each construct was visualized in the presence (upper panel) or absence (lower panel) of 1.0 μg/ml Dox. All images were captured under similar conditions. (C) Quantitative measurement of Tet-mediated gene regulation. β-Galactosidase enzyme activity was measured by the ONPG assay. Each construct was measured in the presence (filled bar) or absence (open bar) of 1.0 μg/ml Dox. Each bar represents at least three independent samples (mean ± SEM). Numbers indicate mean Miller unit values.

Tet-responsive promoter. LacZ induction values from the TetO[0]-minCYC promoter was close to the background level (Figure 2C), indicating that the minCYC promoter by itself has minimal promoter activity. The TetO[1] or TetO[7] motifs induced high levels of reporter gene expression. In the absence of Dox, the CMV, SV40 and CYC1-tTA constructs showed a difference of only ~1.5-fold in the lacZ reporter expression level in spite of the 100-fold difference in promoter potency. This indicates that very low levels of tTA expression were sufficient to achieve a nearly maximal TetO-minCYC promoter induction. Seven repeats of the TetO motif showed an increase in potency of ~45% compared to a single TetO motif (Figure 2C). In the presence of 1.0 μg/ml Dox, a marked reduction of reporter gene expression was observed, but a low level of leakage activity remained in all constructs (Figure 2B and C). In this condition, seven TetO motifs caused an increase in leakage activity of ~160% over a single TetO motif construct (Figure 2C). Significant leakage activity was also observed in EGFP fluorescence, especially in SV40 and CYC1-tTA constructs (Figure 2B). In the control pBP-X-CYC1 constructs that do not have tTA or TRE elements (Figure 1A), 1.0 μg/ml Dox did not cause any significant changes in

reporter gene expression (Figure 2B and C). Taken together, the combination of tTA under a weak CMV promoter and TRE with a single TetO motif could achieve sufficient reporter gene induction with minimal leakage activity. The pBT-[bait]-TetO[1]-[3rd protein]-CMV-tTA construct was used for further analysis.

Dox dose-dependent gene regulation

To characterize the details of Tet-mediated gene regulation, yeast cells with pBT-TetO[1]-[reporter]- CMV-tTA constructs and control pBP-CYC1-[reporter] constructs were cultured in the presence of various concentrations of Dox (Figure 3). In the pBT constructs, reporter gene expression was suppressed in a Dox dose-dependent manner as shown by EGFP fluorescence (Figure 3A), FlagEGFP western blotting (Figure 3B) and lacZ activity (Figure 3C). Half-maximum activity was observed at ~0.05 μg/ml Dox (Figure 3C). Control pBP constructs showed no significant change up to 2.0 μg/ml Dox (Figure 3). In both constructs, overall growth rates were not affected by up to 2.0 μg/ml Dox (data not shown).

Three-hybrid assay

To test the Tet-regulated Y3H system, a well-studied epidermal growth factor receptor (EGFR) protein complex (24,25) was used as an example. pBT-EGFRc-TetO[1]-Grb2-CMV-tTA was constructed, which has an EGFR C-terminal cytosolic domain as a bait and growth factor receptor-bound protein 2 (Grb2) as a 3rd gene under the control of the Tet-responsive promoter. Using this construct, a mouse brain cDNA library in the pACT2 prey vector was screened. A total of 1.8×10^8 colonies were screened and 350 colonies were isolated. Colonies were duplicated onto selection plates with or without 5.0 μg/ml Dox. Of these, 31 colonies confirmed activation of the HIS3, ADE2 and X-gal reporter genes in the absence of Dox and 10 of them showed significant reductions of reporter gene activity in the presence of Dox. Among the Dox-irresponsive group, Grb2, Src homology 2 domain containing transforming protein 1 (SHC1), activated Cdc42-associated kinase 1 (Ack1) and Crk fragments were found. These proteins are known to interact directly with the EGFR C-terminal domain (26–29). In the Dox-responsive group, son of sevenless homolog 1 and 2 (Sos1 and Sos2), modifier of cell adhesion protein (MOCA) and dynamin were found. These proteins are known to bind directly with Grb2 and interact with EGFR indirectly (25,30,31). No known direct interactor of EGFR was included in the Dox-responsive group. A control study confirmed that the Sos1 prey caused reporter gene activation only when the EGFRc bait and Grb2 coexisted, showing a functional Y3H interaction (Figure 4A). Reporter gene activation through EGFRc-Grb2-Sos1 Y3H interaction was suppressed in a Dox dose-dependent manner in pBT, in contrast to the Dox-independent constitutive interaction in pBP (Figure 4B). Quantitative measurements of lacZ reporter activity also showed that EGFRc-Grb2-Sos1 Y3H interaction is under the control of the Tet-mediated regulation in the pBT system (Figure 4C, filled circle). Half-maximum reporter activation was observed at ~1.0 μg/ml Dox (Figure 4C), where the 3rd protein expression should be <5% of the initial level (Figure 3C). This means that there may be an excess of the 3rd protein at 0.0 μg/ml Dox. Reducing the expression level of 3rd protein to a half or one-third may induce sufficient Y3H reporter activation with improved

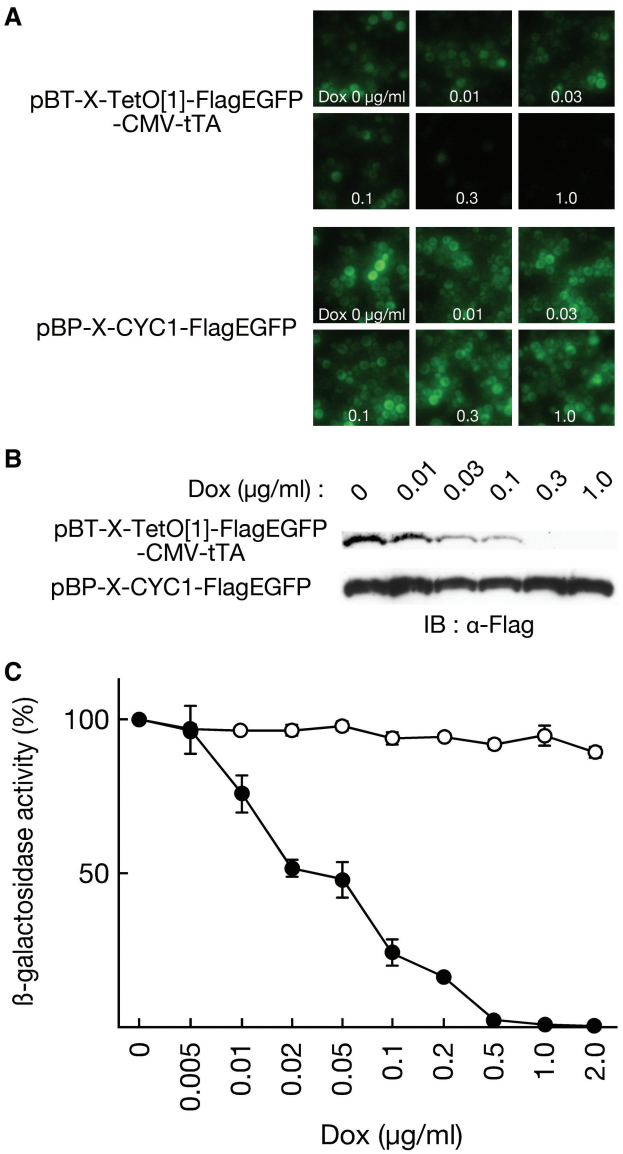

Figure 3. Dox dose-dependent gene expression. Flag-EGFP (**A** and **B**) or lacZ (**C**) reporter was inserted as the 3rd gene downstream of the TetO[1]-minCYC promoter (Figure 2A). The CMV promoter was used to drive tTA expression. pBP-X-CYC1 vector (Figure 1) was used as a control vector without a Tet regulation system. (A) Semi-quantitative monitoring of FlagEGFP expression under a fluorescent microscope. Each construct was cultured in the presence of 0.0–1.0 μg/ml Dox. All images were captured under similar conditions. (B) FlagEGFP expression was also monitored by western blotting. FlagEGFP protein was detected by an anti-Flag M2 antibody. (C) Quantitative measurement of Dox dose-dependent lacZ expression. Cells with pBT-X-TetO[1]-lacZ-CMV-tTA (filled circle) or pBP-X-CYC1-lacZ (open circle) were cultured in the presence of 0.0–2.0 μg/ml Dox. β-Galactosidase enzyme activity was measured by the ONPG assay. Each point represents at least three independent samples (mean ± SEM).

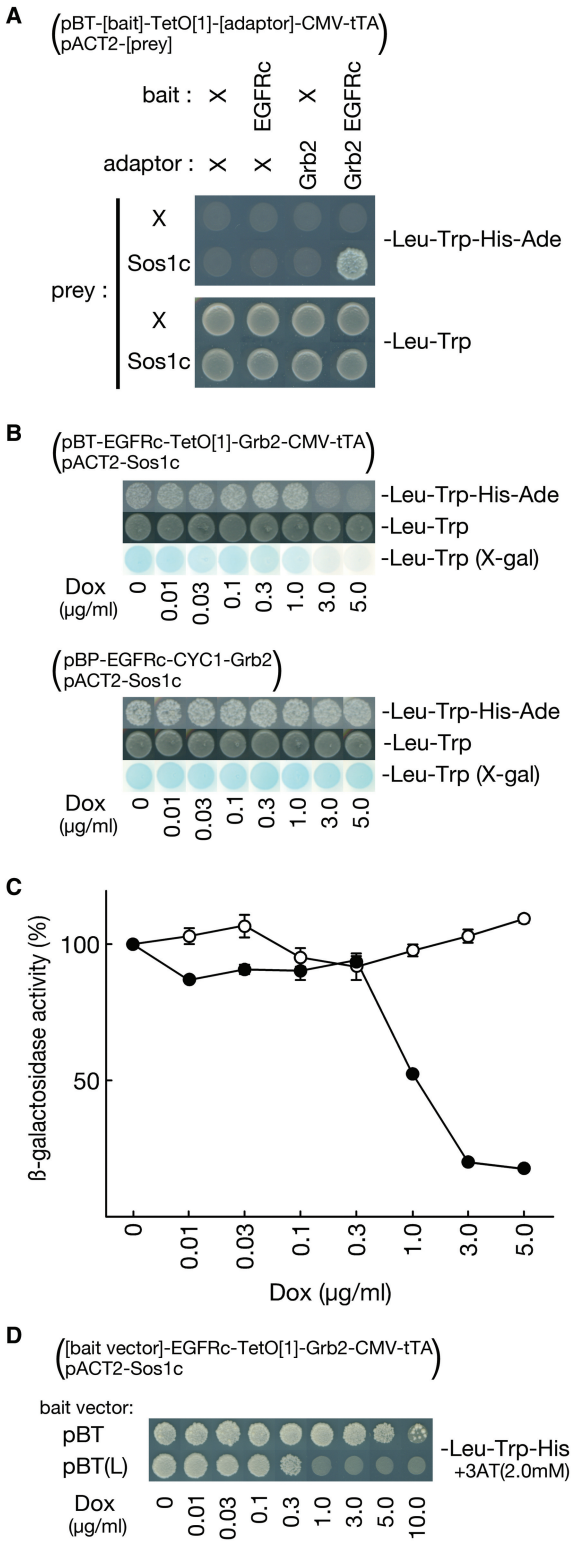

Figure 4. Y2H and Y3H interactions using the pBT vector. (**A**) Confirmation of Y3H interaction. Cells carrying indicated combinations of bait, prey and 3rd genes (X indicates no corresponding gene) were spotted onto selection plates. Cells could grow on an SD/-Leu/-Trp/-His/-Ade selection plate only when a triplex was formed. All cells harbored both of pBT and pACT2 plasmids, as shown by their effective growth on SD/-Leu/-Trp plates. (**B** and **C**) Dox dose-dependent EGFRc-Grb2-Sos1c triplex formation. Grb2 was under the control of the TetO[1]-minCYC promoter and the CMV promoter was used to drive tTA expression (Figure 2A). pBP-X-CYC1 vector (Figure 1) was used as a control vector without a Tet regulation system. Cells were cultured in the presence of 0.0–5.0 μg/ml Dox. HIS3 and ADE2 reporter activation was monitored by cell growth on SD/-Leu/-Trp/-His/-Ade selection plates (B, top row). lacZ reporter activation was assessed by the X-gal agarose overlay (B, bottom row), and quantitative ONPG (C) assays. Each point in (C) represents at least three independent samples (mean ± SEM). (**D**) Plasmid copy number and leakage activity under a low-stringent condition. Cells harboring either a pBT(high-copy-number, 2 μm origin) or a pBT(L) (low-copy-number, CEN6/ARSH4 origin) construct in combination with a pACT2 plasmid were grown on SD/-Leu/-Trp/-His/ + 3AT (2.0 mM) selection plates containing 0.0–10.0 μg/ml Dox.

signal-to-background ratio. To test this hypothesis, a low-copy-number version vector, pBT(L), was constructed for reduced protein expression (Figure 2A). Under a low-stringent condition selected only by HIS3 in the presence of 2.0 mM 3-amino-1,2,4-triazole (3AT), the pBT construct showed significant reporter activation even at 10.0 μg/ml Dox (Figure 4D, top row). In the pBT(L) construct, reporter activation was completely suppressed at a dose of ~1.0 μg/ml Dox (Figure 4D, bottom row). This result indicates that the optimization of the tTA/3rd protein expression level raises the signal-to-background ratio of the Y3H assay.

DISCUSSION

The present study describes the development of a novel pBT Y3H vector system. Previously established Y3H systems utilize a constitutive promoter (2,4) or an inducible Met25 promoter (3,5) for driving 3rd protein expression. Constitutive promoter system requires plasmid isolation and retransformation to confirm the three-hybrid interaction, which makes its application to large-scale screening difficult. The Met25 promoter system could regulate three-hybrid formation by controlling methionine in the medium. However, significant colony formation was observed under a restrictive condition (in the presence of 1 mM methionine) (3,5), indicating leakage activity of the Met25 promoter. It was also found that methionine depletion severely hampered the growth of the commonly used AH109 yeast strain (unpublished data). To overcome these limitations, the pBT system utilizes Tet-mediated gene regulation for driving the 3rd protein, thus enabling the control of Y3H interaction without affecting cell metabolism by adding or removing Dox. This feature makes the Y3H screening more efficient since the Y3H interaction can be easily distinguished from the background Y2H interaction by a simple colony duplication onto Dox(–) and Dox(+) plates, as described in this article.

One advantage of pBT is that it contains the GAL4BD-bait, tTA and 3rd protein within a single vector, which means that it is fully compatible with any existing GAL4AD-prey libraries such as the pACT2 cDNA library used in this study. Investigators who have already utilized the GAL4 Y2H system can easily upgrade their screening to a Y3H mode by simply replacing their bait vector with pBT.

In the course of vector development, the optimization of protein expression level was found critical to achieve high sensitivity and specificity. Using a weak CMV promoter for tTA expression helped to minimize background leakage expression of the TRE-driven gene (Figure 2). Reducing plasmid copy number suppressed background Y3H interaction (Figure 4). Although the low-copy-number vector pBT(L) was the best in the case of EGFR-Grb2-Sos1 Y3H interaction, it may also cause reduced bait expression and lowered sensitivity for the detection of weaker protein interactions. The high-copy-number pBT should be chosen in such cases. Users can select the appropriate Y3H vector with the best sensitivity and specificity depending on the nature of the protein interaction of interest.

The pBT system would be useful not only for studying higher-order protein complexes but also for isolating protein fragments or peptides that inhibit specific protein interactions. Reverse Y2H systems have been developed to study the dissociation of binding partners induced by mutations in protein sequence or by addition of exogenous reagents (32–34). The Y3H system could monitor protein dissociation induced by a 3rd protein or peptide (3). These principles are applicable to the pBT system. The tightly regulated pBT system has the advantage of being able to eliminate possible false positives in isolating protein interaction inhibitors, and has potential as a tool for biological research or for identifying therapeutic agents.

Further improvements of the pBT system are possible. For example, using the Tet-ON system will be useful in some cases. Although a simple replacement of tTA with reverse Tet transactivator (rtTA) (35) did not work because of its high leakage activity (data not shown), using improved rtTA2^{s}-M2 (36) and Tet-repressor (37) may help to reduce the background. An improved Tet-responsive element (TREmod) with tighter regulation may also be useful.

In summary, a simple-to-use Tet-regulated pBT Y3H system was developed and successfully used to detect native triplex formation. This pBT system will be useful for studying the nature of protein interaction networks and for isolating proteins or peptides that inhibit specific protein interactions.

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