A yeast one-hybrid system to screen for methylated DNA-binding proteins

Shu-Ying Feng¹, Kazuhisa Ota¹,² and Takashi Ito¹,²,*

¹Department of Computational Biology, Graduate School of Frontier Sciences and ²Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo

Received July 19, 2010; Revised August 5, 2010; Accepted August 9, 2010

ABSTRACT

We had previously exploited a method for targeted DNA methylation in budding yeast to succeed in one-hybrid detection of methylation-dependent DNA–protein interactions. Based on this finding, we developed a yeast one-hybrid system to screen cDNA libraries for clones encoding methylated DNA-binding proteins. Concurrent use of two independent bait sequences in the same cell, or dual-bait system, effectively reduced false positive clones, which were derived from methylation-insensitive sequence-specific DNA-binding proteins. We applied the dual-bait system to screen cDNA libraries and demonstrated efficient isolation of clones for methylated DNA-binding proteins. This system would serve as a unique research tool for epigenetics.

INTRODUCTION

Methylation occurs at the C5-position of cytosine in genomic DNA of various eukaryotes, including plants, fungi and animals, each of which displays a characteristic pattern in genomic distribution of 5-methylcytosine (5mC) (1–3). While some organisms such as budding yeast Saccharomyces cerevisiae and nematode Caenorhabditis elegans are devoid of DNA methylation, it is well established that 5mC functions as a critical epigenetic mark in a variety of organisms, and its distribution pattern along with the genome, or the DNA methylome, constitutes a critical layer of epigenomic information. To understand the encoding mechanisms of this layer, it is essential to reveal how the cell targets DNA methylase/demethylase activities to particular genomic loci (4).

Mammals have at least three distinct classes of methylated DNA-binding proteins, namely methyl-CpG binding domain (MBD) proteins, Kaiso-related zinc finger proteins and SET and RING finger-associated (SRA) domain proteins (5). The MBD protein family includes MBD1, MBD2, MBD4 and MeCP2. Intriguingly, MBD4 has been implicated in demethylation, and defects of MeCP2 cause Rett syndrome, both indicating their critical roles. The Kaiso-related family comprises Kaiso, ZBTB4 and ZBTB38, each of which displays a sequence-specific interaction with methylated DNA. The SRA domain protein family includes UHRF1, which binds hemi-methylated DNA and recruits DNMT1 responsible for maintenance methylation. While MBD and SRA domain were found in plants, methylated DNA-binding proteins have not been rigorously explored in organisms other than mammals. It is conceivable that totally different classes of methylated DNA-binding proteins remain to be identified in any eukaryotic species. An efficient method for selective cloning of methylated DNA-binding proteins would accelerate their identification.

A powerful method to clone DNA-binding proteins is the yeast one-hybrid system (6). Taking advantage of the lack of endogenous DNA methylation in S. cerevisiae, we modified the system and successfully detected methylated DNA–protein interactions mediated by MBD1, MeCP2 and Kaiso (7). In this system, we integrated LexA operator (LexAop) at the 5′-flanking region of the bait sequence and expressed LexA-fused SssI CpG methylase (M.SssI), a bacterial CpG methylase (M.SssI), a bacterial de novo DNA methylase. Consequently, LexA-mediated recruitment of M.SssI led to targeted methylation of the bait sequence, to which methylated DNA-binding proteins fused with Gal4 or VP16 activation domain bound and activated the expression of lacZ reporter gene. These results suggested a possible use of the one-hybrid system in screening for methylated DNA-binding proteins. In this study, we pursued the possibility and developed a one-hybrid system to screen cDNA libraries for clones encoding proteins that recognize methylated DNA.

*To whom correspondence should be addressed. Tel: +81 4 7136 3989; Fax: +81 4 7136 3979; Email: ito@bi.s.u-tokyo.ac.jp

© The Author(s) 2010. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
MATERIALS AND METHODS

Construction of strains for one-hybrid screening

We constructed two integration vectors pOHAK and pOHHZ to generate ADE2 and HIS3 reporter genes, respectively (Figure 1). We amplified and cloned a DNA fragment spanning AFI1-GAL1UAS-GAL1TATA-ADE2 from PJ69-2A (Clontech), which contains the 3'-most 350 nt of AFI1 and the 5'-most 455 nt of ADE2, to pT7-Blue (Novagen) and replaced the GAL2UAS with a fragment containing KanMX and an HpaI site to obtain pOHAK. Similarly, we cloned a fragment spanning LYS2-GAL1UAS-GAL1TATA-HIS3 containing the 3'-most 254 nt of LYS2 and the 5'-most 426 nt of HIS3 and replaced the GAL1UAS with a fragment containing ZeoR and a Pmel site to obtain pOHHZ.

Next, we prepared a DNA fragment LexAop8-Sm4 comprised of eight tandemly iterated copies of LexAop followed by four tandemly iterated copies of Sm (CAG CCG CCA ACG CGC CCA ACG CTG GGA; methylatable CpG residues are underlined) (8). We cloned the LexAop8-Sm4 fragment into the HpaI site of pOHAK, digested the obtained plasmid by SalI and KpnI, used the linearized plasmid DNA to transform PJ69-2A cells (Clontech) and selected G418-resistant transformants. Following a PCR-based examination of correct integration of the bait sequence between AFI1 and ADE2, we confirmed that the strain fails to grow in the absence of adenine. We then transformed the obtained strain with BglII/KpnI-linearized pOHHZ plasmid bearing the LexAop8-Sm4 fragment at its PmeI site, and isolated Zeocin-resistant clones. We confirmed the correct integration of the second bait sequence between LYS2 and HIS3 as well as the histidine-dependent growth of the strain.

Finally, we transformed the strain with pALMS, a TRP1-marked 2μ plasmid that expresses LexA-fused M.SssI under the control of ADH1 promoter, and selected the transformants on synthetic complete media (SC) lacking tryptophan (SC–Trp) (9). Consequently, we obtained a single-bait/dual-reporter system. For the construction of a dual-bait/dual-reporter system, we used GAM12 or (GAC)12 (10) as the second bait. To conduct a dual-bait reverse one-hybrid selection, we replaced the ura5-52 allele in the single-bait/dual-reporter strain with a URA3 allele whose promoter bears Sm4 but not LexAop8.

One-hybrid screening of cDNA libraries

Mouse brain and human ovary Matchmaker™ cDNA libraries were purchased from Clontech. The single- and dual-bait strains constructed as above were transformed with the library plasmids using the standard lithium acetate method (9). The transformants were selected on SC–Trp–Leu–His–Ade. On the other hand, an aliquot of the transformants was serially diluted and spread on SC–Trp–Leu agar plates to estimate the size of screening or the number of transformants screened. The library plasmid was isolated from each positive colony and subjected to DNA sequencing.

RESULTS

Construction of a yeast one-hybrid system with auxotrophic reporter genes regulated by a methylatable bait sequence

It is essential for a large-scale yeast one/two-hybrid screening to use auxotrophic markers (e.g. ADE2, HIS3) as reporter genes, since transformants with auxotrophic reporters can be spread on agar plates at much higher density than those with colorimetric reporters (e.g. lacZ). Thus, we developed a host strain in which a promoter carrying a methylatable bait sequence regulates the expression of ADE2 and HIS3 (Figure 2A). Note that these reporter genes were integrated into the host chromosomes.

Figure 1. Integration vectors to construct one-hybrid strains with ADE2 and HIS3 reporter genes. (A) Integration vector pOHAK for ADE2 reporter gene. We cloned a fragment spanning AFI1-GAL2UAS-GAL2TATA-ADE2 from PJ69-2A and replaced the GAL2UAS with a fragment containing KanMX and an HpaI site to obtain pOHAK. (B) Integration vector pOHHZ for HIS3 reporter gene. We cloned a fragment spanning LYS2-GAL1UAS-GAL1TATA-HIS3 from PJ69-2A and replaced the GAL1UAS with a fragment containing ZeoR and a Pmel site to obtain pOHHZ. (C) Schematic representation of reporter gene construction in the case of ADE2 as an example. A methylatable bait sequence is cloned at the HpaI site of pOHAK, and the plasmid linearized by SalI-KpnI digestion is used for transformation of PJ69-2A. Homologous recombination occurs within ORFs for AFI1 and ADE2.
in contrast with the episomal lacZ reporter used in the previous work (7). In this strain, we used Sm4 or four tandemly iterated copies of Sm as the bait (8). We placed eight tandemly iterated copies of LexAop (LexAop8) at the 5′-flanking region of the Sm4 bait sequence to tether LexA-fused M.SssI CpG methyltransferase (LexA-M.SssI) for targeted methylation (7). If a methylated DNA-binding protein fused with Gal4 activation domain (GAD) binds the methylated Sm4 bait, it activates the expression of ADE2 and HIS3. Indeed, transformation with the plasmid that expresses LexA-M.SssI β-galactosidase activity in the extract of each transformant was measured using a standard method (9).

Identification of methylation-dependent and -insensitive DNA-binding proteins by the single-bait one-hybrid system

Confirming that the single-bait/dual-reporter strain described above behaved as intended, we exploited it to screen a GAD-fusion cDNA library constructed from mouse brain mRNA. We screened ∼3.22 × 10⁶ clones to identify 77 positives capable of growing in the absence of adenine and histidine. We sequenced the plasmids in positive clones to reveal that all of them encode DNA-binding proteins (Table 1). Note that we regarded six PLZF clones as a noise inherent to the system, because PLZF was shown to bind LexAop (11). Of the 71 clones remained, 26 (37%) were derived from MBD2 and judged as true positives. We also screened 2.76 × 10⁶ clones from a human ovary cDNA library and identified 158 positive clones, 49 of which were turned out be PLZF (Table 1). Of the 109 clones remained, 20 (18%) were judged as true positives, including 19 and 1 clones for MBD2 and MBD4, respectively. The other 89 clones were derived from various transcription factors.

To examine whether or not the identified proteins other than MBD2 and MBD4 bind the bait in a methylation-dependent manner, we examined their one-hybrid interaction with the Sm4 sequence using a lacZ reporter plasmid in the absence and presence of pALMS that expresses LexA-M.SssI β-galactosidase activity (Fig. 2B). As shown in Figure 2B, these proteins bound the bait regardless of its methylation status, thereby failing to show a methylation-dependent interaction mode.

These results demonstrated that the simple single-bait screening can identify methylated DNA-binding proteins but suffers from background clones derived from methylatioin-insensitive DNA-binding proteins.
Discrimination between methylation-dependent and -insensitive DNA-binding proteins by a dual-bait reverse one-hybrid system

Inspired by a dual-bait reverse two-hybrid system to select separation-of-function alleles (12), we intended to examine whether or not the reverse one-hybrid selection (13) can eliminate the backgrounds. We modified the single-bait/dual-reporter strain described above to bear an additional Sm4 bait sequence that is notably preceded by no LexAop to escape methylation and followed by URA3 to enable reverse one-hybrid selection (Figure 3A). If a GAD-fused protein binds the Sm4 bait in a methylation-insensitive manner, it would induce the expression of both ADE2/HIS3 and URA3. Such cells can be eradicated in the presence of 5-fluoro-orotic acid (5-FOA), because URA3 encodes orotidine-5'-phosphate decarboxylase that converts 5-FOA to 5-fluorouracil, a potent cytotoxin to kill the yeast (9,13). In contrast, if a GAD-fused protein binds the Sm4 bait in a methylation-dependent manner, it would induce the expression of ADE2/HIS3 but not URA3, making the cell viable in the presence of 5-FOA and uracil. In other words, 5-FOA-resistant Ade+/His+ cells should bear plasmids encoding methylation-dependent DNA-binding proteins.

To test the performance of the dual-bait reverse one-hybrid selection, we used MBD2 and KLF6 as representatives of methylation-dependent and -insensitive DNA-binding proteins, respectively. The strain bearing MBD2, but not KLF6, grew in the medium devoid of adenine and histidine but supplemented with uracil and 5-FOA (Figure 3B). These results provide a proof-of-concept for the dual-bait reverse one-hybrid selection of methylation-dependent DNA-binding proteins. Unfortunately, the selection was not perfect, as we observed leaky growth of a KLF6 clone, which might lead to backgrounds in library screening. The system would be useful rather for examining the methylation dependence of DNA-binding proteins identified by one-hybrid screening.

High-specificity screening for methylated DNA-binding proteins by a dual-bait one-hybrid system

We developed a third system that concurrently uses two independent bait sequences, based on the following simple rationale. If a protein leading to background clones binds the Sm4 bait in a methylation-insensitive, but sequence-dependent manner, it would fail to bind a second bait sequence that lacks any similarity to Sm4, because it is highly unlikely that a single sequence-specific DNA-binding protein can interact with two totally unrelated sequences. To test this idea, we integrated Sm4 and GAM12 upstream of ADE2 and HIS3, respectively, to develop a third system (Figure 3C) and confirmed that the expression of MBD2, but not KLF6, made the cell viable in the absence of adenine and histidine (data not shown).

We used this system to screen the same cDNA libraries as those that we screened using the single-bait system. Screening of 0.32×10^6 clones from the mouse brain library led us to isolate six non-PLZF positives, five (83%) of which were clones for MBD2 or true positives.
We developed a yeast one-hybrid system for selective cloning of methylated DNA-binding proteins. Although our previous method detected methylated DNA–protein interactions via lacZ reporter activity, it could not screen libraries (7). A single-bait dual-reporter system, which was basically constructed by simply replacing lacZ with ADE2 and HIS3, could screen libraries but suffered high backgrounds (Figure 2 and Table 1). We found that the dual-bait approach was critical to suppress background clones, which were almost always derived from methylation-insensitive sequence-specific DNA-binding proteins (Figures 2, 3 and Table 2). While it is formally possible that proteins showing two-hybrid interactions with LexA or M.SssI lead to backgrounds even in the dual-bait system, we have so far encountered no such clones and, even if we do any, we will be able to discriminate them from true positives through appropriate control experiments.

We recommend the dual-bait system with two independent bait sequences for initial screening (Figure 3C). Each positive clone should be retransformed to the same strain in the absence and presence of LexA-M.SssI as a second screening to confirm methylation dependence of the interaction. Alternatively, one may use the dual-bait reverse one-hybrid system for this purpose (Figure 3A). This two-step approach is critical to select candidates for methylated DNA-binding proteins, especially when screening libraries from organisms with poorly annotated genomes. Finally, the interaction has to be biochemically verified, typically, by an electrophoretic mobility shift assay using methylated and unmethylated DNA.

A matter of concern with this system would be low sensitivity leading to false negatives. For instance, our screening of a brain cDNA library identified MB2 but not MeCP2 abundantly expressed in neurons (14). Of note, these two proteins show different preferences for methylated DNA; MeCP2 requires an A/T-rich sequence adjacent to methyl-CpG for efficient DNA binding, whereas MB2 does not (15). While new methods and strategies may help to overcome the problem of low sensitivity, we recommend establishing a second screening step to confirm methylation dependence of the interaction.

We recommend the dual-bait system described here and its variants would provide valuable research tools for epigenetics.
ACKNOWLEDGEMENTS

We thank Kazuyuki Mizushima for his contribution to vector construction.

FUNDING

Genome Network Project and Cell Innovation Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (to T.I.). Funding for open access charge: MEXT.

Conflict of interest statement. None declared.

REFERENCES

1. Suzuki, M.M. and Bird, A. (2008) DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet., 9, 465–476.
2. Feng, S., Cokus, S.J., Zhang, X., Chen, P.Y., Bostick, M., Goll, M.G., Hetzel, J., Jain, J., Strauss, S.H., Halperrn, M.E. et al. (2010) Conservation and divergence of methylation patterning in plants and animals. Proc. Natl Acad. Sci. USA, 107, 8689–8694.
3. Zemach, A., McDaniel, I.E., Silva, P. and Zilberman, D. (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science, 328, 916–919.
4. Law, J.A. and Jacobsen, S.E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat. Rev. Genet., 11, 204–220.
5. Sasai, N. and Defossez, P.A. (2009) Many paths to one goal? The proteins that recognize methylated DNA in eukaryotes. Int. J. Dev. Biol., 53, 323–334.
6. Li, J.J. and Herskowitz, I. (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science, 262, 1870–1874.
7. Feng, S.Y., Ota, K., Yamada, Y., Sawabu, N. and Ito, T. (2004) A yeast one-hybrid system to detect methylation-dependent DNA–protein interactions. Biochem. Biophys. Res. Commun., 313, 922–925.
8. Prokhortchouk, A., Hendrich, B., Jørgensen, H., Ruzov, A., Wiln, M., Georgiev, G., Bird, A. and Prokhortchouk, E. (2001) The β20 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev., 15, 1613–1618.
9. Adams, A., Gottschling, D.E., Kaiser, C.A. and Steams, T. (1997) Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Nan, X., Mechein, R.R. and Bird, A. (1993) Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res., 21, 4886–4892.
11. Sitterlin, D., Tiollais, P. and Transy, C. (1997) The RARα-PLZF chimera associated with Acute Promyelocytic Leukemia has retained a sequence-specific DNA-binding domain. Oncogene, 14, 1067–1074.
12. Yamaguchi, Y., Ota, K. and Ito, T. (2007) A novel Cde42-interacting domain of the yeast polarity establishment protein Bem1: implications for modulation of mating pheromone signaling. J. Biol. Chem., 282, 29–38.
13. Vidal, M., Brachmann, R.K., Fattaey, A., Harlow, E. and Boeke, J.D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein–protein and DNA–protein interactions. Proc. Natl Acad. Sci. USA, 93, 10315–10320.
14. Skene, P.J., Illingworth, R.S., Webb, S., Kerr, A.R., James, K.D., Turner, D.J., Andrews, R. and Bird, A.P. (2010) Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state. Mol. Cell, 37, 457–468.
15. Klose, R.J., Sarraf, S.A., Schmiedeberg, L., McDermott, S.M., Stancheva, I. and Bird, A.P. (2005) DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. Mol. Cell, 19, 667–678.
16. Clouaire, T., de Las Heras, J.I., Merusi, C. and Stancheva, I. (2010) Recruitment of MBD1 to target genes requires sequence-specific interaction of the MBD domain with methylated DNA. Nucleic Acids Res., 38, 4620–4634.
17. Sasai, N., Nakao, M. and Defossez, P.A. (2010) Sequence-specific recognition of methylated DNA by human zinc-finger proteins. Nucleic Acids Res., doi:10.1093/nar/gkq280; April 19, 2010 [Epub ahead of print].
18. Kladde, M.P., Xu, M. and Simpson, R.T. (1996) Direct study of DNA–protein interactions in repressed and active chromatin in living cells. EMBO J., 15, 6290–6300.
19. Lister, R., Pelizzola, M., Down, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M. et al. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature, 462, 315–322.
20. Gerasimenia, R., Villkaitis, G. and Klimasauskas, S. (2009) A directed evolution design of a GCG-specific DNA hemimethylase. Nucleic Acids Res., 37, 7332–7341.
21. Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, I.L.M., Liu, D.R., Aravin, L. et al. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science, 324, 930–935.
22. Jin, S.G., Kadam, S. and Pfeifer, G.P. (2010) Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Res., 38, e125.