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Human DNA methyltransferase gene-transformed yeasts display an inducible flocculation inhibited by 5-aza-2′-deoxycytidine

Kei-ichi Sugiyama *, Makiko Takamune, Hiroko Furusawa, Masamitsu Honma

Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

Mammalian DNA methyltransferases (DNMTs) play an important role in establishing and maintaining the proper regulation of epigenetic information. However, it remains unclear whether mammalian DNMTs can be functionally expressed in yeasts, which probably lack endogenous DNMTs. We cotransformed the budding yeast *Saccharomyces cerevisiae* with the human *DNMT1* gene, which encodes a methylation maintenance enzyme, and the *DNMT3A/3B* genes, which encode *de novo* methylation enzymes, in an expression vector also containing the *GAL1* promoter, which is induced by galactose, and examined the effects of the DNMT inhibitor 5-aza-2′-deoxycytidine (5AZ) on cell growth. Transformed yeast strains grown in galactose- and glucose-containing media showed growth inhibition, and their growth rate was unaffected by 5AZ. Conversely, 5AZ, but not 2′-deoxycytidine, dose-dependently interfered with the flocculation exhibited by *DNMT*-gene transformants grown in glucose-containing medium. Further investigation of the properties of this flocculation indicated that it may be dependent on the expression of a Flocculin-encoding gene, *FLO1*. Taken together, these findings suggest that *DNMT*-gene transformed yeast strains functionally express these enzymes and represent a useful tool for *in vivo* screening for DNMT inhibitors.

1. Introduction

The methylation of DNA, an epigenetic modification, is of central importance for normal cellular development, and changes in DNA methylation may affect the development of cancer [1]. The methylation of cytosine residues in nuclear DNA, a common form of DNA methylation found predominantly in cytosine–phosphate–guanine (CpG) dinucleotides, is an important epigenetic event [2]. Three catalytically active DNA methyltransferases (DNMTs) are involved in the establishment and maintenance of DNA methylation patterns in mammalian cells. DNMT1, which preferentially methylates hemi-methylated DNA, is critical for maintaining DNA methylation patterns [3]. Conversely, DNMT3A and DNMT3B, members of the DNMT3 family of *de novo* DNA methyltransferases, are involved in establishing DNA methylation patterns during normal embryonic and germ cell development and mainly act on cytosines within unmethylated CpGs [4,5].

5-Aza-2′-deoxycytidine (decitabine; 5AZ), an analog of the natural nucleoside 2′-deoxycytidine, is known to inhibit DNA methylation and is approved for the treatment of myelodysplastic syndrome [6,7]. 5AZ exerts an inhibitory effect on the activity of DNMTs, consequently promoting the reactivation of tumor suppressor genes silenced by DNA methylation [8]. 5AZ taken into cells is phosphorylated and incorporated into the DNA by DNA polymerase [9,10]. Subsequently, DNMTs become trapped by covalently binding to 5AZ, resulting in the depletion of DNMTs [11]. Therefore, generation of the phenotypes associated with 5AZ may facilitate the identification of novel DNMT inhibitors.

In most eukaryotes, DNA methylation occurs exclusively at cytosine residues. Reportedly, 80% of CpG dinucleotides in mammalian genomes are methylated [12]. In contrast, no *DNMT*-like genes are found in the genome of the budding yeast *Saccharomyces cerevisiae*, a model organism for eukaryotic cells [13]. The ease of genetic manipulation of *S. cerevisiae* predisposes it for use as a platform for the functional analysis of mammalian DNMTs. The development of a yeast cell-based system for detecting DNA methylation levels may provide an *in vivo* screening system to identify novel DNMT inhibitors. In this study, we created yeast strains

Abbreviations: 5AZ, 5-aza-2′-deoxycytidine; ACT1, β-actin; CpG, cytosine–phosphate–guanine; DNMT, DNA methyltransferase; OD_{600}, optical density at 600 nm; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction.

* Corresponding author. Fax: +81 3 3700 9847.
E-mail address: sugiyama@nihs.go.jp (K.-i. Sugiyama).

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transformed with plasmids encoding the human *DNMT1* and *DNMT3A*/*3B* cDNAs and revealed that these transformants displayed an inducible flocculation phenotype that could be inhibited by 5AZ. Furthermore, we discovered that significantly high mRNA transcript levels of *FLO1*, a Flocculin-encoding gene that causes nonsexual flocculation in yeast [14], are present in the transformants in a glucose-based medium, whereas the upregulation of *FLO1* mRNA is repressed by 5AZ. Here, for the first time, we report the creation of engineered yeasts that have gained the ability to respond to 5AZ.

2. Materials and methods

2.1. Strains, culture conditions, and plasmids

S. cerevisiae YPH250 (*MAT*a *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2–801 ade2–101 ura3–52*) was obtained from the Yeast Genetic Stock Center (University of California at Berkeley, CA, USA). Yeast cells were cultivated at 30 °C in a synthetic medium with a 0.67% yeast nitrogen base containing the appropriate amino acids and carbon sources (final concentration 2%). For 5AZ treatment, yeast cells were incubated with specified amounts of 5AZ. To construct human DNMT1, DNMT3A, and DNMT3B expression plasmids, the open-reading frame (ORF) regions of each gene were amplified by polymerase chain reaction (PCR) and inserted into the multiple cloning site of the expression vectors pYES2/CT or pYES3/CT (Invitrogen, Carlsbad, CA, USA). Construction of the expression plasmids for each DNMT proceeded as follows. The DNMT1 expression plasmid, pY2CThD1, was constructed by cloning a fragment encoding the *DNMT1* ORF, amplified from *DNMT1* cDNA (BC144093), into the pYES2/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGCCGGCGCGTACCGCCCCAGC-3′ and 5′-GGGAATTCCTAGTCCTTAGCAGCTTCCT-3′. The DNMT3A expression plasmid, pY3CThD3A, was constructed by cloning a fragment encoding the *DNMT3A* ORF, amplified from *DNMT3A* cDNA (BC043617), into the pYES3/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGCCCGCCATGCCCTCCAGCGG-3′ and 5′-GGGAATTCTTACACACACGCAAAATACT-3′. The DNMT3B expression plasmid, pY3CThD3B, was constructed by cloning a fragment encoding the *DNMT3B* ORF, amplified from *DNMT3B* cDNA (BC111933), into the pYES3/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGAAGGGAGACACCAGGCATCT-3′ and 5′-GGGAATTCTCACATGCAAAGTAGTCCTT-3′. The constructed plasmids were verified by DNA sequencing.

2.2. Western blotting analysis

Transformed yeast cells were grown to the late logarithmic phase (approximate optical density at 600 nm [OD_{600}] = 4) in synthetic glucose-containing medium lacking uracil and tryptophan, then the cells were collected by centrifugation, washed, and resuspended at an OD_{600} of 0.4 in synthetic galactose-containing medium supplemented with appropriate requirements. Yeast cells harboring plasmids were cultivated for 0, 16 and 24 h. After centrifugation, cell pellets were rinsed once with 0.9% NaCl. Pellets were suspended in phosphate-buffered saline containing a protease inhibitor cocktail for yeast (Sigma–Aldrich Corporation, St. Louis, MO, USA). The suspension was vortexed with zirconium beads at 4 °C for 5 min, then centrifuged at 3500×*g* for 10 min to remove cell debris. To examine the expression of DNMT1, DNMT3A, DNMT3B, and β-actin (ACT1), we conducted Western blotting analysis using rabbit polyclonal anti-DNMT1 (ab19905; Abcam, Cambridge, UK), rabbit polyclonal anti-DNMT3A (SC-20703; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), mouse monoclonal anti-DNMT3B (IMG-184A; Imgenex Corporation, San Diego, CA, USA), and mouse monoclonal anti-β-actin (Abcam) antibodies. β-Actin was used as a loading control. Horseradish peroxidase-conjugated anti-rabbit/mouse Immunoglobulin G antibodies from Jackson Immunoresearch Labs (West Grove, PA, USA) were used as the secondary antibodies. The signals were visualized using Immunostar LD (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.3. Measurement of growth rate and flocculation

Transformed yeast cells were cultured in synthetic glucose- or galactose-containing minimal medium, with or without 100 μM 5AZ. Cell growth was monitored by measuring OD_{600}. To measure flocculation ability, culture tubes allowed to settle for 5 min were photographed from beneath and the diameters of both floc (F) and tube (T) were measured. Relative flocculation activity was calculated using the following equation:

$$\text{Relative flocculation activity} = 100 \times (F/T)$$

2.4. Semi-quantitative reverse-transcription polymerase chain reaction

Yeast cells grown to the late logarithmic phase in the glucose-containing medium were harvested. Total RNA was prepared by the glass bead method using an RNeasy Kit (Qiagen N.V., Venlo, Limburg, The Netherlands) with RNase-free DNase treatment, according to the manufacturer's instructions. Total RNA (0.5 μg) was subjected to reverse-transcription (RT)-PCR using the SuperScript® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). The following primer pairs were used: *FLO1*, 5′-CTCATCGCTATATGTTTTTGG-3′ (forward) and 5′-CGAGTAAACAACCTTCATTGG-3′ (reverse); *FLO11*, 5′-GTCACGACGGCTATTCCAACCACAGTTATTACC-3′ (forward) and 5′-GAATACAACTGGAAGAGCGAGTAGCAACCAC-3′ (reverse); and *ACT1*, 5′-ATTCTGAGGTTGCTGCTTTGG-3′ (forward) and 5′-GAAGATTGAGCAGCGGTTTGC-3′ (reverse).

2.5. Statistical analysis

Statistical tests comparing multiple groups were performed using one-way analysis of variance followed by Dunnett's *post hoc* test. Data are expressed as means ± the standard error of the mean.

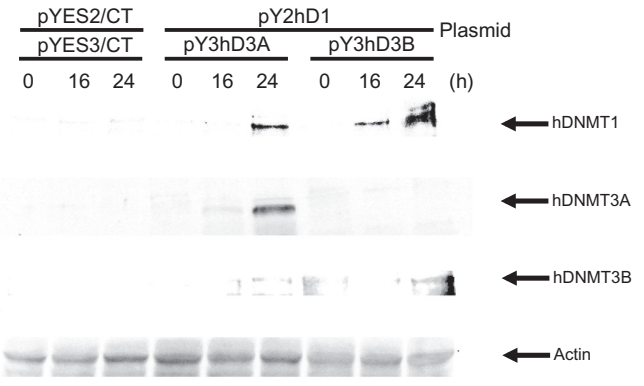

Fig. 1. Levels of expression of DNA methyltransferases. Cells were grown to the late logarithmic phase (an optical density at 600 nm [OD_{600}] of 3–4) in liquid synthetic glucose-containing medium lacking uracil and tryptophan. Cells were collected by centrifugation, washed, and adjusted to an OD_{600} of 0.4 with synthetic galactose-containing medium lacking uracil and tryptophan. The cells were further cultured for 16 or 24 h. The extracts were prepared and analyzed for DNA methyltransferase (DNMT) 1, DNMT3A, DNMT3B, and β-actin proteins by Western blotting. The results are representative of three independent experiments.

3. Results

3.1. DNA methyltransferase expression in yeast cells

It is thought that DNMT1 acts as a "maintenance" methyltransferase, whereas DNMT3A and DNMT3B act as "*de novo*" enzymes [15,16]. To coexpress human DNMT1 and DNMT3A/3B in yeast cells, the coding regions of *DNMTs* were placed in expression vectors under the control of the *GAL1* promoter. Yeast cells transformed with the *DNMT1* and *DNMT3A*/*3B* cDNAs were grown in a synthetic glucose-containing medium to the late logarithmic phase and were subsequently transferred to a synthetic galactose-containing medium to induce DNMT expression. DNMT expression was analyzed by Western blotting using antibodies for each DNMT. As shown in Fig. 1, DNMT1 and DNMT3A/3B were expressed in yeast cells cotransformed with these genes 24 h after induction. This result indicates that DNMT-gene transformants possess the ability to express these DNMTs.

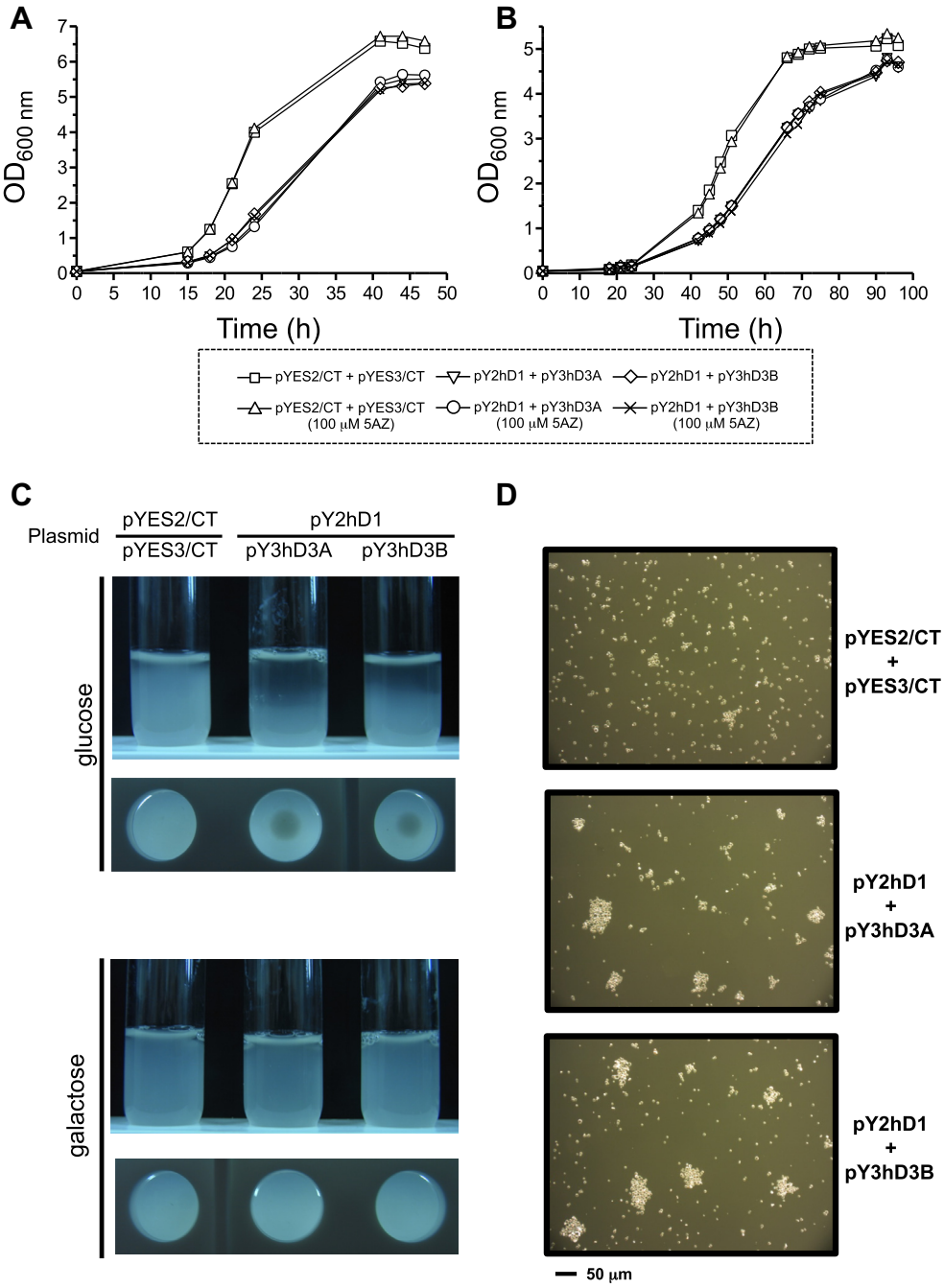

Fig. 2. Phenotypic analysis of transformants. (A and B) Effect of 5-aza-2′-deoxycytidine (5AZ) on the growth of (A) glucose- and (B) galactose-grown transformants. Growth of cells cultured in the presence or absence of 100 μM 5AZ was monitored by measuring optical density at 600 nm (OD_{600}) periodically. Data show the average results of three experiments. (C) Cells were grown to an OD_{600} of 4–6 in glucose- or galactose-containing synthetic dropout liquid medium lacking tryptophan and uracil. Glucose-grown cells cotransformed with pY2hD1 and pY3hD3A/3B flocculate, so that the yeast cells sink to the bottom of the tube. After allowing the yeast to settle for 5 min, flocculation was photographed from the side and underneath. (D) Digital micrographs of glucose-grown transformants. We observed glucose-grown cells to monitor the level of flocculation.

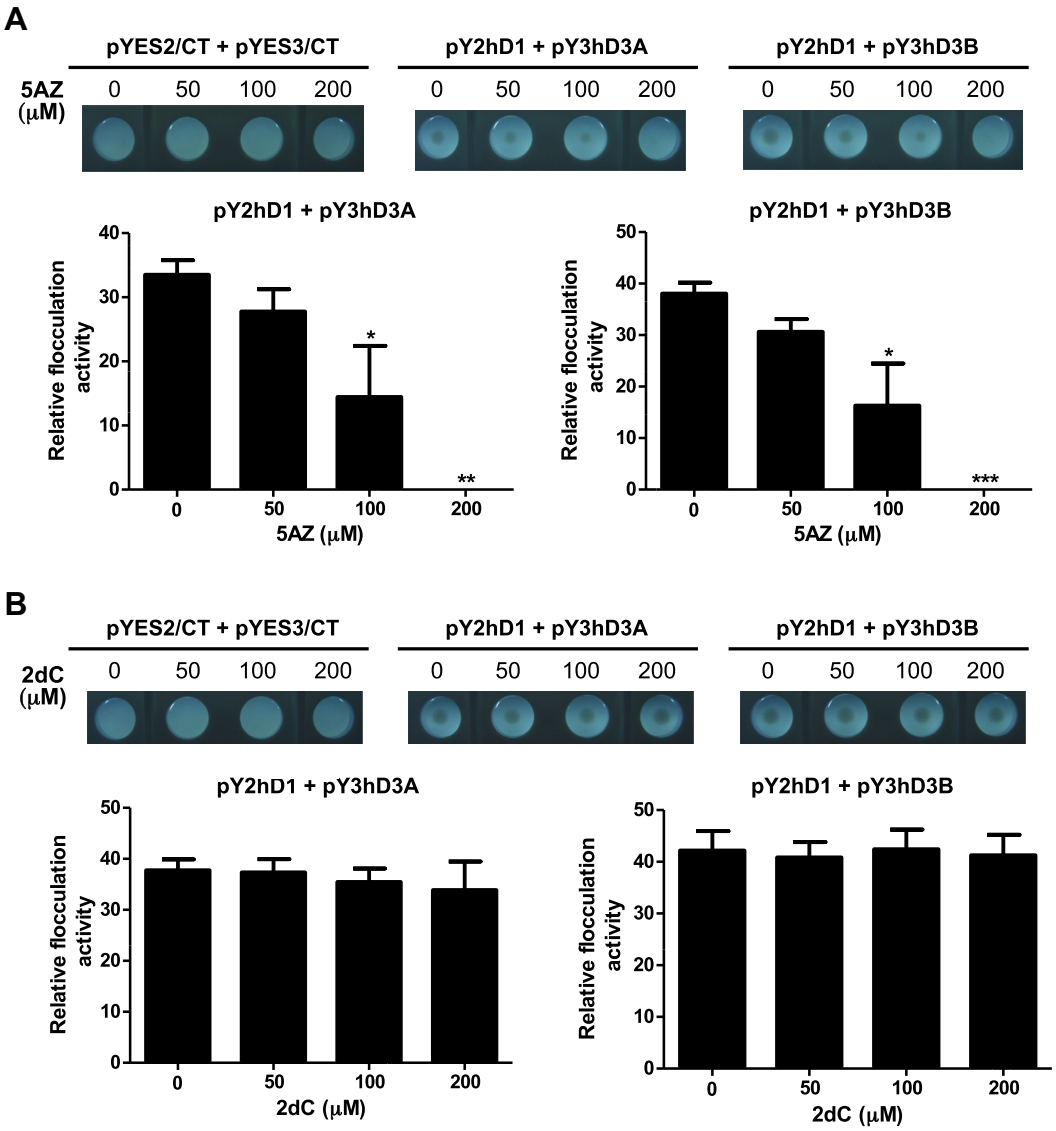

Fig. 3. Effects of 5-aza-2′-deoxycytidine and the natural nucleoside 2dC on the flocculation of glucose-grown transformants. (A and B) Yeast cells treated with 5-aza-2′-deoxycytidine (5AZ; A) and the natural nucleoside 2dC (B) were grown to an optical density at 600 nm (OD_{600}) of 4–7 in glucose-containing synthetic dropout liquid medium lacking tryptophan and uracil. Pictures were taken after allowing the cells to settle at room temperature for 5 min. These experiments were repeated at least three times with similar results, and data from one representative experiment are shown. Histograms show the relative flocculation activity. Data are presented as means ± standard error of the mean from three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's *post hoc* test ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ versus control).

3.2. DNMT-gene transformants grown in glucose-based medium exhibit flocculation

To investigate the effects of the induction of DNMT expression, we first checked the growth of the transformants in the absence and presence of 5AZ, a DNMT inhibitor [17]. As shown in Fig. 2A and B, growth rates on glucose- and galactose-containing media were reduced in yeast cells harboring DNMT expression plasmids compared with yeast cells harboring empty vector. Furthermore, 5AZ had no effect on the growth rate of the transformants. These results suggest that the low growth rate of *DNMT*-gene transformants grown in both galactose- and glucose-containing media is one reason for the expression of DNMTs and that the decrease in the proliferation of these transformants is not reversible by 5AZ.

Reportedly, the tendency of yeast cells to flocculate is related to a growth-arrested phenotype [18]. Because we observed growth inhibition of *DNMT*-gene transformants in both glucose- and galactose-containing media, we examined the glucose- and galactose-based culture media of each transformant. Yeast strains harboring DNMT expression plasmids showed flocculation when grown in glucose-containing medium, but were nonflocculent in the galactose-containing medium (Fig. 2C). Furthermore, examination of the flocculating cells by microscopy revealed aggregation of these strains (Fig. 2D). These results indicate that an inducible flocculation phenotype is generated in *DNMT*-gene transformants grown in glucose-containing media, but not galactose-containing media.

3.3. Flocculation in DNA methyltransferase-gene transformants is repressed by 5-aza-2′-deoxycytidine

Our discovery of an inducible flocculation phenotype in glucose-grown transformants carrying DNMT1 and DNMT3A/3B expression plasmids led us to investigate the effects of 5AZ on this morphogenetic event. In the glucose-containing medium, yeast cells cotransformed with cDNAs encoding *DNMT1* and *DNMT3A*/

3B underwent flocculation, which was significantly suppressed by the addition of 5AZ in a dose-dependent manner. In particular, this flocculation did not occur in the presence of 200 μM 5AZ. Conversely, the flocculation phenotype conferred resistance to the natural nucleoside 2dC, which has a molecular structure similar to that of 5AZ (Fig. 3). Moreover, 5-azacytidine, a DNMT inhibitor, caused a reduction in flocculation activity similar to that affected by 5AZ in *DNMT*-gene transformants grown in the glucose-containing medium in a dose-dependent manner (Supplementary Fig. 1). Taken together, these results led us to speculate that cotransformants carrying the *DNMT1* and *DNMT3A*/*3B* genes functionally express these DNMTs and gain the ability to respond to DNMT inhibition.

3.4. Effect of 5-aza-2′-deoxycytidine on the induction of FLO1 mRNA in DNA methyltransferase-gene transformants

The experimental data in the literature is obtained with YPH250, which is derived from S288C as a reference strain [19]. The *FLO8* gene, which encodes a transcriptional activator of the dominant flocculation genes *FLO1* and *FLO11*, harbors a nonsense mutation in S288C [20,21]. To investigate *FLO1* and *FLO11* mRNA levels in the *DNMT*-gene transformants, we performed semi-quantitative RT-PCR analysis on total RNA. In these cotransformants (derivatives of S288C), although *FLO1* and *FLO11* mRNA were detectable, *FLO1*, but not *FLO11*, mRNA levels were increased in *DNMT*-genes transformants (Fig. 4A).

To investigate whether 5AZ affects the level of *FLO1* mRNA in *DNMT*-genes transformants, we analyzed the level of *FLO1* mRNA expression. We found that 5AZ (200 μM) treatment repressed the enhanced level of *FLO1* mRNA expression in these transformants (Fig. 4B). Taken together, these results suggest that the inducible flocculation phenotype generated in cotransformants carrying *DNMT1* and *DNMT3A*/*3B* genes can be induced or repressed by altering the level of *FLO1* mRNA expression. Although further experiments are needed to confirm the effect of the level of *FLO1* mRNA expression on flocculation, the results presented here suggest that 5AZ affects mRNA expression of the Flocculin-encoding gene, *FLO1*.

4. Discussion

Although methylation at position 5 of the cytosine moiety catalyzed by DNMT is a heritable epigenetic modification [22], no *DNMT*-like genes are believed to exist in the model eukaryote *S. cerevisiae* [13]. In this study, we demonstrated that transformation with the *DNMT1* and *DNMT3A*/*3B* cDNAs causes yeast cells to acquire a flocculation phenotype that can be inhibited by the DNMT inhibitor, 5AZ [17]. These results suggest that these transformants represent a useful tool for the identification of DNMT inhibitors.

A previous study [23] reported that 5AZ has no effect on the growth rate of yeast expressing mouse DNMT1 or DNMT3A and that the presence of both enzymes affected no fundamental properties of yeasts. However, our findings indicate that yeast cotransformants carrying the *DNMT1* and *DNMT3A*/*3B* genes experienced growth inhibition and exhibited inducible flocculation (Fig. 2). One possible explanation for this discrepancy is that these two apparently different phenotypes are linked to the background genome of each strain; therefore, the strain used in this study may represent a good model for the study of DNMT function *in vivo*. We speculate that exogenously expressed DNMTs will affect the regulation of cellular growth and/or metabolism in the yeast strain YPH250.

Although *GAL1* gene expression in *S. cerevisiae* is repressed in the glucose-based medium [24], our results indicate that 5AZ inhibits an inducible flocculation phenotype in *DNMT*-gene transformants grown in the synthetic glucose-containing medium. This implies that this flocculation is caused by expression of DNMTs. This observation is consistent with a previous report [25] that states that the *GAL1* promoter causes leaky expression in glucose-based media. Furthermore, transformants with low levels of DNMTs seem to be more sensitive to DNMT inhibitors, suggesting that the glucose-induced promotion of flocculation is useful for the detection of novel DNMT inhibitors.

We showed that glucose-grown cells cotransformed with cDNAs encoding *DNMT1* and *DNMT3A*/*3B* displayed an inducible flocculation phenotype inhibited by 5AZ and that the *FLO1* gene, which encodes a cell-surface Flocculin [26], was upregulated in these cells compared with control cells (transformed with empty vector; Figs. 3A and 4A). These results suggest that the inducible flocculation is coordinated by *FLO1*, the expression of which is regulated through DNMT-mediated epigenetic effects on the *FLO1* promoter. Indeed, the epigenetic modifying agent 5AZ (a DNMT inhibitor) inhibited the induction of mRNA encoding FLO1 in cotransformants (Fig. 4B). 5AZ incorporated into genomic DNA exerts DNMT inhibitory activity through covalent adduct formation with DNMTs, triggering proteasome-mediated degradation [17,27]. Therefore, it is also possible to state that DNMTs function-

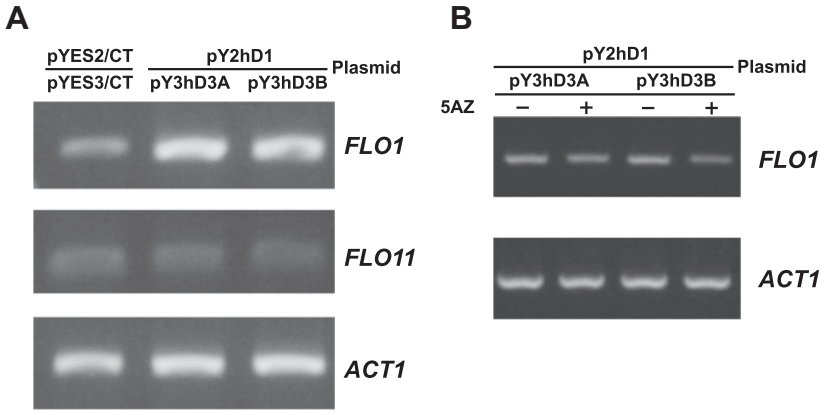

Fig. 4. mRNA levels of flocculation genes in glucose-grown transformants. (A) Yeast cells were grown to an optical density at 600 nm (OD_{600}) of 4–7 in glucose-containing synthetic dropout liquid medium lacking tryptophan and uracil. Total RNA was extracted from transformants, and mRNA levels of *FLO1*, *FLO11*, and *β-actin* (*ACT1*) were monitored by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). The results are representative of three independent experiments. (B) Yeast cells were treated with the indicated concentration of 5-aza-2′-deoxycytidine for 40 h. Total RNA was extracted from the transformants, and mRNA levels of *FLO1* and *ACT1* were monitored by semi-quantitative RT-PCR. The results are representative of three independent experiments.

ally expressed in the nuclei of transformants display a 5AZ-inhibitable behavior, namely flocculation. We are now working to analyze the methylation level in the promoter region of the *FLO1* gene to characterize the epigenetic alterations of yeast transformants carrying the *DNMT1* and *DNMT3A*/*3B* genes.

Further experiments involving the identification of yeast CpG site methylation by heterogeneous DNMTs and the analysis of the relationship between histone modification and DNA methylation will help us to understand the principles of epigenetic modification in *S. cerevisiae*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.032.

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