TRANSCRIPTIONAL REGULATION AND CHROMATIN REMODELING MECHANISMS AT *PHO5*

A Dissertation

by

CHRISTOPHER DUMAS CARVIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Biochemistry

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Approved as to style and content by:

Michael Kladde (Chair of Committee)	Gregory Reinhart (Member)
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ABSTRACT

Transcriptional Regulation and Chromatin Remodeling Mechanisms at *PHO5*

(May 2004)

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Chair of Advisory Committee: Dr. Michael Kladde

Regulation of gene expression is vital for proper growth and prevention of disease states. In eukaryotes this regulation occurs in the context of chromatin which creates an inherent barrier for the binding of *trans*-acting factors, such as transcription factors and RNA polymerase. This dissertation focuses on the role of transcriptional activators and chromatin remodeling coactivators in the regulation of the repressible acid phosphatase gene *PHO5*. Our studies show that histone methylation at lysine 4 of histone H3 is required for the full repression of *PHO5* and *GAL1-10*. We show that bromodomains, a domain conserved in chromatin remodeling coactivators, may function to stabilize binding. Finally, we present a strategy using DNA methyltransferases as *in vivo* probes to detect DNA-protein interactions and examine chromatin structure. We extend this strategy to zinc-finger proteins which can be engineered to bind to any desired DNA sequence as a means of targeting methylation with potential use in epigenetic silencing.

DEDICATION

I would like to dedicate this dissertation to my future wife. Ingrid, without you, none of this would have been possible.

ACKNOWLEDGMENTS

I would first like to acknowledge my graduate advisor, Dr. Michael Kladde, for his support and guidance. Mike has been a very supportive mentor both in and out of the laboratory. I would also like to thank my committee members Drs. Geoffrey Kapler, Yi Wei Jiang, and Greg Reinhart for their continual support. I would also like to thank Drs. Mary Bryk and John Mueller for their assistance with strains and reagents. I am also appreciative of their support during my graduate work and in my future endeavors.

I would like to thank past and present members of the Kladde lab, soon to be Drs. Dan Neef and Walter Jessen as well as Laurie Friesenhahn, April Risinger, Biji Oommen, Jason McKinnie, and Alison Pirozzo for their support both professionally as well as personally. I am especially grateful to my fellow "PCR twin" Archana Dhasarathy. Her help has been invaluable to me and most of all she always has been supportive of me. I could not ask for a better friend.

I would like acknowledge the many friends that I have made here in Aggieland, especially Sarah Aubert, Kelly Vaughan, Peter Cornish, Mario Pennella, Rachel Idol, Angela Domitrovich, Lisa Forrester, and Audrey Nicholson.

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CHAPTER I

INTRODUCTION

SCOPE

The primary scope of this dissertation is to explore the role of chromatin remodeling enzymes in transcriptional regulation using the *PHO5* gene from *Saccharomyces cerevisiae* as a model. I will present evidence for a role of histone methylation in transcriptional repression of *PHO5* and *GAL1-10*. Next, the role of bromodomains in transcriptional activation will be further characterized. This dissertation will also demonstrate the use of targeted cytosine methylation to detect protein-DNA interactions, chromatin structure and to introduce *de novo* methylation to allow for epigenetic silencing in higher eukaryotes.

The first part of the introduction provides background information in the field of chromatin structure with an emphasis on its repressive role in gene expression. The middle will detail the myriad of chromatin remodeling enzymes which are utilized to effect transcriptional activation and repression. The last part will introduce the yeast repressible acid phosphatase gene *PHO5* as an ideal model system for the study of transcriptional regulation.

This dissertation follows the style and format of Cell.

SIGNIFICANCE

The packaging of DNA into chromatin creates an inherent repressive environment for the binding of *trans*-acting factors, such as transcription factors and the DNA replication machinery. Hence, eukaryotes have a variety of chromatin remodeling enzymes which remodel local chromatin structure to make it more accessible. These remodeling enzymes are highly conserved throughout all eukaryotic organisms and defects in these complexes have been correlated with a variety of diseases, including cancer. This dissertation will focus on the roles of histone methylation and bromodomains in gene expression. It will also introduce the use of DNA methyltransferases to characterize chromatin structure and detect protein-DNA interactions *in vivo*.

CHROMATIN STRUCTURE

In order for DNA to fit into the nucleus of a cell it must be heavily compacted. If the DNA sequence that comprises the human genome was stretched out end to end, it would reach three meters in length. This must be compressed to fit inside a nucleus that is five micrometers in diameter; thus a greater than 10,000-fold compaction is needed. This packaging of DNA is referred to as chromatin structure and is a widely-studied process with connections to a myriad of biological processes and diseases.

While chromatin is heavily studied, there is a lot that remains unknown. Most of what is known is at the first level of compaction. 146 bp of DNA is wrapped up by a histone octamer protein complex consisting of two copies each of histone H2A, H2B, H3, and H4 to form the nucleosome (Fig. 1-1), the basic repeating unit of chromosome organization (Richmond et al., 1988). An array of nucleosomes separated by small stretches of histone-free regions called linkers make up the "beads on a string" form and it is this form that most research focuses on. From here on, higher forms of chromatin organization occur. In mitotic condensation, a DNA molecule is packaged greater than 50,000 fold.

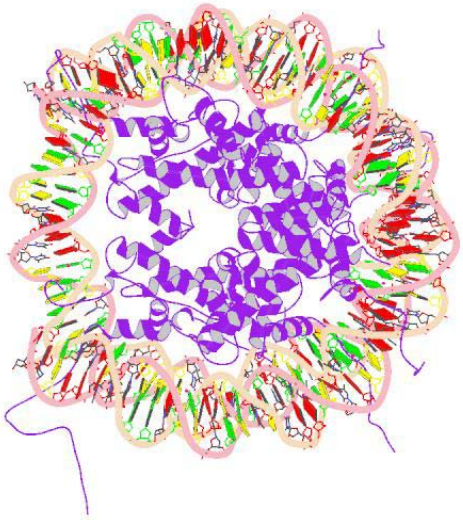

Figure 1-1. Crystal structure of the nucleosome core particle.

Ribbon diagram of the crystal structure at 2.5 Å resolution of 146 bp of DNA wrapped around histone octamer to form the nucleosome core particle (Harp et al., 2000). Random coils protruding from the nucleosome represent parts of the N-terminal tails of histones that yield high electron density in the crystal structure. The bulk of the N-terminal tails are not visible.

This heavy compaction creates a naturally repressive environment for almost all proteins including transcription factors, RNA polymerase, and the DNA replication/repair machinery. Chromatin is classified into two types based on the degree of compaction: euchromatin and heterochromatin. Euchromatin is less compacted than heterochromatin and is considered transcriptionally active chromatin while heterochromatin is generally silent. Some elegant studies using DNA methyltransferases as probes *in vivo* have shown that DNA packaged into a nucleosome is most inaccessible near the center or dyad. Within the first two helical turns of the nucleosome edge, there is modest accessibility but it is still significantly less than that seen in the linker region (Kladde and Simpson, 1994; Kladde et al., 1996).

A large percentage of *trans*-acting factor binding sites, such as TATA boxes and upstream activating sequences (UAS) are found in nucleosome-containing regions. This may be a result of evolutionary pressure as a means of controlling gene expression or just simply due to the fact that most linker regions are very small and the vast majority of DNA is packaged into nucleosomes. The actual size of linker regions is variable in different eukaryotes, ranging from zero to 100 bp with the shortest linker lengths in lower eukaryotes and the longest in animals (Wolfe, 1993). In any case, local chromatin remodeling is required for these sites to be utilized. Eukaryotes have developed

a highly conserved series of protein complexes which function to remodel chromatin.

CHROMATIN REMODELING

It is still unclear as to what precise mechanism(s) are involved in chromatin remodeling. In some instances, nucleosomes physically slide from one DNA region to another. A study showed that during activation of IFN-β, a single nucleosome is repositioned downstream by 36 bp, which exposes the TATA box (Lomvardas and Thanos, 2001). Further, the same authors showed that when the nucleosome is artificially positioned such that the TATA box is exposed prior to activation, IFN-β induces at a faster kinetic rate and obviates the need for certain chromatin remodeling enzymes (Lomvardas and Thanos, 2002). Additionally, recent evidence suggests that nucleosomes may be physically removed from DNA (Reinke and Hörz, 2003; Boeger et al., 2003), however, this is difficult to distinguish from a third potential mechanism where nucleosomes remain fixed but instead render the DNA accessible by conformational changes in the nucleosome structure.

Chromatin remodeling is an active process and requires a myriad of highly conserved protein complexes. There are two general classes of chromatin remodeling enzymes. The first class is called ATP-dependent chromatin remodelers (reviewed in Becker and Hörz, 2002). These complexes are defined

by their use of ATP hydrolysis in nucleosome remodeling. The most widely studied complex is the Swi-Snf complex in yeast, named for its role in mating-type switching as well as sucrose fermentation (reviewed in Martens and Winston, 2003). Defects in the human Swi-Snf complex are linked to a variety of disorders and diseases, including cancer. There are other related ATP-remodeling chromatin complexes in yeast, including RSC, Ino80.com, and ISWI, which regulate a variety of biological processes. It is important to note that while ATP-dependent chromatin remodelers are typically thought to remodel chromatin to mediate transcriptional activation, they are involved in transcriptional repression as well (Martens and Winston, 2002).

A second class of chromatin remodeling enzymes involves the post-translational modification of histones. At present, histones have been shown to be modified by acetylation, methylation, phosphorylation, sumoylation and ubiquitination. These modifications mediate a complex signaling pathway to distinguish between active *versus* inactive chromatin that is referred to as the "histone code" (reviewed in Fischle et al., 2003).

Of all the known modifications, histone acetylation is by far the most extensively studied. Acetylation of certain lysines in the N-terminal tails of histones H3 and H4 is associated with transcriptional activation. During transcriptional activation of most genes, increased acetylation is observed in the promoter region (reviewed in Kurdistani and Grunstein, 2003). Histone

acetylation mediates chromatin remodeling by two main mechanisms. First, the acetylation of lysine residues neutralizes the positive charge of the N-terminal tails and increases the alpha-helical content which may reduce the affinity of the tail for DNA and/or histone-histone interactions (reviewed in Hansen et al., 1998). Additionally, histone acetylation may serve as a target for the recruitment of other transcriptional activators. This concept will be explored further in a later section (see bromodomains).

Histone acetylation is catalyzed by a class of protein complexes called histone acetyltransferases (HATs). The first such HAT, HAT A, was discovered in the ciliated protozoan *Tetrahymena thermophila* (Brownell et al., 1996). HAT A is homologous to the *Saccharomyces cerevisiae* protein Gcn5 and is highly conserved in higher eukaryotes. Gcn5 is the catalytic subunit of SAGA and is required for its transcriptional activation and the ability to acetylate histones *in vivo* (Gregory et al., 1998; Kuo et al., 1998; Wang et al., 1998; Krebs et al., 1999; Syntichaki et al., 2000). Conversely, there are histone deacetylase complexes (HDACs) which counteract HAT activity to silence gene expression (reviewed in Kurdistani and Grunstein, 2003). The regulation of HATs and HDACs is key to proper gene expression.

Another emerging histone modification is histone methylation. Like histone acetylation, the methylation state plays a key role in determining active and inactive chromatin. However, whereas acetylation is correlated strictly with

active chromatin, methylation is utilized to demarcate euchromatin as well as heterochromatin. The difference lies not in whether the nucleosome is methylated, but rather in which residue is methylated (Noma et al., 2001).

In higher eukaryotes, heterochromatic silencing is mediated by histone methylation at lysine 9 of histone H3 (Rea et al., 2000). Defects in the histone methyltransferase Suv39h responsible for K9 methylation impairs proper heterochromatin formation and increases genomic instability (Peters et al., 2001). The heterochromatic coating protein HP1 selectively recognizes and binds to K9-methylated nucleosomes (Bannister et al., 2001; Lachner et al., 2001).

In contrast, euchromatin is marked by methylation at lysine 4 of histone H3. All K4 methylation in yeast is mediated by the COMPASS complex (Miller et al., 2001), with the catalytic subunit being the histone methyltransferase Set1 (Briggs et al., 2001). A recent study looking at several genes demonstrated, that Set1-dependent methylation primarily occurs within the promoter and 5' portion of coding regions (Ng et al., 2003b) and is required for full expression of several euchromatic genes (Nislow et al., 1997; Santos-Rosa et al., 2002).

Recent publications have begun to elucidate the regulation of Set1 and its role in transcription. Set1-dependent methylation requires histone ubiquitination of histone H2B at lysine 123 via the Rad6-Bre1 complex. Rad6-deficient strains or strains in which lysine 123 of histone H2B has been mutated to arginine

contain no detectable K4 methylation (Sun and Allis, 2002). This is the first example where a modification on one histone is required for the modification on another. Histone modifications can also regulate other modifications on the same histone, *e.g.* histone phosphorylation at serine 10 leads to increased histone acetylation of lysine 14 of histone H3 (Lo et al., 2000). Interestingly, loss of histone ubiquitination leads to loss of histone methylation; however, Set1 is still recruited (Ng et al., 2003b).

The Paf1-Rtf1 complex is required for histone ubiquitination by Rad6-Bre1 and hence it is required for K4 methylation as well. This complex is involved in transcriptional elongation and interacts with the phosphorylated C-terminal domain of RNA polymerase II (Krogan et al., 2003a; Ng et al., 2003b). As seen before, loss of Paf1 prevents histone ubiquitination but Rad6 is still recruited, however, no recruitment of the COMPASS complex is observed. The mechanism which prevents enzyme activity despite factor recruitment is not known.

Based on these observations, a model has been proposed in which K9 methylation is the signal for transcriptional repression while K4 methylation signals activation; however, other evidence suggests that the true mechanism is more complicated. Defects in Set1 cause loss of telomeric and rDNA silencing (Briggs et al., 2001; Bryk et al., 2002; Krogan et al., 2002a). Likewise, the Rad6-Bre1 and Paf1-Rtf1 complexes also have roles telomeric silencing (Sun and Allis, 2002; Krogan et al., 2003a; Ng et al., 2003a). It has been shown that

these complexes have a repressive role on genes found in active chromatin. Additionally, another histone methyltransferase, Set2, which is also involved in transcriptional elongation, appears to have positive and negative roles in transcription.

Chapter II of this dissertation will explore the role of Set1 in the regulation of the yeast repressible acid phosphatase gene *PHO5*. I will present evidence that Set1 can also be a repressor of genes in active chromatin regions.

TRANSCRIPTIONAL ACTIVATION BY RECRUITMENT

In order to properly control gene expression, the cell must overcome the repressive structure of chromatin using an array of chromatin remodelers as detailed previously. The extent of chromatin remodeling must be restricted to localized regions of the desired gene's promoter to minimize effects on expression of neighboring genes.

The most current model is referred to as the transcriptional activation by recruitment model (Fig 1-2). According to this model, site-specific DNA-binding transcription factors initially occupy their binding site(s) in DNA. These activators contain activation domains which can bind to and recruit both coactivators and the general transcription machinery (reviewed in Fry and Peterson, 2001). Recruitment of the chromatin remodeling coactivators perturbs chromatin structure which allows increased accessibility for general transcription factors

and RNA polymerase. Additionally, it has been demonstrated that chromatin remodeling enzymes can also interact with the general machinery, such as TBP (Sterner et al., 1999; Dudley et al., 1999; Bhaumik and Green, 2002). Thus, the primary activator and the recruited coactivators both directly assist in the recruitment of RNA polymerase. We and others have also shown that the primary activator binding is also dependent on the recruited coactivators (Dhasarathy, Carvin, Jessen and Kladde, manuscript in preparation; Duina and Winston, 2004). This may be a result of the protein-protein interactions of the activator with its coactivators which in turn may cooperatively stabilize the initial DNA-protein interaction. It may also be a result of chromatin remodeling which increases the accessibility of DNA.

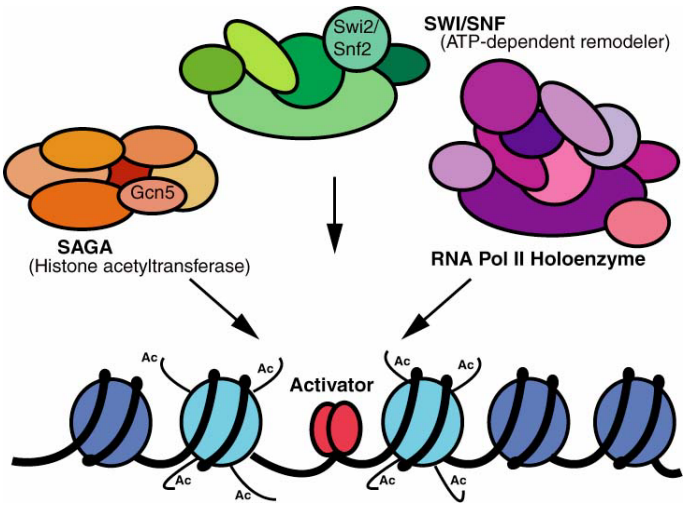

Figure 1-2. Transcriptional activation by recruitment.

In this model the primary activator initially binds to its cognate DNA site. Upon binding, chromatin remodeling coactivators are recruited by the activator's activation domain. These coactivators may stabilize primary activator binding, remodel chromatin structure, and help recruit RNA polymerase. Adapted with permission from Archana Dhasarathy.

An earlier study showed that yeast genes could be classified into three distinct classes based on their requirement for the chromatin remodelers Swi-Snf and SAGA: 1) those genes which require both Swi-Snf and SAGA, 2) those in which either SWI-SNF or SAGA is required but not both, and 3) those which are independent of both Swi-Snf and SAGA (Biggar and Crabtree, 1999). The class where only one of the complexes is required suggests that, while these two complexes perform distinct functions, their overall activity is functionally redundant to one another. This is reinforced by the observation that overexpression of subunits in SAGA can compensate for Swi-Snf defects (Wallberg et al., 2000). One of the ongoing projects in the laboratory is currently investigating the differences between genes which are strictly dependent on chromatin remodelers *versus* those which are largely independent of individual chromatin remodelers.

BROMODOMAINS

In the previous section, the interplay between coactivators in transcriptional activation was discussed. Some recent studies have begun to elucidate the temporal order of recruitment of factors during activation. An elegant study by Nasmyth and colleagues determined the temporal order of recruitment of chromatin remodelers at the cell cycle-regulated *HO* gene (Cosma et al., 1999). In this case, the ATP-dependent chromatin remodeler Swi-

Snf was required for the stable association of the histone acetyltransferase SAGA and both complexes remained stably bound to the promoter after dissociation of the activator that recruited them, Swi5. However, at other genes such as α_1 antitrypsin and IFN-β, the histone acetyltransferase precedes Swi-Snf recruitment (Agalioti et al., 2000; Soutoglou and Talianidis, 2002). *In vitro* studies have shown that histone acetylation stabilizes the association of Swi-Snf to nucleosomal arrays (Hassan et al., 2001).

Several chromatin remodeling coactivator complexes, including Swi-Snf and SAGA, contain a highly conserved domain which was first discovered in the *Drosophila* protein brahma (Tamkun et al., 1992; Haynes et al., 1992) and hence named bromodomain(s) (reviewed in Jeanmougin et al., 1997). Further, the general transcription factor TAFII250 contains two bromodomains (Jacobson et al., 2000). It has been suggested that bromodomains function by recognizing acetyl-lysines in the N-terminal tails of histones (Dhalluin et al., 1999; Ornaghi et al., 1999; Hudson et al., 2000; Owen et al., 2000). The structure of the bromodomain has been solved and is shown in Figure 1-3. Thus, the presence of histone acetylation may lead to increased binding of factors necessary for transcription.

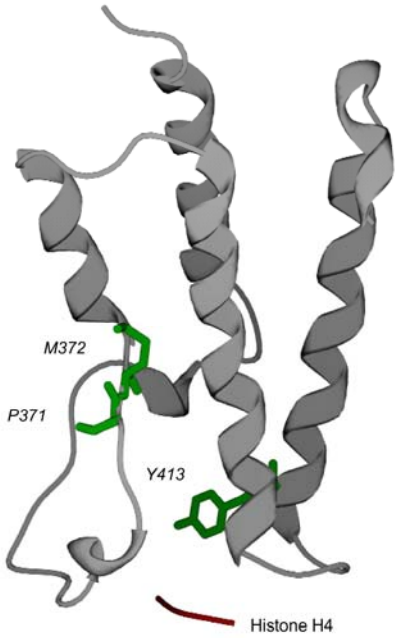

Figure 1-3. Structure of Gcn5 bromodomain complexed with acetylated H4 peptide.

Crystal structure of Gcn5 bromodomain bound to histone H4 that is acetylated at lysine 16 (Owen et al., 2000). Three conserved residues P371, M372, and Y413 which were shown to be important for its function (Syntichaki et al., 2000) are indicated.

A number of studies have tried to determine the extent to which bromodomains affect transcriptional activation. An *in vitro* study found that the bromodomains in Gcn5 of SAGA and Swi2 in Swi-Snf were essential for their stabile interaction with nucleosomal arrays, respectively (Hassan et al., 2002). In contrast, deletion of the bromodomain in the Spt7 subunit of SAGA had no effect on the binding of SAGA to nucleosomal arrays. Interestingly, fusion of the Spt7 bromodomain to Gcn5 could complement a deletion in the Gcn5 bromodomain. The Gcn5 bromodomain was required for *in vivo* chromatin remodeling and Swi-Snf recruitment in an artificial reporter construct (Syntichaki

et al., 2000); however, it had no effect at the endogenous *PHO5* gene which utilizes both Swi-Snf and SAGA for activation.

It is difficult to determine the contribution of a bromodomain in binding *in vivo* since coactivators which do not bind DNA directly show only very modest enrichments in recruitment when analyzed by chromatin immunoprecipitation (ChIP). In Chapter III, we will present evidence that the fusion of the Gcn5 bromodomain to the transcriptional activator Pho4 significantly enhances levels of *PHO5* gene expression, consistent with the bromodomain increasing the binding affinity of Pho4 for the *PHO5* promoter.

PHO5 AS A MODEL SYSTEM

PHO5 is a stress response gene that is activated when the cell is starved for phosphate. It encodes the major acid phosphatase in yeast that is secreted to the periplasmic space to scavenge phosphate from phosphate esters that are present in the media. *PHO5* is just one of 22 genes that are induced in phosphate-limiting media (Ogawa et al., 2000). *PHO5* has been well characterized and serves as a primary model for the study of transcriptional activation and chromatin remodeling.

The pathway that regulates PHO-responsive genes has been determined (Fig. 1-4; reviewed in (Lenburg and O'Shea, 1996). Most PHO-responsive genes (21 of 22) contain putative binding site(s) for the basic helix-loop-helix

transcription factor Pho4 (Ogawa et al., 2000). In high phosphate, the PHO cluster is repressed. This occurs by inactivation of Pho4 by phosphorylation by the cyclin/cyclin-dependent kinase Pho80-Pho85 (Kaffman et al., 1994). In phosphate-limiting conditions, the cyclin-dependent kinase inhibitor Pho81 inactivates Pho80-Pho85 which allows for full activation of Pho4 (Schneider et al., 1994). Pho4 and Pho2 cooperatively bind to their cognate DNA sites to activate transcription (Barbaric et al., 1996; Barbaric et al., 1998). It is important to note that phosphate starvation also increases *PHO81* expression through Pho4 binding (Yoshida et al., 1989b; Creasy et al., 1993), which provides a positive feedback loop during PHO activation.

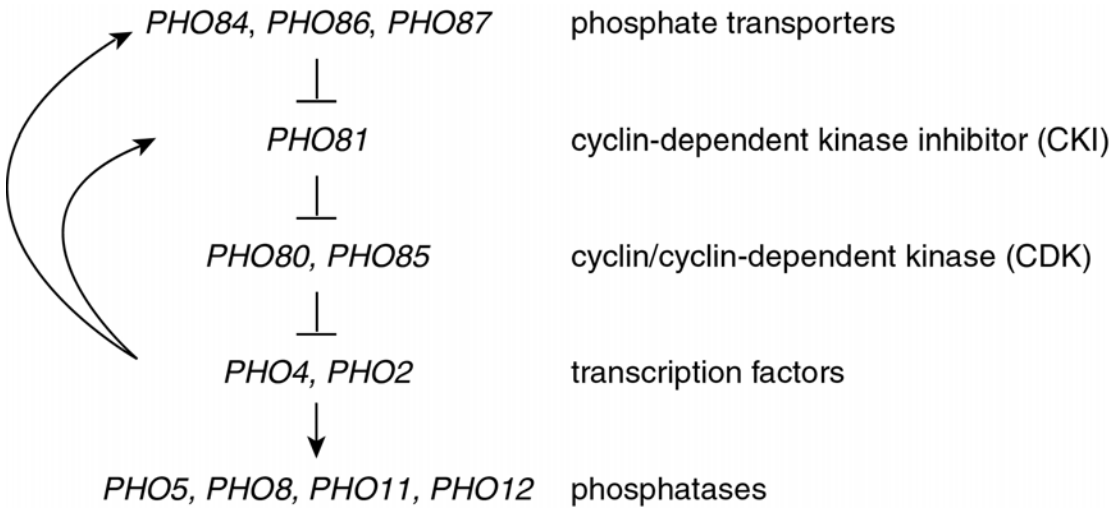

Figure 1-4. Regulatory pathway of PHO-responsive genes.

The repressible acid phosphatases *PHO5* and *PHO8* are regulated by a signal transduction cascade. In repressed conditions these phosphatases are repressed by the inactivation of the primary activator Pho4 by the cyclin/cyclin-dependent kinase Pho80/Pho85. In activating conditions, Pho80/Pho85 is inhibited by the cyclin-dependent kinase inhibitor Pho81 which allows for full Pho4-dependent activation. Pho4 regulates Pho81 transcription which provides positive feedback on activation.

O'Shea and colleagues have determined that inactivation of Pho4 occurs through phosphorylation of five serine residues each which regulate a different inhibitory mechanism. Though Pho4 is constitutively expressed (Lemire et al., 1985; Yoshida et al., 1989a), it is regulated by cellular localization (Fig. 1-5). In high phosphate, Pho4 is found almost exclusively in the cytoplasm (O'Neill et al., 1996). Newly synthesized Pho4 enters the nucleus via the Pse1/Kap121 nuclear importer (Kaffman et al., 1998b). Pho80-Pho85 phosphorylates Pho4 at five different sites; two of those sites cause Pho4 to be exported from the nucleus by the nuclear receptor Msn5 (Kaffman et al., 1998a). A third phosphorylation site prevents re-entry into the nucleus. A fourth phosphorylation site controls the transcriptional activation potential of Pho4 by preventing its interaction with Pho2, which binds DNA cooperatively with Pho4 (Komeili and O'Shea, 1999). When shifted to no phosphate, Pho81 inhibits Pho80-Pho85 which allows Pho4 to rapidly become almost fully nuclear within 1 hour (Komeili and O'Shea, 1999; Barbaric et al., 2001).

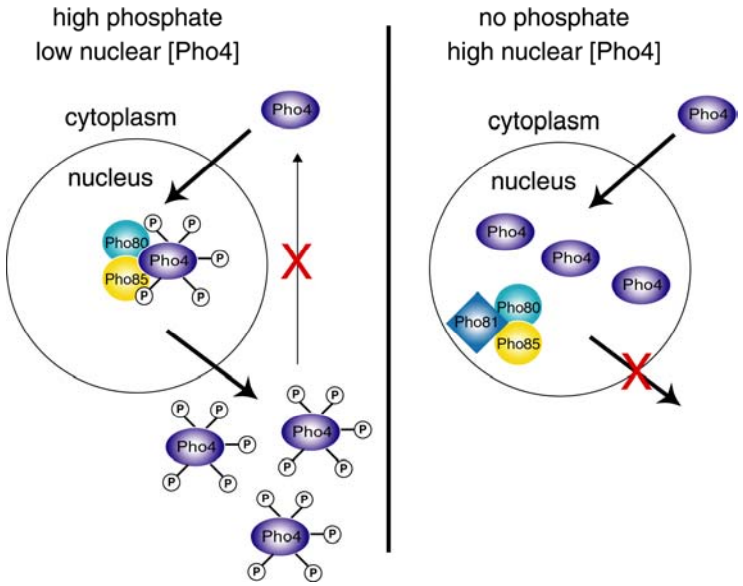

Figure 1-5. Phosphorylation of Pho4 regulates its nucleo-cytoplasmic localization.

Pho80-Pho85 regulates Pho4 nuclear import by phosphorylation. Upon phosphoryation Pho4 is exported from the nucleus via the Msn5 nuclear exporter and its import via Pse1/Kap121 is inhibited. In activating conditions, Pho81 inactivates Pho80-Pho85 which allows for constitutive nuclear import of Pho4.

When a cell is starved for phosphate, it induces a number of phosphatases which try to salvage any environmental phosphate. The two main repressible phosphatases are the acid phosphatase Pho5 and the alkaline phosphatase Pho8. Pho5 is secreted to the periplasmic space, while Pho8 is localized to the vacuole. There are also two minor repressible acid phosphatases (rAPases) Pho10 and Pho11, which have little to no effect on overall rAPase activity (Neef and Kladde, unpublished observations). *PHO5* expression requires Pho4 and Pho2, while at *PHO8* only Pho4 is necessary (Münsterkötter et al., 2000). While both genes are co-regulated, their expression levels are quite different. *PHO5* is highly expressed; rAPase levels are induced greater than 200-

fold in no phosphate *versus* high phosphate media (Neef and Kladde, 2003). *PHO8* activation is approximately 10-times weaker than *PHO5* (Münsterkötter et al., 2000). In addition, *PHO5* only requires the chromatin remodelers SAGA and Swi-Snf during early times of induction or when the nuclear concentration of Pho4 is limiting (Dhasarathy, Carvin, Jessen and Kladde, manuscript in preparation; (Barbaric et al., 2001; Neef and Kladde, 2003). However, *PHO8* expression is strictly dependent on both Swi-Snf and SAGA (Gregory et al., 1999). Thus, the PHO system provides an ideal system for investigations into the reasons for disparate requirements of chromatin remodeling coactivators.

The promoter structures of *PHO5* and *PHO8* have been well characterized (Almer et al., 1986; Barbaric et al., 1992). The *PHO5* promoter contains five positioned nucleosomes and two upstream activating sequences where Pho4 and Pho2 bind (Fig. 1-6). UASp1 is contained in a hypersensitive site which is accessible in high phosphate (Almer et al., 1986; Fascher et al., 1990; Carvin et al., 2003a; Carvin et al., 2003b). UASp2 and the TATA box are located in nucleosomes -2 and -1, respectively, and are inaccessible in repressed conditions. Thus, chromatin remodeling is required for full activation as well as for recruitment of the transcription machinery. Previous work had identified remodeling of four nucleosomes upon phosphate starvation; however work in our laboratory has shown that a fifth nucleosome is remodeled as well (Jessen, Dhasarathy, Carvin, McKinnie, and Kladde, manuscript in preparation).

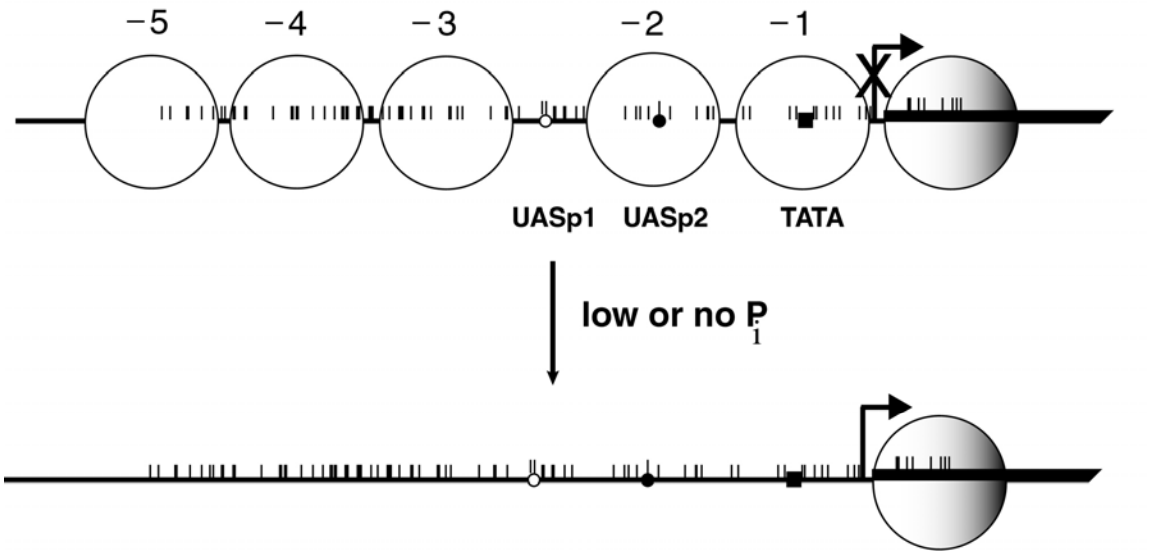

Figure 1-6. The promoter structure of *PHO5.*

The *PHO5* promoter contains six positioned nucleosomes and two Pho4-Pho2 UAS sites. Note that UASp2 and the TATA box are located in the center of nucleosomes and hence block binding of *trans*-acting factors. Upon activation all five nucleosomes are remodeled. Tick marks indicate binding sites for the DNA methyltransferases M.CviPI and M.SssI.

Another advantage in studying *PHO5* is that the Pho5 protein serves as its own reporter. Pho5 protein levels can be measured qualitatively by a plate assay using α-naphthyl-phosphate (Fig. 1-7); and quantitatively by a standard colorimetric phosphatase assay. *PHO5* expression can be modulated by growing cells in different concentrations of inorganic phosphate (Fig. 1-8). Work in our laboratory has determined that varying phosphate concentration regulates the nuclear concentration of Pho4 and the amount of Pho4 bound at the *PHO5* promoter (Dhasarathy, Carvin, Jessen and Kladde, manuscript in preparation).

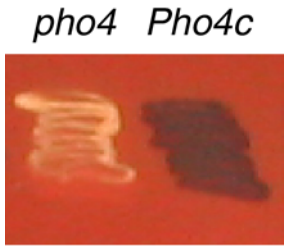

Figure 1-7. *PHO5* expression requires the transcription factor Pho4.

Plate assay detects Pho5 levels. In assay cells which express Pho5 protein are stained red. Cells lacking Pho4 (*pho4*) are white while cells which contain a constitutively active mutant of Pho4 (O'Neill et al., 1996) leads to dark red color.

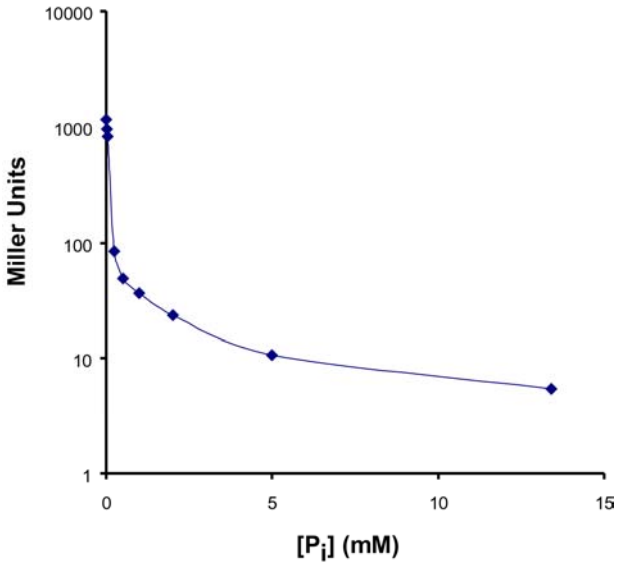

Figure 1-8. *PHO5* expression *versus* phosphate concentration.

Repressible acid phosphatase assay of wild-type cells grown in varying concentrations of inorganic phosphate. High phosphate (13.4 mM) shows a very low amount of rAPase activity. Conversely, Pho5 is induced greater than 200-fold in no phosphate. Activities are reported in Miller units {(A_{420} x 1,000)/(OD_{600} x volume of cells assayed in mL x 10 min)}.

In conclusion, the PHO system is an ideal system for the study of transcriptional regulation and chromatin structure. In this dissertation, I present evidence that histone methylation plays a regulatory role in the repression of

PHO5. I provide a more detailed study on the ability of bromodomains to affect transcription factor binding. Our laboratory has developed the use of DNA methyltransferases which allow for the *in vivo* probing of chromatin structure (reviewed in Kladde et al., 1999). This dissertation has extended the use of DNA methyltransferases to allow for the detection of protein-DNA interactions, called targeted gene methylation (TAGM). Finally, I demonstrate that TAGM can be applied to target methylation via engineered zinc-finger proteins which can be altered to bind to any desired sequence. These chimeric proteins can be used in the further study of the effects of DNA methylation and/or establish heritable transcriptional silencing.

CHAPTER II

SET1 IS A NEGATIVE REGULATOR OF *PHO5* AND *GAL1-10*

OVERVIEW

Post-translational modifications of histone amino-terminal tails are a key determinant in gene expression. In most eukaryotes, histone methylation plays a dual role in gene regulation. Methylation of lysine 9 of histone H3 associates with heterochromatin while methylation of lysine 4 correlates with active chromatin. K4 methylation via Set1, a component of the COMPASS complex, is regulated by the transcriptional elongation complex Paf1-Rtf1 and is required for expression of a subset of genes. This suggests that K4 methylation may play an activating role in transcription. However, we here show that K4 methylation negatively regulates gene expression as well. Strains that are deficient in Set1 show enhanced expression of *PHO5*. Defects in the Paf1-Rtf1 complex show a greater derepression than that observed in defects in COMPASS. *PHO84* and *GAL1-10* are also derepressed in *set1*Δ cells. These results suggest that K4 methylation, in conjunction with transcriptional elongation, may function in a negative feedback pathway for basal transcription of some genes while being a positive effector at others.

INTRODUCTION

In eukaryotes, DNA is packaged with histone proteins to form nucleosomes which are further condensed into higher-order chromatin structure. This compaction serves as a barrier for the binding of factors important in cellular processes such as transcription and DNA replication. Thus, genes found in heavily condensed regions, such as heterochromatin, are typically transcriptionally silent. Expression of genes located in euchromatic regions, which are generally less compacted is also regulated by chromatin structure.

Post-translational modifications of the amino-terminal tails of histone proteins are a key determinant in defining active (accessible) and repressed (inaccessible) chromatin. These modifications may alter chromatin structure directly by affecting histone-DNA and histone-histone interactions (reviewed in Hayes and Hansen, 2001). Further, they also allow for the recruitment of transcriptional activators or repressors. Acetylation of histone H3 at lysines 9 and 14 is strongly correlated with transcriptionally active and accessible chromatin. Treatment of cells with histone deacetylase inhibitors, such as trichostatin A, leads to active chromatin states (Yang et al., 2000). Phosphorylation of serine 10 of histone H3 is also observed in transcriptional activation (Lo et al., 2000; Cheung et al., 2000) and has an unknown role in mitotic condensation (reviewed in Prigent and Dimitrov, 2003).

Histone methylation is correlated with both active and repressed chromatin states. In eukaryotes other than budding yeast, heterochromatic silencing is marked by methylation of histone H3 at lysine 9. Conversely, euchromatic regions are associated with histone methylation at lysines 4 and 79 of histone H3 by the histone methyltransferases Set1 and Dot1, respectively (Noma et al., 2001). Set1 is the catalytic subunit of a large complex named COMPASS (Miller et al., 2001) and is responsible for all K4 methylation observed in yeast (Briggs et al., 2001). It is required for full activation of a subset of euchromatic genes, including *RAM2, HAS1, INO1, PPH3,* and *MET16* (Nislow et al., 1997; Santos-Rosa et al., 2002). Paradoxically, defects in the Set1 or other components of COMPASS also lead to loss of rDNA (Briggs et al., 2001; Bryk et al., 2002) and telomeric (Krogan et al., 2002a) silencing.

Set1-dependent methylation requires histone ubiquitination of lysine 123 of histone H2B via the Rad6-Bre1 complex (Sun and Allis, 2002). Set1 is still recruited to promoter regions in *RAD6* deletion strains; however no resulting K4 methylation is observed (Ng et al., 2003b). This is the first evidence where a modification on one histone regulates the modification of another histone. Recent reports have also indicated that Set1 methylation is associated with transcriptional elongation (reviewed in Hampsey and Reinberg, 2003). The Paf1-Rtf1 complex, which has been observed to be associated with RNA polymerase II, is required for K4 methylation as well as recruitment of the COMPASS

complex (Krogan et al., 2003a). Deletions in Paf1 lead to loss of histone ubiquitination by Rad6, however, Rad6 is still recruited (Wood et al., 2003). As seen with *set1Δ* mutants, strains lacking *PAF1*, *RTF1* or *RAD6* show loss of telomeric silencing (Sun and Allis, 2002; Krogan et al., 2003a; Ng et al., 2003a).

In this report, we explore the role of Set1 in the transcriptional regulation of the phosphate-repressible PHO cluster. We find that loss of Set1 leads to increased levels of expression of the repressible acid phosphatase *PHO5* in both repressed and active conditions. The expression of the high affinity phosphate transporter *PHO84* is also higher in *set1*Δ than in wild-type strains. Deletions in critical components of the Rad6-Bre1 and Paf1-Rtf1 complexes, which are required for Set1-dependent methylation, also exhibit derepression of *PHO5*. Finally, we also observe derepression of the *GAL1-10* locus. Our results suggest that histone methylation at K4 of histone H3 may be a repressive signal at some euchromatic genes while an activating one at others.

MATERIALS AND METHODS

Yeast strains

The genotypes of the *Saccharomyces cerevisiae* strains used are listed in Table 2-1. The *SET1* open reading frame was completely replaced in the diploid strain CCY694 (Neef and Kladde, 2003) with the *kanMX4* selectable marker by a PCR-based method using the plasmid pRS400 as described (Brachmann et al.,

1998). Gene replacement was confirmed by PCR and the resulting diploid was sporulated and tetrads were dissected to obtain wild-type (CCY1467 and CCY1468) and *set1*Δ haploids (CCY1471 and CCY1472). MBY1198 and MBY1217 are gifts from Mary Bryk and are described elsewhere (Bryk et al., 2002). Yeast deletion strains were obtained from the homozygous deletion panel (Research Genetics).

Growth conditions

For *PHO5* expression experiments, strains were pregrown in minimal media containing 0.7 g yeast nitrogen base with ammonium sulfate, phosphate, and amino acids (Bio 101), 2 g glutamine (Sigma), 20 g dextrose (Fisher), and 3.9 g 2-*N*-morpholino ethanesulfonic acid (JT Baker), pH 5.5, per liter supplemented with 13.4 mM KH_2PO_4. Cells were then washed and resuspended in minimal media containing either 13.4 mM KH_2PO_4 or 13.4 mM KCl and incubated at 30^{o}C with shaking for 6 h. *PHO5* activity was measured by either repressible acid phosphatase activity assays or Northern hybridization as described (Neef and Kladde, 2003). Cells were also grown in rich YPD medium supplemented with 13.4 mM KH_2PO_4 (YPPD) overnight at 23^{o}C without shaking. For *GAL1-10* experiments, strains were pregrown overnight in YPD and then resuspended in YPD or YP galactose (YPG) + 0.5% glucose and incubated at 30^{o}C with shaking for 4 h. Northern hybridization probes were generated using

PCR amplification using the oligonucleotides listed in Table 2-2. mRNA levels were quantified by Storm 860 phosphorimager analysis.

Table 2-1. Yeast strains.

Strain	*Parent*	*Genotype*
CCY1467	NA	*MAT***a** *leu2Δ0 lys2Δ0 ura3Δ0 pho3Δ::R*
CCY1471	NA	*MAT***a** *leu2Δ0 lys2Δ0 ura3Δ0 pho3Δ::R set1Δ::kanMX4*
MBY1198	NA	*MATα his3Δ200 ade2Δ::hisG leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 Ty1his3AI-236 Ty1ade2AI-515 cir^{0}*
MBY1217	NA	*MATα his3Δ200 ade2Δ::hisG leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 Ty1his3AI-236 Ty1ade2AI-515 cir^{0} set1Δ::TRP1*
MBY1499	NA	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hht1-hhf1::LEU2 hht2-hhf2::HIS3 pRS414-HHT2-HHF2*
MBY1500	NA	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hht1-hhf1::LEU2 hht2-hhf2::HIS3 pRS414-hht2K4R-HHF2*
BY4743	NA	*MAT***a***/MATα his3Δ1/ his3Δ1 leu2Δ0/ leu2Δ0 MET15/met15Δ0 LYS2/lysΔ0 ura3Δ0/ura3Δ0*
CCY2895	BY4743	*set1Δ::kanMX4*
31570	BY4743	*bre2Δ::kanMX4*
32773	BY4743	*lge1Δ::kanMX4*
33771	BY4743	*bre1Δ::kanMX4*
34425	BY4743	*rad6Δ::kanMX4*
34611	BY4743	*rtf1Δ::kanMX4*
35727	BY4743	*paf1Δ::kanMX4*

Table 2-2. Primers used for generation of Northern hybridization probes.

Probe	Sequence	Primer name
ACT1	GGCATCATACCTTCTACAAC	DNO455
ACT1	CGATGTTACCGTATAATTCC	DNO456
GAL1[a]	CTCATTCAGAAGAAGTGATTGTAC	CCO369
GAL1	AGCACTGGCAAACCTTTC	CCO370
GAL10	CTCAGTTACAAAGTGAAAGTA	CCO1135
GAL10	GCTACTTGAGCCATATATGG	CCO1136
PHO5	TCTTTCCCTGGCGA	DNO425
PHO5[a]	GTCATCCAAGTAGGTTGTGT	DNO426
PHO84	ATGAGTTCCGTCAATAAAGAT	MKO928
PHO84	TTATGCTTCATGTTGAAGTTG	MKO929
PPH3	ATGATGGACTTAGATAAGATTATAG	CCO1138
PPH3	TAAGAAATAGTCCATTTGAGATTT	CCO1139

[a]Primer contains a 5' tail with core T7 promoter sequence.

Chromatin immunoprecipitation (ChIP) assay

To analyze histone H3 K4 methylation levels, antibodies specific for di- and tri-methylated forms of histone H3 K4 were used to immunoprecipitate chromatin from MBY1198 and MBY1217 strains grown in YPD. Quantitative PCR amplification was performed using primers ADO236 and LFO740 as described (Carvin et al., 2003a).

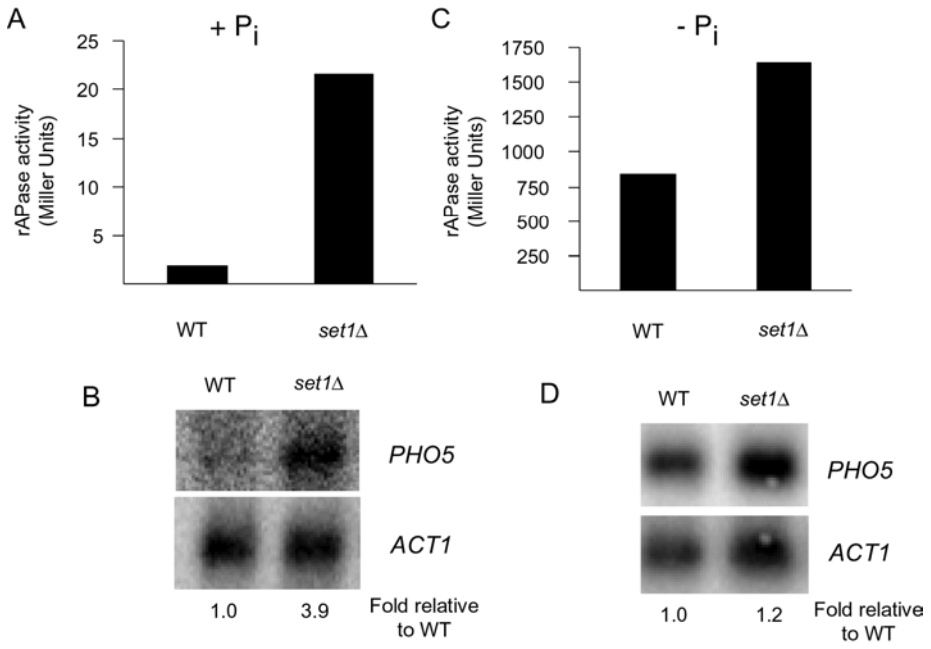

Figure 2-1. Loss of Set1 leads to higher levels of *PHO5* in repressed and activated conditions.

(A) Total repressible acid phosphatase (rAPase) activities from the CCY1467 wild-type strain and the CCY1471 *set1*Δ mutant strain grown in minimal high phosphate media for 6 h. Activities are reported as Miller Units as described previously (Neef and Kladde, 2003). Results are representative of five independent experiments. Similar results are observed using two independent wild-type and mutant segregants.
(B) Northern analysis of RNA internally isolated in (A) for *PHO5* and *ACT1* mRNA levels. For quantification (fold relative to WT), *PHO5* transcript levels in each lane are normalized to *ACT1* mRNA levels.
(C) Total rAPase activities of strains grown in minimal no phosphate media for 6 hours.
(D) Northern analysis of RNA internally isolated from cells in (C).

RESULTS AND DISCUSSION

Deletion of Set1 leads to increased levels of Pho5

To determine the role that histone H3 K4 methylation has on *PHO5* gene, we analyzed *PHO5* expression levels in wild-type and *set1Δ* strains under both repressed and activating conditions. In order to distinguish repressible acid phosphatase levels as well as potential cross-hybridization in Northern analysis from that of the constitutive acid phosphatase Pho3, we used strains in which

the entire coding sequence of *PHO3* was deleted. In repressed conditions of minimal media supplemented with phosphate, *set1*Δ strains show significantly higher levels of rAPase activity than wild-type cells (Fig. 2-1A). This increased rAPase activity correlates with higher *PHO5* mRNA levels in *set1Δ* cells, suggesting that this derepression is due to increased transcription (Fig. 2-1B). We also observed enhanced *PHO5* expression in fully activating conditions of minimal media lacking phosphate (Fig. 2-1C). Although the fold-increase is lower, the increase by nearly 1000 Miller Units is substantial. Similarly, under no phosphate conditions, a modest but reproducible increase in *PHO5* transcript was observed (Fig. 2-1D). To better quantify the level of derepression due to the deletion of *SET1*, we grew cells under conditions of higher basal expression, in rich medium supplemented with phosphate at 23^{o}C. Under these conditions rAPase activity is approximately 10-fold higher than when grown in minimal media containing phosphate at 30^{o}C (compare levels observed for wild-type in Fig. 2-1A to that observed in Fig. 2-2A). *PHO5* is still further enhanced in cells lacking Set1 (Fig. 2-2A-B). *PHO5* expression is noticeably higher throughout a time course of phosphate starvation and is repressed slower in a *set1*Δ strain (data not shown). Additionally, yeast in which lysine 4 is mutated to arginine and hence cannot be methylated, show increased basal expression of *PHO5* (Fig. 2-2C). The lower fold derepression observed in this H3 K4R strain is likely due to the high basal expression that results from expression of the histone on a

plasmid. Nevertheless, K4 methylation contributes to the regulation of *PHO5* and suggests that Set1 plays a role in the repression of the euchromatic gene *PHO5*.

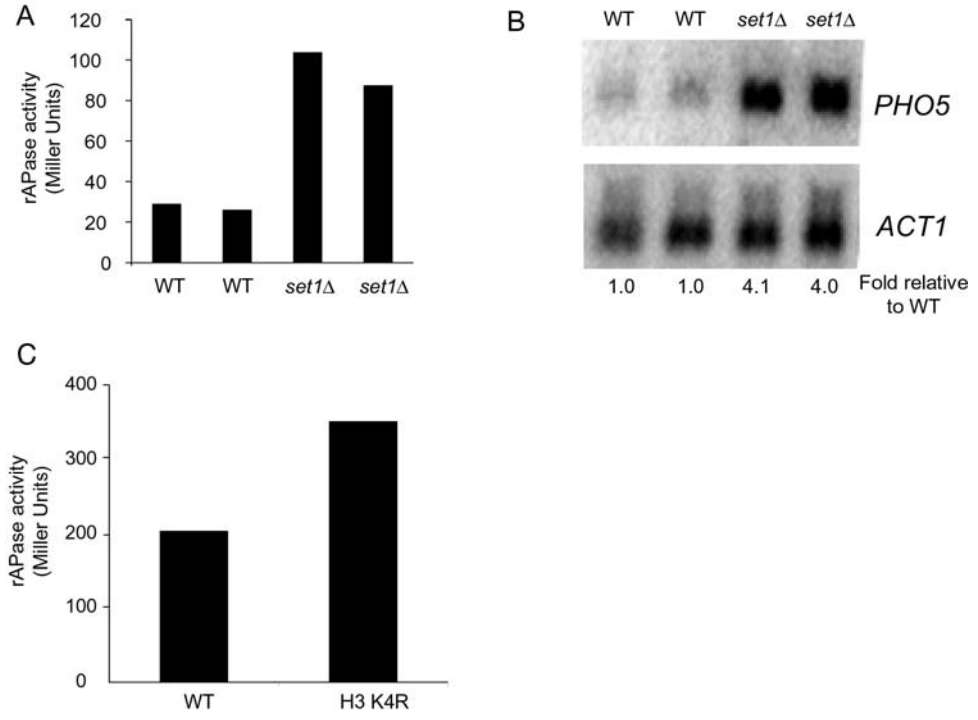

Figure 2-2. Derepression of *PHO5* is observed in *set1*Δ cells when grown in YPPD.

(A) Total rAPase activities from two independent wild-type and *set1*Δ strains grown in YPPD at 23°C. rAPase activity results are representative of three independent experiments.
(B) Northern analysis of RNA internally isolated from cells in (A). For quantification (fold relative to WT), *PHO5* transcript levels in each lane are normalized to *ACT1* mRNA levels.
(C) rAPase activities of MBY1499 wild-type and MBY1500 histone H3 K4R mutant strains.

Methylation of K4 of histone H3 is present at the *PHO5* promoter

In order to see if Set1 is regulating *PHO5* directly, we investigated the methylation state of histone H3 at the *PHO5* promoter. Chromatin immunoprecipitation (ChIP) was performed using antibodies specific for di- and

tri-methylated lysine 4 of histone H3 (Santos-Rosa et al., 2002). When cells are grown in YPD, which is limiting for inorganic phosphate and hence leads to significant *PHO5* expression (Neef and Kladde, 2003), considerable amounts of both di- and tri-methylated forms of K4 are present at the *PHO5* promoter (Fig. 2-3). This enrichment is abolished in a strain that lacks Set1. This is consistent with results reported previously (Reinke and Hörz, 2003) and suggests that Set1 is directly associated with *PHO5*.

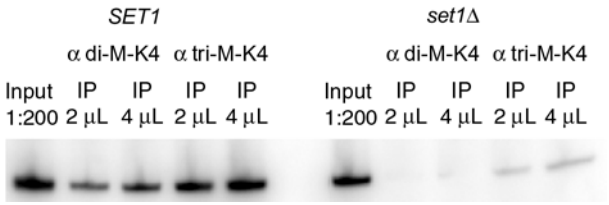

Figure 2-3. Set1-dependent K4 methylation is enriched at the *PHO5* promoter.

Chromatin immunoprecipitation analysis of wild-type MBY1198 and *set1*Δ MBY1217 strains grown in YPD media using antibodies specific for di- and tri- methylated forms of histone H3 K4. PCR amplifications of input and immunoprecipitated DNA samples using primers specific for the *PHO5* promoter region are shown.

Upstream regulators of Set1 also regulate *PHO5*

Histone ubiquitination of histone H2B at lysine 123 by the Rad6-Bre1 complex is required for Set1 to methylate histone H3 K4. Additionally, recent reports have linked Set1-dependent methylation to transcriptional elongation via the Paf1-Rtf1 complex. Defects in Paf1 or Rtf1 lead to loss of K4 methylation. Since these complexes are necessary for K4 methylation, we surmised that defects in these complexes should have a similar phenotype to that observed in

*set1*Δ. Deletions of both *RTF1* and *PAF1* cause significant increased expression of *PHO5* with *paf1*Δ having a larger effect than *rtf1*Δ (Fig. 2-4A). This is consistent with previous results that have shown that a *paf1Δ* strain has a larger transcriptional effect than *rtf1Δ* (Squazzo et al., 2002). Further, *PAF1* null strains reduce the association of Set1 with coding regions more than does a deletion of *RTF1* (Ng et al., 2003b). Deletions in *RAD6*, *BRE1* or *LGE1*, encoding components of the Rad6-Bre1 complex (Hwang et al., 2003), lead to increased *PHO5* levels (Fig. 2-4B). Finally, deletions of *SET1* or *BRE2* of the COMPASS complex (Miller et al., 2001) also show enhanced rAPase expression (Fig. 2-4C). It is interesting to note that the Paf1-Rtf1 complex, which is genetically upstream of the Rad6-Bre1 and COMPASS complexes, has the largest effect on derepression. This suggests that Paf1-Rtf1 may recruit other components that regulate *PHO5* expression.

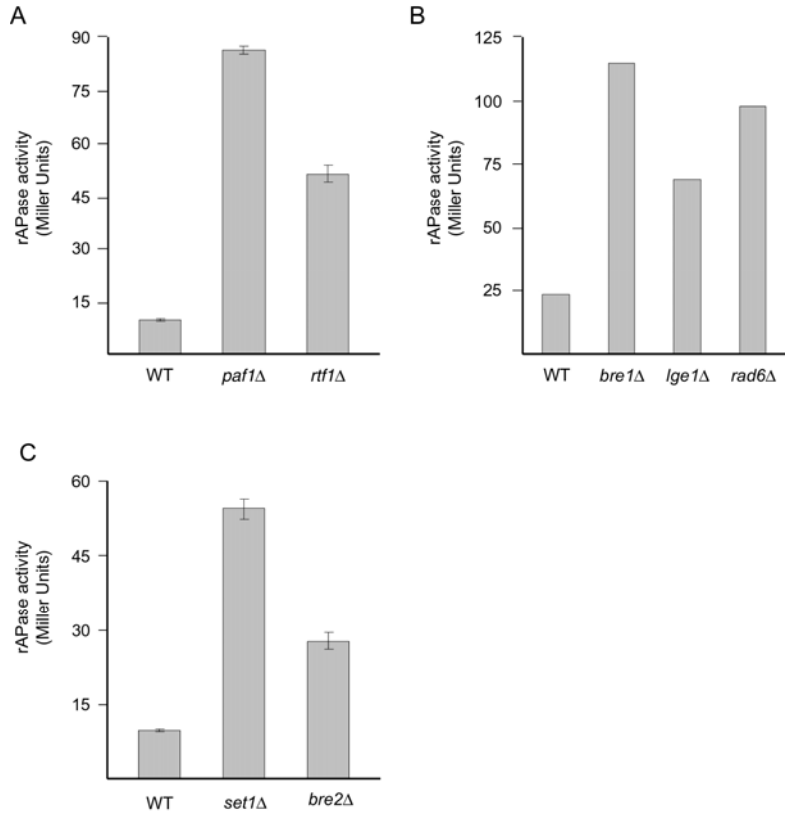

Figure 2-4. Upstream regulators of Set1 also regulate *PHO5* expression.

(A) rAPase activities of wild-type, *paf1*Δ, and *rtf1*Δ strains grown in minimal high phosphate media. The means ± 1 standard deviation from three independent experiments are shown.
(B) rAPase activities of wild-type, *bre1*Δ, *lge1Δ*, and *rad6*Δ strains.
(C) rAPase activities of wild-type, *bre2Δ*, and *set1Δ* strains. The means ± 1 standard deviation from three independent experiments are shown.

Set1 is a negative regulator of *PHO84* expression

To test if other genes of the PHO cluster are regulated by Set1, we examined the expression of *PHO84*, which codes for the high affinity phosphate transporter. Like *PHO5*, *PHO84* is only minimally expressed in high phosphate conditions and is highly expressed in media where phosphate is limiting. *PHO84* mRNA levels were studied in wild-type and *set1*Δ cells grown in YPPD. As seen

previously for *PHO5*, the strain lacking Set1 shows higher expression of *PHO84* than wild-type (Fig. 2-5A). Conversely, a reduction in the mRNA levels of the constitutive protein phosphatase *PPH3* is observed in *set1*Δ (Fig. 2-5B) as has been shown previously (Santos-Rosa et al., 2002). The *PHO84* results confirm the above-mentioned *PHO5* results and demonstrates that Set1 is a repressor of other PHO genes while is required for full expression at others.

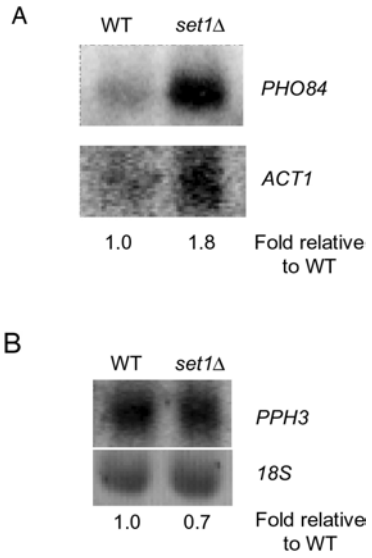

Figure 2-5. Set1 regulates other PHO-responsive genes.

(A) Northern analysis of *PHO84* expression of wild-type and *set1*Δ strains grown in YPPD. For quantification (fold relative to WT), *PHO84* transcript levels in each lane are normalized to *ACT1* mRNA levels.
(B) Northern analysis of *PPH3* expression in minimal media normalized to 18S RNA.

GAL1-10 is also negatively regulated by Set1

To determine if Set1 is involved in the repression of other genes not under phosphate control, we examined the *GAL1-10* locus. Wild-type and *set1Δ* strains were grown in repressed conditions, YPD, and semi-activating conditions

YPG + 0.5% glucose and *GAL1* and *GAL10* mRNA expression was measured. No detectable *GAL1* or *GAL10* transcript was observed in YPD; however, both *GAL1* and *GAL10* are expressed more in a *set1*Δ strain in YPG + 0.5% glucose (Fig. 2-6). These data are consistent with two previous microarray analyses which also indicated that *GAL1* has increased mRNA levels in a *set1*Δ deletion strain (Bernstein et al., 2002; Boa et al., 2003). A recent study has also shown that *GAL10* is expressed significantly higher in a *rad6*Δ null as well as in *set1*Δ at early times of induction (Daniel et al., 2004). Thus, Set1 may negatively regulate a myriad of genes with different functions and regulatory mechanisms.

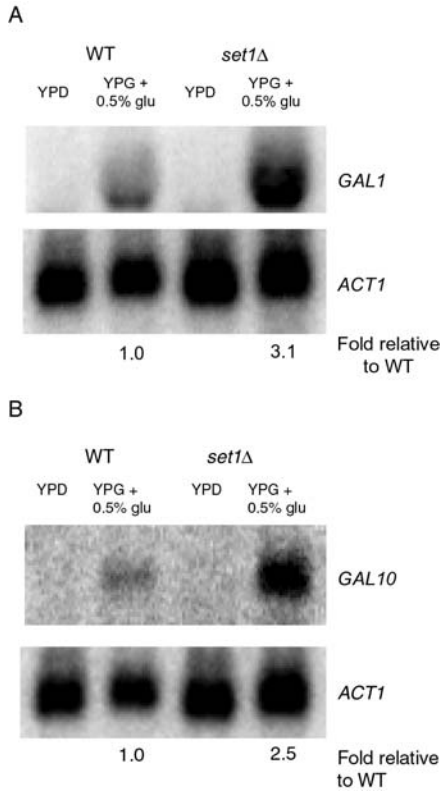

Figure 2-6. Set1 represses *GAL1-10* expression.

(A) Northern analysis of *GAL1* expression of wild-type and *set1Δ* strains grown in YPD or YPG + 0.5% glucose. For quantification (fold relative to WT), *GAL1* transcript levels in each lane are normalized to *ACT1* mRNA levels.
(B) Northern analysis of *GAL10* mRNA levels. For quantification (fold relative to WT), *GAL10* transcript levels in each lane are normalized to *ACT1* mRNA levels.

CHAPTER III

THE ROLE OF BROMODOMAINS IN TRANSCRIPTIONAL ACTIVATION

OVERVIEW

Post-translational modifications of histones are key regulatory events in transcription coordination. During transcriptional activation, the primary activator recruits a variety of coactivators which function to remodel chromatin as well as to recruit the transcriptional machinery. Histone acetylation is a determinant of transcriptionally active chromatin regions; however, its functional role has not been clearly defined. A number of chromatin remodeling coactivators contain a highly conserved bromodomain which selectively recognizes acetylated histones. Since chromatin remodeling coactivators do not bind DNA directly, it is difficult to quantify the promoter association accurately, and it is even harder to determine the individual contributions of each subunit in promoter interaction. In order to determine the function that the bromodomain motif may contribute in transcriptional activation, we fused the Gcn5 bromodomain up to the transcriptional activator Pho4. This fusion leads to significant enhancement of *PHO5* expression. Mutations in essential residues of the bromodomain alleviate this effect. We can use this strategy to measure accurately the effect of individual bromodomains on promoter binding and transcriptional activation.

INTRODUCTION

In eukaryotes, the process of transcription requires the interplay of DNA-binding transcriptional activators, chromatin remodeling coactivators, and RNA polymerase. The primary activator is able to independently recruit all of these activities (reviewed in Fry and Peterson, 2001). At the yeast *HO* gene, the primary activator Swi5 recruits Swi-Snf and SAGA in a temporal manner and Swi-Snf and SAGA remain associated with the promoter after Swi5 has dissociated. An *in vitro* system using nucleosomal arrays has shown that histone acetylation stabilizes the binding of Swi-Snf (Hassan et al., 2001). This suggests a connection between the activity of one chromatin remodeling enzyme and the recruitment of another.

A number of chromatin remodeling complexes in yeast contain subunits which contain a highly conserved bromodomain which has been shown to be an acetyl-lysine binding domain (Dhalluin et al., 1999). Since histone acetylation usually increases at promoters during activation, the bromodomain may provide a functional link in the recruitment and stabilization of transcriptional coactivators. However, it is difficult to determine the relative contributions particular domains may have in binding using chromatin immunoprecipitation, since coactivators often cross-link poorly as they do not bind DNA directly.

Here, we show that fusion of the bromodomain from the Gcn5 histone acetyltransferase to the transcriptional activator Pho4 significantly increases the

ability of Pho4 to activate transcription. We observe a greater than eight-fold enhancement of *PHO5* expression in repressed conditions. This effect is not seen when critical residues that make up the acetyl-lysine binding pocket of the bromodomain are mutated. This strategy provides a convenient way to test the effect that individual bromodomains may have on the binding affinity of coactivators to promoter regions.

MATERIALS AND METHODS

Yeast strains, plasmid construction, and growth conditions

All strains used in this study are derived from CCY880 (*MAT***a** *leu2Δ0 lys2Δ0 ura3Δ0 pho3Δ*::R). All bromodomain fusions were constructed by tagging Pho4 with 3xHA-$(his)_6$-GPGS$(G)_6(SGG)_2$GLGST (linker)-BD fusion at its C-terminus with the selectable marker *URA3* immediately downstream. All constructs were integrated at the endogenous *PHO4* locus so that the chimeric proteins would be expressed by the endogenous *PHO4* promoter (Legrain et al., 1986). Proper integration was screened by PCR. *URA3*, which was flanked by *Zygosaccharomyces rouxii* recombinase sites, was then recovered by homologous recombination (Roca et al., 1992). Bromodomain mutants were created by site-directed mutagenesis using the mutagenic primers described in (Syntichaki et al., 2000). All plasmids were sequence-verified prior to integration.

Strains were pre-grown in minimal media containing 0.7 g yeast nitrogen base with ammonium sulfate, phosphate, and amino acids (Bio 101), 2 g glutamine (Sigma), 20 g dextrose (Fisher), and 3.9 g 2-*N*-morpholino ethanesulfonic acid (JT Baker), pH 5.5, per liter supplemented with 13.4 mM KH_2PO_4. Cells were reseeded to OD_{600} = 0.2 in minimal media containing either 13.4 mM KH_2PO_4 or 13.4 mM KCl and incubated at 30^{o}C with shaking for 6 h. *PHO5* activity was measured by repressible acid phosphatase activity assays (Neef and Kladde, 2003).

RESULTS AND DISCUSSION

Fusion of the Gcn5 bromodomain to Pho4 enhances *PHO5* expression in repressed conditions

To test whether the bromodomain of Gcn5 may affect the ability of Pho4 to activate transcription, we fused it in-frame to C-terminus of Pho4 (Pho4-Gcn5BD). The fusion was integrated at the endogenous Pho4 locus and its expression was regulated by the *PHO4* promoter. We analyzed *PHO5* expression levels by acid phosphatase assays as described previously (Neef and Kladde, 2003). The Pho4-Gcn5BD fusion showed a dramatic increase in the expression of the repressible acid phosphatase gene *PHO5* in repressed conditions (Fig. 3-1). Though Pho4 is mainly localized to the cytoplasm in high phosphate, some

transient Pho4 binding is observed (Carvin et al., 2003a) and the addition of the bromodomain makes Pho4 a more potent activator.

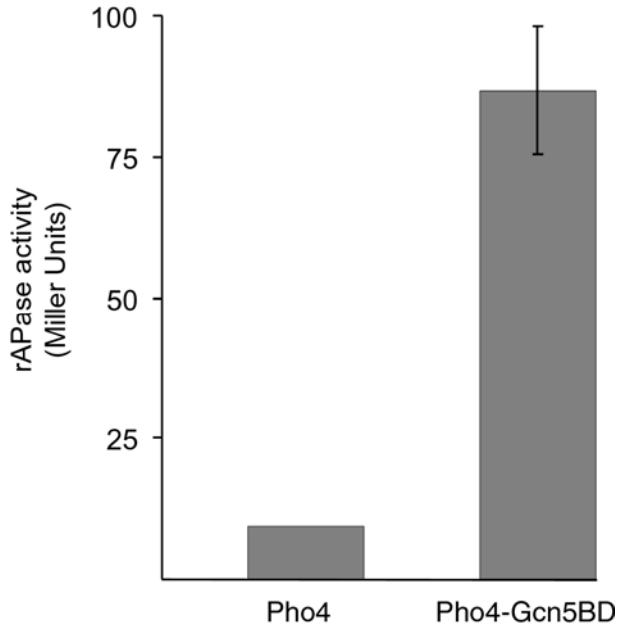

Figure 3-1. Fusion of the Gcn5 bromodomain to Pho4 increases *PHO5* expression in high phosphate medium.

rAPase activity assays of wild-type (Pho4) and bromodomain fusion (Pho4-Gcn5BD) strains grown in high phosphate media. The means ± 1 standard deviation from seven independent Pho4-Gcn5BD strains are shown.

Under fully activating conditions, no significant enhancement of expression is observed in the Pho4-Gcn5BD fusion strain (Fig. 3-2). Previous work has shown that histone acetylation levels are lower in phosphate-limiting media which has been attributed to complete nucleosome displacement (Reinke and Hörz, 2003; Boeger et al., 2003). The loss of acetylated histones would prevent the bromodomain from providing an additional binding contact.

Alternatively, Pho4 binding at the *PHO5* promoter or *PHO5* expression may have achieved saturated.

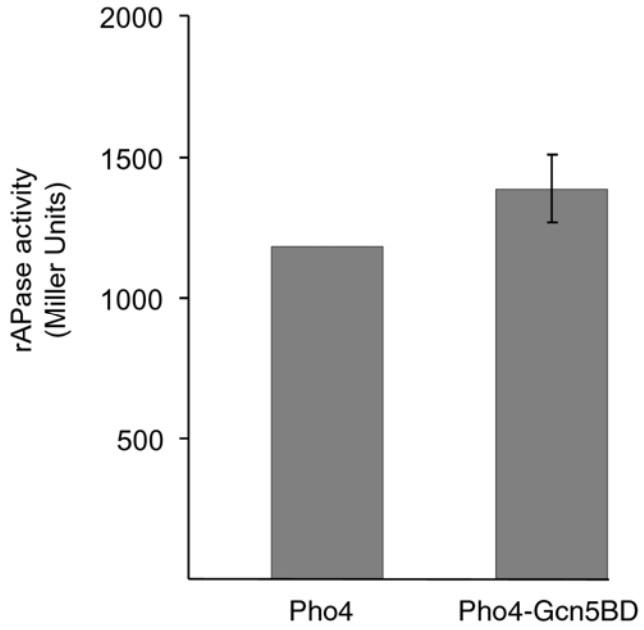

Figure 3-2. Pho4-Gcn5BD does not show increased expression in the absence of phosphate. rAPase activity assays of wild-type (Pho4) and bromodomain fusion (Pho4-Gcn5BD) strains grown in no phosphate media. The means ± 1 standard deviation from seven independent Pho4-Gcn5BD strains are shown.

Bromodomain mutants cannot increase *PHO5* expression

It is formally possible that the enhanced expression seen is a result of higher Pho4 concentrations in the nucleus, however, previous work has shown that deletion of *MSN5*, which leads to constitutive Pho4 nuclear localization, does not lead to higher *PHO5* expression (Kaffman et al., 1998a). Additionally, we have that other Pho4 C-terminal fusions do not lead to derepression (Carvin

et al., 2003a). It is also possible that the bromodomain, which contains a number of acidic residues, contains a cryptic activation domain. To demonstrate that the additional induction seen in the Pho4-Gcn5BD fusion is a true result of the bromodomain, we mutated the bromodomain at two essential residues which were shown to be required for its function (Syntichaki et al., 2000). The P371T and M372A mutations completely abolish the enhanced expression observed (Fig. 3-3). This demonstrates that the bromodomain itself and it is not merely the result of a fusion protein which leads to the enhanced expression.

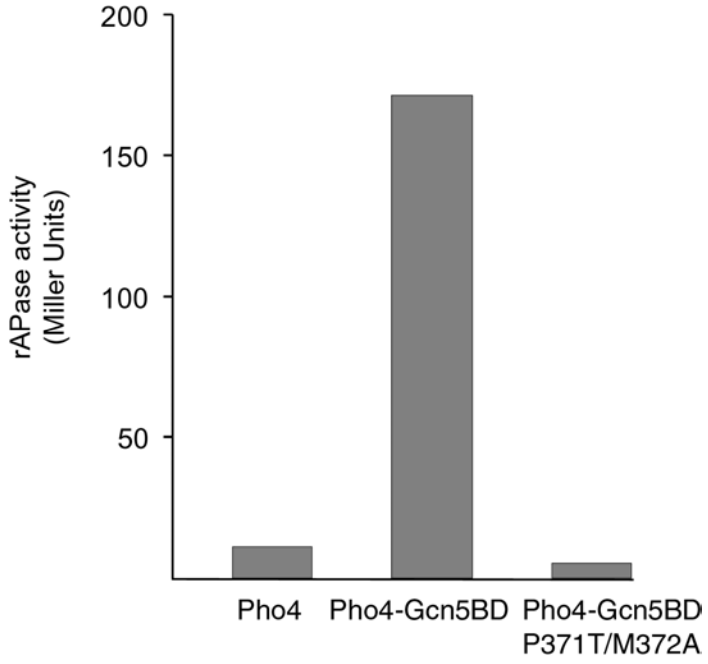

Figure 3-3. Mutations in the Gcn5 bromodomain impair its function.

rAPase activity assays of wild-type (Pho4), bromodomain fusion (Pho4-Gcn5BD), and mutated bromodomain fusion (Pho4-Gcn5BD P371T/M372A strains grown in high phosphate media.

CHAPTER IV

TARGETED CYTOSINE METHYLATION FOR *IN VIVO* DETECTION OF PROTEIN-DNA INTERACTIONS[†]

OVERVIEW

We report a technique, named targeted gene methylation (TAGM), for identifying *in vivo* protein binding sites in chromatin. M.CviPI, a cytosine-5 DNA methyltransferase recognizing GC sites, is fused to a DNA-binding factor enabling simultaneous detection of targeted methylation, factor footprints, and chromatin structural changes by bisulfite genomic sequencing. Using TAGM with the yeast transactivator Pho4, methylation enrichments of up to 34-fold occur proximal to native Pho4 binding sites. Additionally, significant, selective targeting of methylation is observed several hundred nucleotides away, suggesting the detection of long-range interactions due to higher-order chromatin structure. In contrast, at an extragenic locus lacking Pho4 binding sites, methylation levels are at the detection limit at early times following Pho4 transactivation. Notably, substantial amounts of methylation are targeted by Pho4-M.CviPI under repressive conditions when most of the transactivator is excluded from the nucleus. Thus, TAGM enables rapid detection of DNA-protein

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interactions even at low occupancies and has potential for identifying factor targets at the genome-wide level. Extension of TAGM from yeast to vertebrates, which use methylation to initiate and propagate repressed chromatin, could also provide a valuable strategy for heritable inactivation of gene expression.

INTRODUCTION

The interaction of proteins with chromosomal target sites, either directly or through recruitment by DNA-bound factors, is central to many processes, including transcriptional activation and repression, replication and repair of DNA, recombination, and chromosome segregation. Therefore, strategies are needed that can efficiently identify specific chromosomal sites at which factors act. Few techniques are capable of demonstrating these interactions in the context of native chromatin in living cells, and these methods have limitations (Simpson, 1999). For example, with footprinting techniques, protection against chemical (*e.g.*, dimethyl sulfate) or enzymatic probes expressed in cells, *e.g.*, DNA methyltransferases (DMTases) (Gottschling, 1992; Singh and Klar, 1992; Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b) or DNase I (Wang and Simpson, 2001), requires close proximity of the interacting factor to DNA sites that are modified or cleaved by the footprinting agent. Footprinting methods also require that the factor resists displacement by the enzymatic or chemical probe. Moreover, as many proteins can exclude probe access, a

footprint does not provide an unequivocal identity of the bound protein (Rigaud et al., 1991). To circumvent this latter problem, proteins have been fused to an endonuclease (Lee et al., 1998), however, the resulting DNA damage alters chromatin structure and activates checkpoint controls. Another method, chromatin immunoprecipitation (ChIP), employs *in situ* fixation with formaldehyde followed by immunoselection of DNA-bound complexes. The requirements for large numbers of cells and highly specific antibodies as well as low fixation efficiencies (*ca*. 0.1-0.5%) (Tanaka et al., 1999; Reid et al., 2000) present distinct disadvantages of ChIP analysis. The approach of tethering chromatin proteins to the Dam DMTase, which methylates GATC sites near their sites of chromosomal association, overcomes the above problems (van Steensel and Henikoff, 2000). This method has been used to detect factors bound at chromosomal regions containing multiple factor binding sites, *e.g.,* 14 Gal4 (van Steensel and Henikoff, 2000) and 112 TetR sites (Lebrun et al., 2003); however, it is not known if it can detect a factor bound at a single binding site. In addition, sensitive quantification of methylation frequencies can only be performed for one *dam* site at a time and requires real-time PCR analysis.

We report the specific targeting of cytosine methylation to promoters in living eukaryotic cells. Our strategy (Fig. 4-1), named TAGM, capitalizes on fusing chromatin-associating factors to M.CviPI, a cytosine-5 DNA methyltransferase (C5 DMTase) that methylates the C of a 2-bp GC site. This

short specificity provides a M.CviPI recognition site, on average, once every 27 bp, increasing the frequency of DMTase sites at least 10-fold over DMTases that recognize 4-bp sites. Bisulfite genomic sequencing is used to provide a positive display of 5-methylcytosine (m^5C) levels at many GC sites on a standard sequencing gel. We find that fusion of M.CviPI to a DNA-binding factor leads to substantial increases in, or targeting of, m^5C proximal to factor binding sites that are accessible in chromatin. Moreover, m^5C is selectively targeted distal to the site of the bound factor, suggesting detection of higher-order chromatin structure. Thus, TAGM is sensitive, requiring small numbers of cells to monitor the interaction of a factor with a single, native binding site. Since DNA methylation is a primary signal for establishing and maintaining repressive chromatin structures in vertebrates (Bird, 2002), our demonstration of targeting m^5C in a eukaryote is a critical step toward achieving heritable, methylation-dependent gene silencing in such organisms.

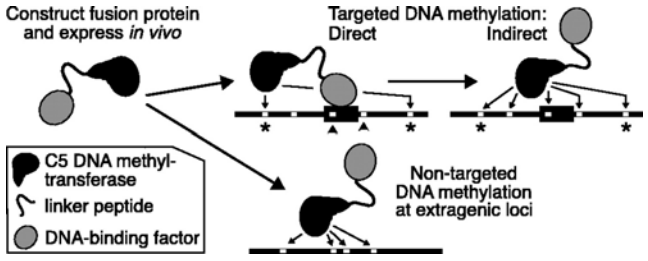

Figure 4-1. The TAGM strategy for identifying DNA–protein interactions *in vivo*.
Hypothetical sites protected against methylation (arrowheads) or directly methylated (asterisks) are indicated.

MATERIALS AND METHODS

Yeast strains, plasmid construction, and growth conditions

All yeast strains used in TAGM analyses have the S288C background and were derived from YPH500ΔL (*MATα ade2-101 ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-Δ1*)(Kladde et al., 1996). Mutated Zif268 (mut Zif), which contains a single amino acid mutation (H58E) (Nardelli et al., 1991) that abolishes DNA binding, was cloned as an in-frame fusion to M.CviPI into pMPK1 under the control of the *GAL1* promoter and integrated at *LYS2* as previously described (Kladde et al., 1996). M.CviPI and mut Zif are separated by a linker peptide, $GS(G)_4SG_4SG_3LGST$ (Xu and Bestor, 1997). Pho4-M.CviPI was constructed by tagging Pho4 with 3HA-$(his)_6$-$GPGS(G)_6(SGG)_2$GLGST (linker)-M.CviPI at its C-terminus under control of the constitutively-expressed, endogenous *PHO4* promoter (Legrain et al., 1986). *URA3*, which was flanked by *Zygosaccharomyces rouxii* recombinase sites, was then deleted by homologous recombination (Roca et al., 1992).

For ChIP analysis, strains LFY2152 (S288C; *MATα leu2-Δ0 lys2-Δ0 ura3-Δ0 pho3Δ*) with the endogenous *PHO4* locus tagged at its N-terminus with a triple myc epitope or ADY2398 with wild-type *PHO4* (no tag control), were used. Both strains also contain a mutated copy of the *PHO5* promoter (deletion of both UASs, from −401 to −352 and −258 to −209) integrated at the extragenic

CAN1 locus.

Strains were pre-grown in minimal media (2% raffinose, 20 mM 2-*N*-morpholino ethanesulfonic acid [MES], pH 5.5, 14 mM *L*-glutamine, and 0.7 g yeast nitrogen base [YNB] without $(NH_4)_2SO_4$, phosphate, or amino acids [Bio101]) that was brought to 13.4 mM KH_2PO_4. Cells were then washed and resuspended to an OD_{600} of 0.2 with the same minimal media containing either 13.4 mM KH_2PO_4 ($+P_i$, repressive conditions) or 13.4 mM KCl ($-P_i$, activating conditions) that also contained 2% galactose.

Bisulfite genomic sequencing

Genomic DNA was rapidly isolated and analyzed by bisulfite genomic sequencing (Frommer et al., 1992; Clark et al., 1994) as modified (Kladde et al., 1996). PCR products amplified from bisulfite-deaminated DNA using Jumpstart Taq DNA polymerase (Sigma) were purified and subjected to primer extension as described previously (Kladde et al., 1996), except that the final concentrations of dNTPs (A, C, T) and ddGTP were 50 μM and 150 μM, respectively. Exclusion of dGTP from the PCR product primer extension reactions yields high termination efficiencies (>96%)(Kladde et al., 1996) at template cytidines (m^5C residues *in vivo*). Absolute frequencies of site methylation are calculated by dividing the intensity of a given band by all summed product intensities, including the run-off product at the top of the gel generated by

extension on non-methylated templates. Oligonucleotides used for the bisulfite genomic sequencing analysis of m^5C levels are listed in Table 4-1.

Table 4-1. Bisulfite genomic sequencing primers.

Primer name	Sequence	Figure
Oligonucleotides for PCR amplification.		
*CAR1*b1-60	CCATTTaAaaaACTCaaaACAATaTaaaAC	4-2D
*CAR1*b2-61	TAtGGAATTAGAGtttTtAATGGAtGAG	4-2D
*PHO5*a1-22	CCAAATaaaTATATaCCTTaCCAAaTAAaaTaACC	4-3
*PHO5*a2-21	TAtAtATtGGAtTGATAAGTTAtTAtTGtAtATTGG	4-3
*PHO5*b1-922	TTCAATTaCTAAATACAATaTTCCTTaaT	4-2, 4-4
*PHO5*b2-924	GAAAAtAGGGAttAGAATtATAAATTTAGTtT	4-2, 4-4
*PHO8*b1-246	ATAACCaCACCTaCAATaACaaTA	4-5A
*PHO8*b2-247	TtGAGTtAGATttAGGAAtAAGAtGT	4-5A
*PHO84*a1-918	ATaTTACCACCTTCaaTAAaaTaTTCTTTATaAA	4-5B
*PHO84*a2-920	AGATGAtTTtAAAtGAtTtGGTATAtTtTG	4-5B
Oligonucleotides that were ^{32}P-end-labeled for primer extension		
*CAR1*b1-60	CCATTTaAaaaACTCaaaACAATaTaaaAC	4-2D
*PHO5*b1-751	TaTTTTCTCATaTAAaCaaACaTCaTCT	4-2AB (upper panel), 4-2C
*PHO5*b1-969	AACaCAACTaCACAATaCCAA	4-2B (lower panel)
*PHO5*a1-22	CCAAATaaaTATATaCCTTaCCAAaTAAaaTaACC	4-3 (UASp1)
*PHO5*a1-20	aaCTAaTTTaCCTAAaaaAATaaTACCTaCATTaaCC	4-3 (UASp2)
*PHO5*b1-768	ATATATCTCGAGGACTAATAaAAaAAAACAAaAaACTCCaT	4-4
*PHO8*b1-248	AaAATCAAaTAAaACCTCAAaA	4-5A
*PHO84*a1-918	ATaTTACCACCTTCaaTAAaaTaTTCTTTATaAA	4-5B

Pairs of 'a' (a1 and a2) or 'b' (b1 and b2) are PCR amplification primers for the upper and lower DNA strands, respectively, from bisulfite-treated DNA. Nucleotides in lower case represent either G to a or C to t transitions.

Chromatin immunoprecipitation (ChIP) analysis

Strains LFY2152 (3Myc-*PHO4*) and ADY2398 (*PHO4*) were grown for 4 h in the above minimal medium (2% glucose) containing the indicated concentrations of P_i before treatment with 1% paraformaldehyde for 15 min at room temperature. ChIP analysis was performed as previously described (Hecht and Grunstein, 1999) using 2 μl rabbit A-14 anti-Myc antibody (Santa Cruz Biotechnologies). Two microliters of immunoselected and input DNA (1:2000 dilution) were amplified in the presence of 10 μCi [α^{32}P]dCTP by quantitative, competitive PCR with primers ADO236 (CATGTAAGCGGACGTC) and LFO740 (GCCTTGCCAAGTAAGGTGAC), which simultaneously amplify both the wild-type (298 bp product) and mutant (198 bp product) copies of the *PHO5* promoter. Radiolabeled PCR products were analyzed by 4% native PAGE.

RESULTS AND DISCUSSION

Targeting of cytosine methylation by Pho4 *in vivo*

m^5C has been selectively targeted to oligonucleotides *in vitro* by fusing C5 DMTases to heterologous DNA-binding factors (Xu and Bestor, 1997; McNamara et al., 2002). To date, however, attempts to reproduce this capability *in vivo* have been unsuccessful (McNamara et al., 2002). As a first step toward targeting C5 DNA methylation *in vivo*, we tested whether a native yeast protein could specifically target a C5 DMTase and hence increase m^5C levels at

promoters in the tractable eukaryote, *S. cerevisiae* (Fig. 4-1). Yeast does not have detectable endogenous m^5C and foreign expression of C5 DMTases is neither deleterious nor has known effects on gene expression (Kladde et al., 1996; Xu et al., 1998b). The sequences coding M.CviPI (Xu et al., 1998a) were integrated at the end of the *PHO4* gene, such that the DMTase is fused to the C-terminus of Pho4 and the fusion protein is constitutively expressed from the endogenous *PHO4* promoter (Legrain et al., 1986). Pho4 is a basic helix-loop-helix transactivator that induces expression of the *PHO* gene cluster after binding as a homodimer to E boxes (CACGTG or CACGTT) when P_i is limiting (Oshima et al., 1996). The factor to which M.CviPI is fused is designated the targeting factor. Acid phosphatase activity is increased in *PHO4*-M.CviPI strains at least 25-fold after 6 h P_i starvation, as has been observed for wild-type strains and those expressing other Pho4 C-terminal fusions (O'Neill et al., 1996; Komeili and O'Shea, 1999). Since fusing foreign proteins to DMTases can decrease the affinity of the DMTase for its recognition site (Xu and Bestor, 1997), as a control, we expressed M.CviPI tethered to a mutated version of the zinc-finger protein, Zif268, that is severely impaired for DNA-binding activity (mut Zif). This 'free', non-targeted DMTase controls for the extent of GC methylation due to DMTase site preferences in protein-free DNA and accessibility in chromatin (Gottschling, 1992; Singh and Klar, 1992; Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b).

We investigated the Pho4-dependent targeting of M.CviPI to the *PHO5* promoter, a well-studied locus of Pho4 binding, in a *PHO4*-M.CviPI/*PHO4* strain. The use of a heterozygote rigorously tests whether Pho4 can target the DMTase in the presence of wild-type Pho4, and more closely approximates the experimental conditions likely to be employed if TAGM were used in vertebrate cells. Relative methylation frequencies at multiple GC sites were determined by bisulfite genomic sequencing (Frommer et al., 1992; Clark et al., 1994; Kladde et al., 1996), where the extent of primer extension termination is directly proportional to the level of m^5C at a given GC site. *PHO4* expression is constitutive (Legrain et al., 1986); in high P_i medium, Pho4 is phosphorylated by the nuclear cyclin-CDK Pho80-Pho85 and is exported to the cytoplasm thereby leading to the repression of *PHO* genes (O'Neill et al., 1996). Consistent with the predominantly cytoplasmic localization of Pho4 under conditions of high P_i, on the lower DNA strand of the nucleosome-free region of the *PHO5* promoter (Almer et al., 1986), C5 methylation by Pho4-M.CviPI of six GC sites (sites 1, 4, 19, 26b, 41, and 43) is at background levels (Fig. 4-2A, lanes 8 and 9).

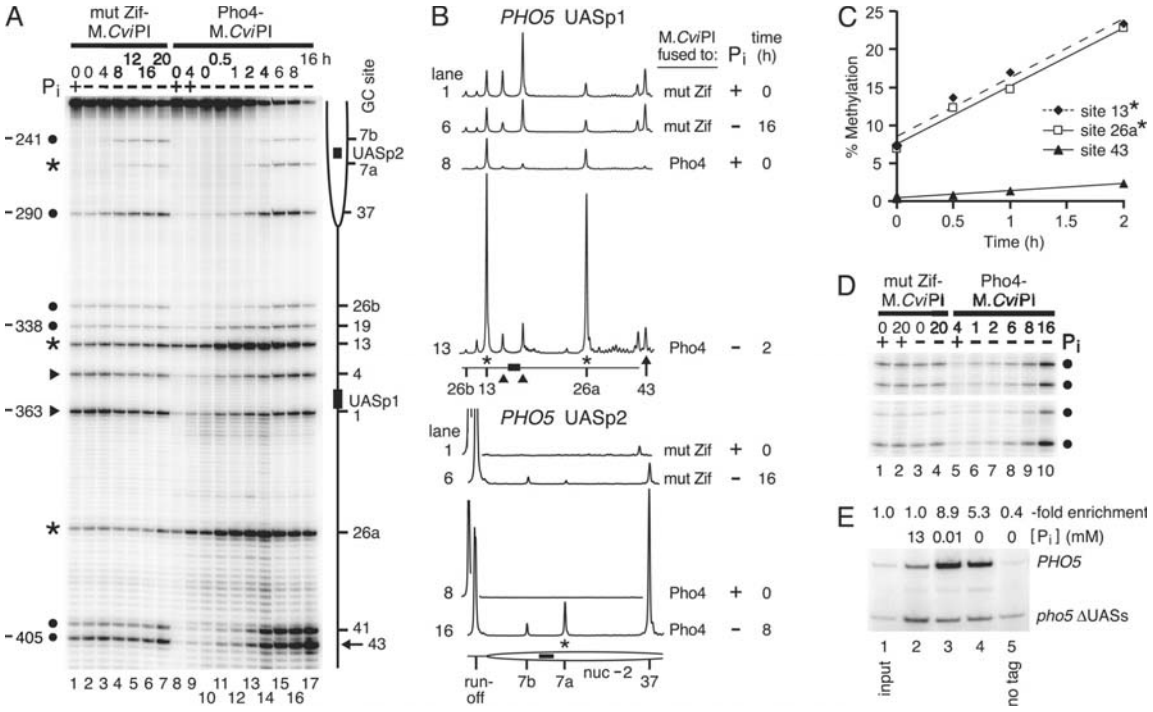

Figure 4-2. Pho4 specifically targets M.CviPI to the *PHO5* promoter.

(A) Cultures expressing Pho4-M.CviPI and Pho4 or mut Zif-M.CviPI as a free DMTase control were grown under repressive conditions in high P_i medium then washed and transferred to P_i-free medium to activate *PHO* genes. Genomic DNA isolated from cells removed at the indicated times was analyzed for m^5C levels at GC sites on the lower strand of the *PHO5* promoter by a bisulfite genomic sequencing. The locations of the two known Pho4-binding sites (filled bars), the UASp1 E box, and UASp2 E box, as well as positioned nucleosome –2 (partial ellipse), are shown. The distance (base pairs) of each GC site from the respective proximal edge of UASp1 in the nuclease hypersensitive region (GC sites from –405 to –331 relative to the *PHO5* ATG) or UASp2 (GC sites from –290 to –241) are also indicated on the right. The same number of total counts was loaded in each lane. In strains expressing either DMTase fusion, the ratios of m^5C between several sites (●) in a given lane were similar, identifying sites to which methylation is nontargeted or targeted indirectly. Normalization of m^5C levels to an accessible histone-free site remote from UASp1, site 43 (⟵), enables lane-to-lane comparisons and demonstrates protection against methylation (►) as well as efficient targeting of M.CviPI to three GC sites (*) by bound Pho4. Selective targeting of m^5C to these latter three sites is highly reproducible, as evidenced in lanes 9–17 and in five additional experiments analyzing one $+P_i$ and a 4-h $-P_i$ sample. Note that, after 2 h, high levels of methylation in the Pho4-M.CviPI samples lead to considerable departure from single-hit kinetics and underestimation of signal intensity.
(B) Quantitative scans of bisulfite genomic sequencing data. (*Upper*) Selected lanes (as indicated) in (A) are scanned (*PHO5* UASp1). Methylation levels can be normalized to that at site 43. (*Lower*) Scans (*PHO5* UASp2) were obtained by re-extension of the same PCR products used in the analysis in (A) with primer *PHO5*b1–969 that anneals between sites 26b and 37.
(C) Initial rates of methylation are linear. Quantification of absolute m^5C frequencies (percentage of total summed product intensities) of the indicated sites from the data in (A), lanes 10–13.
(D) M.CviPI is specifically targeted by Pho4 to *PHO5* and not to *CAR1* at early times after PHO activation. *CAR1* sequences (+159 to +558) were amplified from a subset of the bisulfite-treated samples analyzed in (A) and analyzed for m^5C levels. The ratios among eight additional sites are also identical.
(E) TAGM detects Pho4 binding more sensitively than ChIP analysis. Immunoselected (lanes 2–5) and nonimmunoselected (lane 1, input) samples from either wild-type *PHO4* (lane 5, no tag) or 3Myc-*PHO4* (lanes 2–5) strains that contain a wild-type *PHO5* promoter and a mutated promoter (*pho5* ΔUASs) were analyzed by competitive PCR. The folds of enrichment, normalized to the input ratio, are given.

During a time course of PHO transactivation (Fig. 4-2A, $-P_i$, lanes 11-17), methylation at most nucleosome-free sites (sites 13, 19, 26a, 26b, 41, and 43) in the *PHO5* promoter increased over time in the Pho4-M.CviPI strain, in agreement with the well-known nuclear accumulation of Pho4 under these conditions (O'Neill et al., 1996). In contrast, in the mut Zif-M.CviPI control strain (Fig. 4-2A, lanes 1-7), methylation remained rather constant at most of these sites in this histone-free region, except at sites 1 and 4 adjacent to UASp1, which are probably protected against methylation by bound Pho4 (Kladde et al., 1996; Xu et al., 1998b). Closer analysis of m^5C levels during 0-2 h after activation (Fig. 4-2A, lanes 10-13) indicates that Pho4 predominantly targets M.CviPI to *PHO5* sites 13 and 26a (asterisked), achieving enrichments of up to 20- and 34-fold, respectively, over mut Zif-M.CviPI. Directly targeted methylation is readily identifiable by inspecting for peak areas that are altered relative to other peaks in a given lane with Pho4-M.CviPI as compared to mut Zif-M.CviPI (Fig. 4-2B). Further, in the *PHO4*-M.CviPI strain, methylation frequencies of sites 13 and 26a increase linearly from 7 to 23% between 0 and 2 h (Fig. 4-2A, lanes 10-13; Fig. 4-2C), and plateau at 4 h after induction (Fig. 4-2A, lane 14). By comparison, from 0-2 h activation, m^5C accumulates at an 8-fold slower rate at site 43 than at sites 13 and 26a (Fig. 4-2C). In addition, similar ratios of m^5C levels among GC sites in a given lane at an extragenic locus

(*CAR1*), which lacks Pho4 sites, demonstrate that the enhanced methylation of sites 13 and 26a at *PHO5* is due to site-specific DNA binding by Pho4 (Fig. 4-2D). These results suggest that the frequency of targeted m^5C parallels the increase in Pho4 binding to UASp1 that occurs when cells are starved for P_i (Svaren et al., 1994; Venter et al., 1994). We conclude that M.CviPI is efficiently and directly targeted (see Fig. 4-1) to C residues of GC sites 13 and 26a on the lower strand of the *PHO5* promoter, which agrees well with the optimal distance range of 10-40 bp observed for targeting DNA DMTases to oligodeoxynucleotide substrates *in vitro* (Xu and Bestor, 1997; McNamara et al., 2002). It is likely that the DMTase can reach sites within this distance range when the targeting factor (*i.e.*, Pho4) is specifically bound to its UAS. Interestingly, other sites, *e.g.*, site 19, are not selectively modified by Pho4-M.CviPI (Fig. 4-2A-B).

Indirect targeting of M.CviPI, Pho4-dependent accumulation of m^5C that occurs locally when Pho4 dissociates from its UAS, is also observed (see Fig. 4-1). For instance, methylation at sites 41 and 43 increases abruptly at 4 h $-P_i$ and continues to rise for the remainder of the time course (Fig. 4-2A, lanes 14-17). Moreover, while m^5C amounts introduced by Pho4-M.CviPI at *PHO5* (*e.g.*, sites 41 and 43) surpass those attained with the free DMTase (Fig. 4-2A, compare lanes 14-17 to 3-7), the converse occurs at the extragenic *CAR1* locus at all times until 16 h post-induction (Fig. 4-2D, compare lanes 3-4 to 6-10). This demonstrates that, at early times after induction, Pho4 preferentially targets

M.CviPI to *PHO5*, and, at extragenic loci, at least 4 h more is required to accumulate high levels of m^5C. Therefore, at ≥4 h in P_i-free medium (Fig. 4-2A, lanes 14-17), the significant increases in methylation at *PHO5* sites 41 and 43 are due to indirect targeting of M.CviPI; Pho4-M.CviPI dissociating from either UAS creating a local region of m^5C.

Between 2-16 h induction, Pho4-M.CviPI also increased m^5C levels substantially at *PHO5* sites 7a, 7b, and 37 located in positioned nucleosome –2 (Almer et al., 1986) (Fig. 4-2A, lanes 13-17). Since nucleosomes block accessibility of DMTases (Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b), the increased methylation of these sites by both M.CviPI fusion proteins is indicative of nucleosomal disruption concomitant with *PHO5* activation (Almer et al., 1986). Methylation by mut Zif-M.CviPI at site 37 in the presence of P_i (Fig. 4-2A, lane 1) occurs because DMTases can access two helical turns of DNA that enter and exit nucleosomes (Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b). Note that methylation levels at sites 7a, 7b, and 37 in the Pho4-M.CviPI samples (Fig. 4-2A, lanes 13-17) are substantially underestimated due to high levels of primer extension termination at sites closer to the primer (*i.e.*, the analysis does not satisfy single-hit kinetics at the most primer distal sites). Thus, extension with a primer annealing just downstream of site 26b demonstrates that Pho4-M.CviPI methylates sites 7a, 7b, and 37 more efficiently than mut Zif-M.CviPI (Fig. 4-2B, lower panel). The

extensive methylation of these sites by Pho4-M.CviPI is consistent with the high level of indirect targeting of methylation to the UASp1 region that occurs ≥4 h P_i starvation. In addition, the marked increase in m^5C at site 7a relative to 7b with Pho4-M.CviPI, and not with mut Zif-M.CviPI, strongly suggests that Pho4 targets the DMTase to site 7a after binding UASp2 and/or from a distance when bound at UASp1. Therefore, m^5C is targeted to the central region of nucleosomes, which is inaccessible to DMTases, only when they have been disrupted.

Strikingly, methylation is targeted directly to several GC sites when the majority of Pho4-M.CviPI is expected to be excluded from the nucleus (O'Neill et al., 1996). This is evidenced by the significant level of methylation that is present at sites 13 and 26a in the presence of P_i (Fig. 4-2B, *PHO5* UASp1, compare the scan 8 to scans 1 and 6). After normalization of m^5C levels to a histone-free site, site 43, greater than 20-fold enrichments in targeting of M.CviPI to sites 13 and 26a by Pho4 is observed, as compared to the free DMTase, mut Zif-M.CviPI. Significant methylation is also targeted under repressive conditions to the opposite strand of the *PHO5* promoter (Fig. 4-3, scan 3). A possible explanation for targeted methylation under repressive conditions is that the DMTase fusion impairs the ability of Pho80-Pho85 to phosphorylate Pho4, and hence increases the nuclear retention of Pho4-M.CviPI. This is unlikely as acid phosphatase expression is not derepressed in the Pho4-M.CviPI strain. Nevertheless, we tested this possibility further by comparing the

rate at which *PHO5* transcript levels decrease in wild-type and Pho4-M.CviPI strains after adding P_i back to cultures subjected to 10 h P_i starvation. For both strains, *PHO5* transcript levels decreased by 90% within 20 min of P_i addition, indicating that Pho4 and Pho4-M.CviPI are regulated similarly (data not shown). Thus, TAGM detects Pho4 binding, even under repressive conditions where its nuclear concentration is low (O'Neill et al., 1996), and therefore, promoter occupancy by Pho4 is very low. Repeated attempts to detect Pho4 binding in the presence of P_i by ChIP analysis were unsuccessful (Fig. 4-2E, lane 2); significant immunoselection of Pho4 crosslinked to *PHO5* was only detected upon transactivation (Fig. 4-2E, lanes 3 and 4).

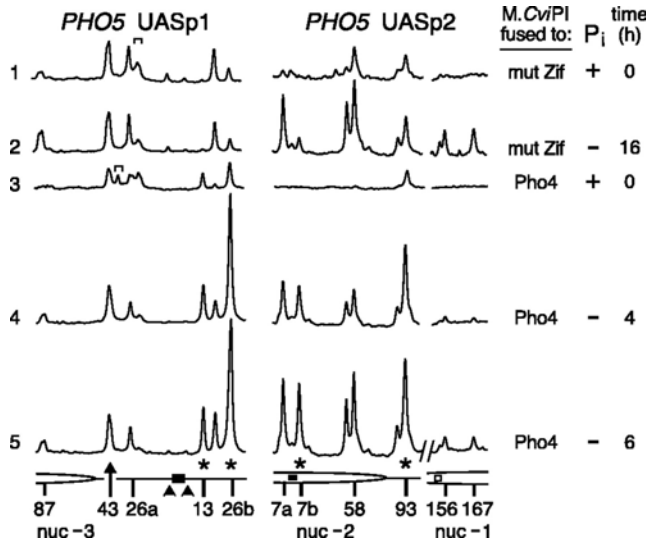

Figure 4-3. Targeting of C5 methylation by Pho4-M.CviPI to the upper strand of the *PHO5* promoter.

The same bisulfite-treated samples used in the analysis in Figure 4-2A were used in the PCR amplification. Scans of the phosphorimage of the gel that was loaded with the same number of total counts per lane are shown. (Left) The brackets above scans 1 and 3 (*PHO5* UASp1) indicate a nonspecific primer extension pause that occurred in samples 1–5 or only sample 3, respectively.

We also analyzed m^5C levels on the upper strand of the *PHO5* promoter (Fig. 4-3). After transactivation, methylation is enhanced at several GC sites near UASp1 (site 87) and UASp2 (sites 7a, 52, 58, 154, 156, and 167), as expected with the increased access of both DMTase fusion proteins that accompanies nucleosome disruption (Almer et al., 1986). Methylation amounts are significantly altered at sites 13, 26b, 7b, and 93 (asterisked) relative to other sites in cells expressing Pho4-M.CviPI (scans 3-5) as compared to the control, mut Zif-M.CviPI (scans 1 and 2), indicating Pho4-dependent targeting of M.CviPI. Interestingly, despite the high level of m^5C targeted to sites 7a and 26a on the lower strand (Fig. 4-2), M.CviPI is not directly targeted to these sites on the upper strand. The reason for this strand-specific, targeting of m^5C to pairs of GC sites that symmetrically flank each Pho4 binding site (7 or 26 bp away) is not understood.

Pho4 targets M.CviPI at a distance

In Figure 4-3, the marked methylation of site 93 as compared to other sites on the upper strand of the *PHO5* promoter suggests that M.CviPI is targeted at distances (93 bp from UASp2 and 202 bp from UASp1) well beyond the optimal targeting distance of 10-40 bp observed *in vitro* (Xu and Bestor, 1997). To investigate this possibility further, we determined methylation levels at GC sites farther upstream in the *PHO5* promoter (Fig. 4-4). m^5C levels at a

number of GC sites increased at the positions of two additional nucleosomes (−3 and −4) that are known to be perturbed upon promoter activation (Almer et al., 1986) (Fig. 4-4A, compare lane 2 to 1 and lanes 6-9 to 3). m^5C was reproducibly enriched at a GC site located 335 bp from UASp1, in the *PHO4*-M.CviPI as compared to the mut Zif-M.CviPI strain, suggesting the formation of long-range interactions stemming from higher order chromatin folding (Fig. 4-4A-B, compare lanes 7-9 to 2). A DNA-bound homodimer of Pho4 similarly targets M.CviPI distally (60, 78, and 91 bp) to a low affinity Pho4 binding site (UAS E) in the *PHO84* promoter (Ogawa et al., 1995) (Fig. 4-5B). The Gal4 DNA-binding domain- and TetR-Dam DMTase bound at 14 and 112 sites, respectively, can also distally target a tethered Dam DMTase (van Steensel and Henikoff, 2000). Thus, in comparison to a free DMTase control, TAGM can discern activation-dependent perturbations in nucleosome structure and preferential DMTase targeting at a distance.

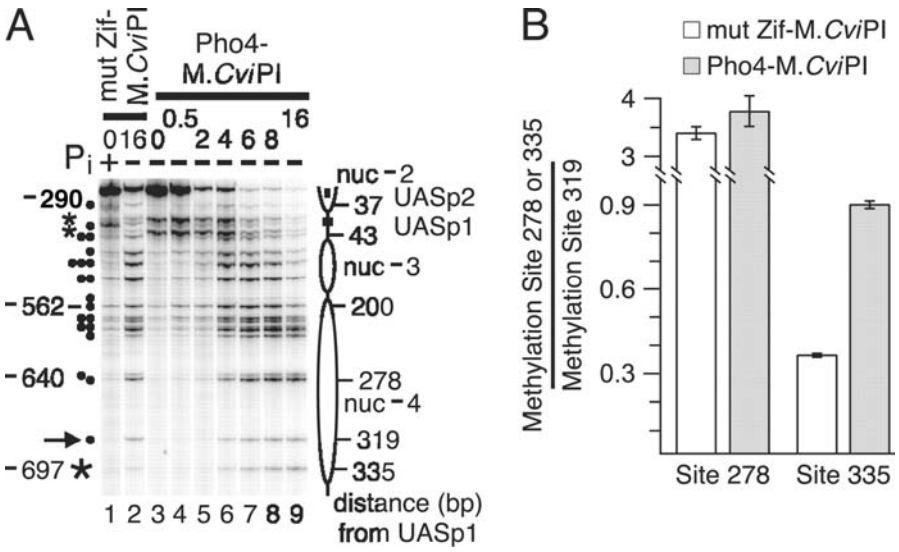

Figure 4-4. Pho4 targets M.CviPI at a distance.

(A) Determination of m^5C levels upstream of *PHO5* UASs. *PHO5* sequences were amplified from a subset of the bisulfite-treated samples analyzed in Fig. 4-2A to assay for m^5C levels. The two asterisks at the top of the gel indicate sites 13 and 26a that are directly targeted by Pho4-M.CviPI near UASp1. Symbols are as in Fig. 4-2A, except that double (●●) and triple GC sites (●●●) that did not resolve during electrophoresis are also indicated. Site 319 used for normalization in (B) is marked as well (→).
(B) Quantification of preferential targeting of M.CviPI by Pho4 to site 335, but not to site 278. The mean ± standard error of m^5C levels for the indicated sites (normalized to site 319) for mut Zif-M.CviPI (n = 3) and Pho4-M.CviPI (n = 6) is shown.

Pho4 targets M.CviPI to additional PHO promoters

Pho4 targeted M.CviPI directly to several GC sites at the *PHO8* and *PHO84* promoters (Fig. 4-5). For example, in contrast to mut Zif-M.CviPI cells, yeast expressing Pho4-M.CviPI exhibited significantly higher levels of m^5C at *PHO8* sites 13, 51, and 54 as compared to site 17, and at *PHO84* sites 19 and 36 relative to site 11 (compare the relative peak areas of scans 3 and 4 in (A) or 3-5 in (B) to those of 1 and 2). Pho4 also significantly targets M.CviPI to each of these sites under repressive conditions when Pho4 binding is very low (scan 3).

In addition, after starving *PHO4*-M.CviPI cells for P_i (scan 4 in (A)), amounts of methylation at *PHO8* sites 24 and 34 in disrupted nucleosome –4 surpass those at site 17. While M.CviPI targeting was evident near UASp2 of *PHO8*, none of four GC sites located 11-42 bp from the putative UASp1 is targeted in the repressed or activated promoter (data not shown). This indicates further that UASp2 is the only functional Pho4 binding site in the *PHO8* promoter (Münsterkötter et al., 2000). After 2 h activation, methylation at *PHO84* sites 60, 78, and 91 exceeds that at neighboring sites 118, 140, and 221 with Pho4-M.CviPI, but not mut Zif-M.CviPI (Fig. 4-5B, compare scan 5 to 2). This suggests that bound Pho4 directly targets M.CviPI to distal *PHO84* sites 60, 78, and 91. We conclude that the native transcription factor Pho4 can efficiently target M.CviPI to each of the endogenous, single-copy *PHO* promoters that we have tested.

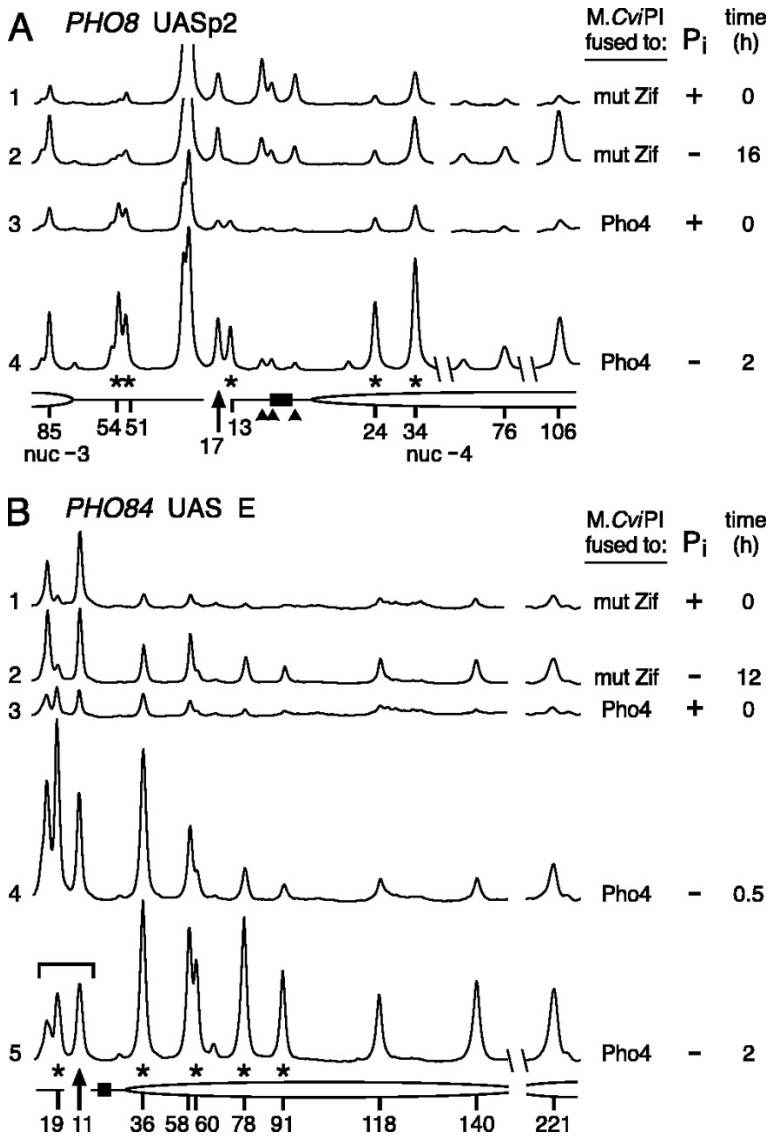

Figure 4-5. M.CviPI is targeted by Pho4 to the *PHO8* and *PHO84* promoters. m^5C levels were determined at *PHO8* (A) and *PHO84* (B) from cells expressing either mut Zif- or Pho4-M.CviPI grown in the presence (+) and absence (-) of P_i, as indicated. Shown are the quantitative scans of the phosphorimage obtained from the gel (same total counts per lane). GC sites to which M.CviPI directly targeted methylation (*), GC sites protected against methylation (▲), and Pho4-binding sites (filled bars), are labeled. m^5C levels can be compared with the sites marked with arrows. The positions of nucleosomes (nuc -3 and nuc -4, partial ellipses), previously mapped at *PHO8* (41), are shown. From the data in (B), we infer the disruption of two nucleosomes in the analyzed *PHO84* region (increased methylation on activation at seven GC sites, 36–221 bp from UAS E; compare scan 2 to scan 1 in (B)). To augment peak heights, quantification of the run-off products has been omitted. A region in scan 5 where the signal is underestimated due to departure from single-hit kinetics is bracketed.

CHAPTER V

SITE-SELECTIVE *IN VIVO* TARGETING OF CYTOSINE-5 DNA METHYLATION BY ZINC-FINGER PROTEINS[‡]

OVERVIEW

Cytosine-5 DNA methylation is a critical signal defining heritable epigenetic states of transcription. As aberrant methylation patterns often accompany disease states, the ability to target cytosine methylation to preselected regions could prove valuable in reestablishing proper gene regulation. We employ the strategy of Targeted Gene Methylation (TAGM) in yeast, which has a naturally unmethylated genome, directing *de novo* DNA methylation to select genomic sites via the fusion of C5 DNA methyltransferases to heterologous DNA-binding proteins. The zinc-finger proteins Zif268 and Zip53 can target DNA methylation by M.CviPI or M.SssI 5-52 nucleotides from single zinc-factor binding sites. Modification at specific GC (M.CviPI) or CG (M.SssI) sites is enhanced as much as 20-fold compared to strains expressing either the free enzyme or a fusion protein with the zinc-finger protein moiety defective for DNA binding. Interestingly, methylation is also selectively targeted as far as 353 nucleotides from the zinc-finger protein binding sites, possibly indicative of

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[‡] The work presented in this Chapter has been published in the following paper: Carvin, C.D., Parr, R.L., and Kladde, M.P. (2003). Site-selective *in vivo* targeting of cytosine-5 DNA methylation by zinc-finger proteins. Nucleic Acids Res. *31*, 6493-6501 by permission of Oxford University Press, Copyright 2003.

higher-order chromatin structure. These data demonstrate that methylation can be targeted *in vivo* to a potentially broad range of sequences using specifically engineered zinc-finger proteins. Further, the selective targeting of methylation by zinc-finger proteins demonstrates that binding of distinct classes of factors can be monitored in living cells.

INTRODUCTION

Methylation of the C5 atom of cytosine in DNA (m^5C) plays an important role in establishing correct patterns of gene expression in vertebrates, usually through repression of transcription. Mechanistically, one way DNA methylation can lead to transcriptional silencing is by decreasing the binding affinity of a transcriptional activator for its site (Attwood et al., 2002). The introduction of m^5C at sites adjacent to a factor binding site can also interfere with binding (Zhu et al., 2003). Perhaps more importantly, symmetrical methylation of CpG sequences (CG) serves as a signal for the recruitment of a family of methyl-CpG binding domain (MBD) proteins, such as MeCP2 and MBD2 (Wade, 2001). In turn, MBDs, either by themselves or as components of complexes, are known to recruit a variety of co-repressors, such as histone deacetylases (Jones et al., 1998; Nan et al., 1998; Wade et al., 1999; Zhang et al., 1999), histone H3 lysine-9 methyltransferases (Jackson et al., 2002), and heterochromatin coating factors like HP1 (Fuks et al., 2003), which can function to establish a local,

repressed region of chromatin (Pikaart et al., 1998; Schubeler et al., 2000; Lorincz et al., 2001; Lorincz et al., 2002; Mutskov et al., 2002; Irvine et al., 2002). This silencing mechanism is also conserved in plants, as the DNA chromomethyltransferase CMT3, which methylates CNG residues, interacts with HP1 to facilitate heterochromatin formation (Jackson et al., 2002).

While regions of m^5C are often associated with hypoacetylation of histones H3 and/or H4 and altered chromatin structure (Pikaart et al., 1998; Schubeler et al., 2000; Lorincz et al., 2001; Lorincz et al., 2002; Mutskov et al., 2002; Irvine et al., 2002), recent evidence suggests DNA methylation- and histone deacetylase-independent modes of silencing. First, trichostatin A (TSA), a specific inhibitor of histone deacetylation, fails to reactivate transcription from densely methylated DNA (Cameron et al., 1999a; Schubeler et al., 2000; Lorincz et al., 2001; Magdinier and Wolffe, 2001; Mutskov et al., 2002; Zhu et al., 2003). Additionally, *mbd2*-null mice are viable and fertile (Hendrich et al., 2001) and *Mecp2*-null mice only display neurological abnormalities (Guy et al., 2001), questioning their global role in m^5C-mediated silencing and cellular differentiation. Moreover, purified MeCP2 itself compacts reconstituted chromatin in the absence of DNA methylation (Georgel et al., 2003).

Although the mechanisms are not yet fully understood, there is a strong correlation between promoter methylation and gene silencing (Robertson, 2001; Jones and Baylin, 2002; Attwood et al., 2002; Bird, 2002). Moreover, once a

methylation state is established, it is maintained heritably after many generations of replication (Stein et al., 1982) by the maintenance DMTase, DNMT1 (Bestor, 2000). An exception includes enhancer sequences that can be passively demethylated on replication and subsequent blockage of DNA methyltransferase (DMTase) access by factor binding (Kladde et al., 1996; Xu et al., 1998b; Hsieh, 1999; Lin et al., 2000; Lin and Hsieh, 2001). However, this enhancer-specific loss of DNA methylation does not lead to derepression (Kladde et al., 1996).

Proper regulation of gene expression is essential for normal cellular functions and the avoidance of disease states. DNA methylation, which occurs almost exclusively at CG dinucleotides in non-diseased cells, is localized to precise regions of the genome, usually in transposons and retroviral elements (Bestor, 2000). In contrast, CG sites in euchromatic regions, most notably when concentrated in CpG islands, are generally unmethylated and are correlated with transcriptional activity. However, in cancer and other diseases, patterns of DNA methylation are frequently aberrant. For instance, the DNA in tumor cells is generally hypomethylated relative to that in normal cells (Feinberg and Vogelstein, 1983), which may lead to genomic instability (Jones and Baylin, 2002). In contrast, a number of tumor-suppressor genes, including BRCA1 and retinoblastoma (Rb), become hypermethylated and transcriptionally inactive (Robertson, 2001). The presence of a single methylated CG site in a gene's

promoter is sufficient to repress its activation (Robertson et al., 1995), although higher m^5C density increases the probability of establishing gene repression (Boyes and Bird, 1991; Boyes and Bird, 1992; Hsieh, 1994; Cameron et al., 1999b; Lorincz et al., 2002). Thus, DNA methylation can be critical in defining the expression state of a gene.

Therefore, directing DNA methylation to improperly regulated loci could be used to reestablish proper gene expression through silencing. Previously, targeting of C5 methylation has been demonstrated *in vitro* (Xu and Bestor, 1997; McNamara et al., 2002), however, selective enrichment of m^5C was not observed *in vivo* (McNamara et al., 2002). Recently, in yeast, using the dinucleotide-specificity DMTase M.CviPI (Xu et al., 1998a) fused to the basic helix-loop-helix activator Pho4, we demonstrated specific targeting of cytosine methylation to promoters containing Pho4 binding sites (targeted gene methylation; TAGM) (Carvin et al., 2003a). Methylation was efficiently targeted to GC sites in nucleosomes that were disrupted on promoter activation, as well as to histone-free regions.

In its present form, targeting DNA methylation is limited to known factors that bind to well characterized DNA binding sites, which are often present in multiple copies in the genome. Toward achieving the ability to methylate one or a small subset of chromosomal regions, herein, we target M.CviPI (GC methylation) and M.SssI (CG methylation) by their fusion to zinc-finger proteins,

Zif268 and its engineered derivative Zip53, which binds p53 sites (Greisman and Pabo, 1997). We detect *de novo* methylation that is enriched at specific CG or GC sites both near and several hundred nucleotides away from their respective binding sites. The ability to use zinc-finger modules, which, in principle, may be selected to recognize any desired DNA sequence, greatly enhances the range of sequences to which m^5C can be directed and could lead to novel therapeutic approaches.

MATERIALS AND METHODS

Plasmids, yeast strains and growth conditions

All yeast strains used in this study were derived from the S288C background strain YPH500ΔL (*MATα ade2-101 ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-Δ1*) (Kladde et al., 1996). Zinc-finger coding sequences were PCR amplified using the primers MKO46 5'-GCACTAGTTAGGCCAGCTGGGCCATGGCTGATATCGGATCTGG-3' and MKO47 5'-GAATAATTCGAGCGCTTTCAAGGTCATGGTGGATCCTAGGCCACCTCCACTCC-3' and cloned between SfiI and AfeI restriction sites as in-frame fusions to either M.CviPI or M.SssI in pMPK1. The fusion proteins are expressed under control of the *GAL1* promoter after integration at *LYS2* as previously described (Kladde et al., 1996). Each *N*-terminal zinc-finger protein is separated from the DMTase by a $G(SGGGG)_2SGGGLGST$ (GS linker) peptide (Xu and Bestor, 1997). As a free

DMTase control, mutated Zif268 (mut Zif), which contains a single amino-acid substitution (H58E) (Nardelli et al., 1991) that ablates DNA binding, was constructed by overlap site-directed mutagenesis using the primers MKO72 5'-CAGTCGTAGTGACgAgCTTACCACCCAC-3' and MKO73 5'-GTGGGTGGTAAGcTcGTCACTACGACTG-3' (mutated residues in *lower case*).

Cells were pre-grown in yeast extract (Difco)/peptone (Difco)/2% dextrose (YPD) medium and then washed and resuspended at an OD_{600} of 0.5 in YP/ 2% galactose (YPG). After resuspension in YPG, cells were incubated at 30° C for 16 h, or for the indicated times.

Bisulfite genomic sequencing

Total genomic DNA was rapidly isolated by the phenol/chloroform lysis method (Adams et al., 1997) and analyzed by bisulfite genomic sequencing (Frommer et al., 1992; Clark et al., 1994) as previously modified (Kladde et al., 1996). PCR amplification from bisulfite-treated genomic DNA with the indicated primer pairs was performed with Jumpstart Taq DNA polymerase (Sigma) and the resulting products were subjected to primer extension using a ^{32}P-labeled oligonucleotide as described previously using final concentrations of 5 μM dATP, dCTP, and dTTP (dGTP omitted) as well as 50 μM ddGTP (Kladde et al., 1996) (Figs. 5-1 and 5-2), or with dNTPs (A, C, T) and ddGTP increased to 50 μM and 150 μM, respectively (Figs. 5-3-5) as recently reported (Carvin et al., 2003a).

Product intensities were determined by ImageQuaNT software (Molecular Dynamics) after subtracting the local background average. Absolute frequencies of cytosine methylation were obtained by dividing the intensity of a given band by all summed product intensities in a given lane, including the run-off product at the top of the gel generated by primer extension on templates lacking cytosine residues (*i.e.*, templates not methylated *in vivo*). Oligonucleotides used for PCR amplification of bisulfite-treated DNA are described in Table 5-1 using the original naming conventions of Frommer et al. (Frommer et al., 1992).

RESULTS AND DISCUSSION

In vivo targeting of C5 DMTases near single, Zif268 binding sites

m^5C has been selectively targeted *in vitro* by fusing C5 DMTases (M.HhaI, M.HpaII, and M.SssI) to zinc-finger DNA-binding factors (Xu and Bestor, 1997; McNamara et al., 2002). However, attempts to use zinc-finger proteins as targeting entities *in vivo* have been unsuccessful (McNamara et al., 2002). As a first step toward targeting DNA methylation *in vivo* using zinc-finger proteins, we tested whether we could increase cytosine methylation levels adjacent to zinc-finger protein binding sites (ZBS) in the genetically tractable eukaryote, *S. cerevisiae*. Yeast genomic DNA does not contain detectable levels of endogenous methylated residues (Proffitt et al., 1984) enabling unambiguous detection of *de novo* DNA methylation. Also, low-level expression of C5 DNA

Table 5-1. Primers used for bisulfite genomic sequencing.

Oligonucleotides for PCR amplification.			^{32}P-end-labeled oligonucleotides for primer extension.		
Primer[a]	Sequence	Figure	Primer	Sequence	Figure
*CAR1*b1-60	CCATTTaAaaaACTCaa aACAATaTaaaAC	5-1 5-5C	*CAR1*b1-60	CCATTTaAaaaACTCaaaACAAT aTaaaAC	5-1, 5-5C
*CAR1*b2-61	TAtGGAATTAGAGtttT tAATGGAtGAG	5-1, 5-5C	*YBR108W*a1-58	aCTaTTaaaaaCATCACCCAAAC aAC	5-2AB
*YBR108W*a1-58	aCTaTTaaaaaCATCAC CCAAACaAC	5-2AB	*YOL019W*b1-54	CCaTAaaAATCAAATTTaCAACA AaTCTaC	5-2C
*YBR108W*a2-59	AAAttTTTtAGAtATGG AtTGATTtTG	5-2AB	*PHO5*b1-751	TaTTTTCTCATaTAAaCaaACaT CaTCT	5-3
*YOL019W*b1-54	CCaTAaaAATCAAATTT aCAACAAaTCTaC	5-2C	*DED1*a1-166	AAaTaACTCTTTaTATTCTTTCA TATTaTTT	5-4
*YOL019W*b2-55	ATAGAttTAGtGTTGtT ATTGAGTTTttAGTTG	5-2C	*YLR016C*a1-1019	CATTCATaAAaATaAaTTCaTAA TCaTTATC	5-5AB
*PHO5*b1-922	TTCAATTaCTAAATACA ATaTTCCTTaaT	5-3			
*PHO5*b2-924	GAAAAtAGGGAttAGAA TtATAAATTTAGTtT	5-3			
*DED1*a1-166	AAaTaACTCTTTaTATT CTTTCATATTaTTT	5-4			
*DED1*a2-165	ttTtAtTTAAGAGGAAA AttAAGAAGT	5-4			
*YLR016C*a1-1020	TaACaaaAATTCAAAAa TTAACACACTaaAT	5-5AB			
*YLR016C*a2-1021	GAtTATGTATGAAtTAG TGATATAtAG	5-5AB			

[a]Pairs of 'a' (a1 and a2) or 'b' (b1 and b2) are PCR amplification primers for the upper and lower DNA strands, respectively, from bisulfite-treated DNA. Nucleotides in lower case represent either G to a or C to t transitions.

methyltransferases in yeast has no known effects on gene expression or growth (Singh and Klar, 1992; Kladde et al., 1996; Xu et al., 1998b).

Since chromatin blocks access of DMTases to their target sites (Gottschling, 1992; Singh and Klar, 1992; Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b), our efforts to target m^5C *in vivo* focus on the use of enzymes that methylate dinucleotide sites. This substantially increases the probability that DMTase target sites located in accessible, histone-free regions will be modified. Either of two C5 DMTases, M.CviPI (GC specificity) (Xu et al., 1998a) or M.SssI (CG specificity) (Renbaum et al., 1990), was tethered to the archetypal zinc-finger protein, Zif268 (Chavrier et al., 1988) and expressed as a single-copy, integrated gene under control of the galactose-inducible *GAL1* promoter. The DNA-binding factor that is fused to the DMTase is designated the targeting factor. As a control, we expressed either the untethered DMTase or a fusion protein in which the DNA-binding activity of Zif268 was severely impaired (Nardelli et al., 1991). Strains expressing these 'free' DMTase controls establish the level of nontargeted methylation due to enzyme site preferences and accessibility in protein-free DNA and chromatin (Gottschling, 1992; Singh and Klar, 1992; Kladde and Simpson, 1994; Kladde et al., 1996; Xu et al., 1998b).

Endogenous yeast Zif268 binding sites (5'-GCGTGGGCG-3') were identified by the PatMatch search engine (Dolinski et al., 2003). We determined the relative methylation frequencies at multiple GC (M.CviPI) and CG (M.SssI)

sites at the *CAR1* locus containing a single, consensus binding site for Zif268 by bisulfite genomic sequencing (see Materials and Methods) (Carvin et al., 2003a). Specific binding by the Zif268 moiety of each fusion protein is supported by protection of multiple CG and GC sites against methylation at the Zif268 site in strains expressing a wild-type Zif268 fusion as compared to its respective free DMTase (*filled bar*; Fig. 5-1A-C; compare lane 1 to 2 and lane 3 to 4). Ratios of m^5C among several sites in a given lane were similar, identifying sites at which nontargeted methylation occurs (*filled circles*), which enable normalization for differences in methylation activity between strains. By this criterion, the mut Zif-M.CviPI strain has approximately 2-fold more methylation activity than cells expressing Zif-M.CviPI. The reason for this activity difference is unclear. DNA methylation increased substantially at several sites (*asterisks*) in cells expressing Zif-M.CviPI and Zif-M.SssI *versus* mut Zif-M.CviPI and M.SssI, respectively, demonstrating enhanced targeting of both DMTases by DNA-bound Zif268. Targeted modification sites (*asterisks*) are readily identifiable by normalizing to sites of nontargeted methylation (*filled circles*). Methylation preferentially accumulates at four GC sites (*asterisks*) over a time course of Zif-M.CviPI induction by galactose (Fig. 5-1C, lanes 1-6). Our data suggest that m^5C accrues with increasing synthesis of Zif-M.CviPI from the *GAL1* promoter, and presumably increased occupancy of the Zif268 site.

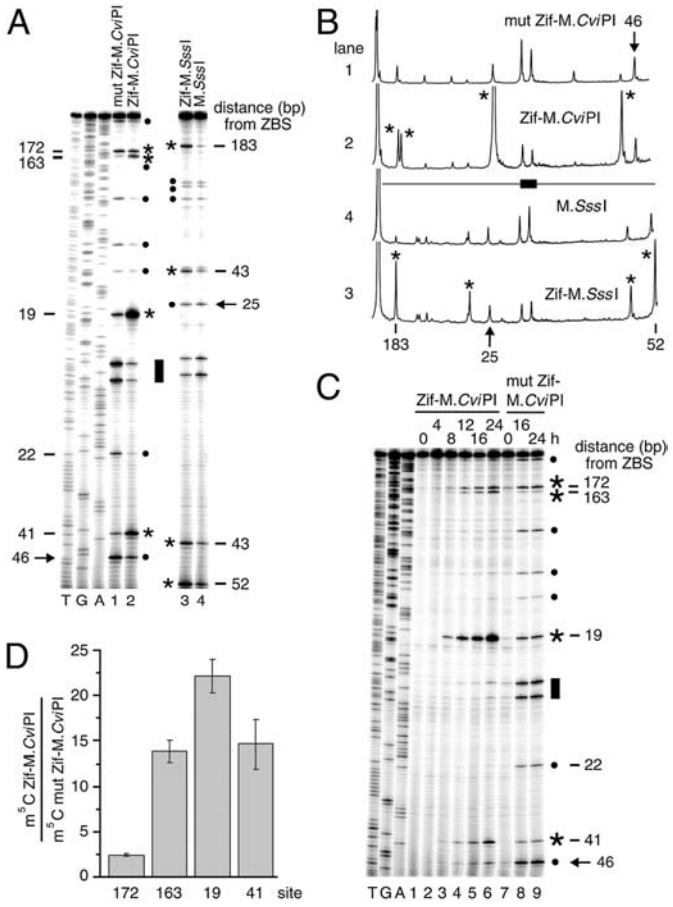

Figure 5-1. Targeting C5 DMTases near a single Zif268 site.

(A) Determination of m^5C levels targeted by Zif268–DMTase fusions. Genomic DNA isolated from strains expressing wild-type Zif268–M.CviPI (Zif–M.CviPI, lane 2), Zif268–M.SssI (Zif–M.SssI, lane 3), or 'free' DMTase controls, a mutated Zif268 fused to M.CviPI (mut Zif–M.CviPI, lane 1) or M.SssI by itself (lane 4), was analyzed by modified bisulfite genomic sequencing of *CAR1* from +558 to +159. Distances (bp) of a subset of sites from the proximal edge of the Zif268 ZBS (filled bar; +438 to +446) are indicated at left and right of the gel. Sites of non-targeted methylation (filled circles). Sites 46 (M.CviPI strains) and 25 (M.SssI strains) (arrows) were chosen for normalization to enable lane-to-lane comparisons [see (B)]. Each DMTase was preferentially targeted to several CG and GC sites (asterisks) by Zif binding as compared with its respective control (compare lanes 1 with 2 and 3 with 4). For site 19, 41% (of all summed intensities) of the templates in the population are methylated. Lanes T, G and A (left) contain sequencing reactions with ddATP, ddCTP and ddTTP, respectively.
(B) Quantitative scans of the phosphoimage in (A). See (A) for definitions of symbols.
(C) Time course of targeting M.CviPI by Zif268. Expression of Zif–M.CviPI (lanes 1–6) and Zif–M.SssI (lanes 7–9) from the *GAL1* promoter was initiated by transferring cells from YPD (dextrose) to YPG (galactose) medium. Genomic DNA was isolated at the indicated times and analyzed as in (A). Symbols are defined in (A).
(D) Quantification of preferential targeting of M.CviPI by Zif268. Ratios of m^5C for the indicated sites (normalized to site 46) for Zif–M.CviPI to mut Zif–M.CviPI are given (mean ± standard error; $n = 3$). Similar values are obtained if the ratios for each site are normalized to other sites of non-targeted methylation (filled circles) or calculated using absolute frequencies of methylation (see Materials and Methods).

M.CviPI is targeted most efficiently to a site located 19 bp from the Zif268 binding site (Fig. 5-1D), which correlates well with the optimal range of 10-40 bp observed when methylating oligonucleotides with other DMTase fusion proteins *in vitro* (Xu and Bestor, 1997; McNamara et al., 2002) and in yeast (Carvin et al., 2003a). This optimal distance for introducing m^5C is likely related to the length and amino acid sequence of the flexible peptide separating Zif268 and the DMTase (McNamara et al., 2002). However, targeting methylation distal to the Zif268 binding site (*e.g.*, sites 163 and 183) is as or more efficient than to some proximal sites (*e.g.*, sites 41, 43, and 52) (Fig. 5-1A-D). Significantly, preferential methylation by M.CviPI and M.SssI at sites 163-183 nucleotidesfrom the Zif268 binding site (Fig. 5-1A-D) suggests that m^5C can be targeted distally, perhaps due to the formation of higher-order chromatin structure. A single, DNA-bound monomer of Zif268 similarly targets both DMTases close to (5-30 bp) and at a considerable distance from (353 bp) a second consensus Zif268 binding site in *YBR108W* (+2067 to +2075; Fig. 5-2A-B). For a third Zif268 binding site (–397 to –389 of *YOL019W*), two GC sites are protected against methylation by Zif-M.CviPI bound at the ZBS, and m^5C is targeted to an additional GC site 39 bp away from the ZBS (Fig. 5-2C). In contrast, the relative levels of CG or GC site methylation at the *PHO5* promoter, which lacks Zif268 sites, show no significant differences between the wild-type Zif268 fusion and its respective free DMTase control (Fig. 5-3, compare lane 1 to 2 and 3 to 4). We

conclude that the targeted methylation is due to site-specific DNA binding by Zif268.

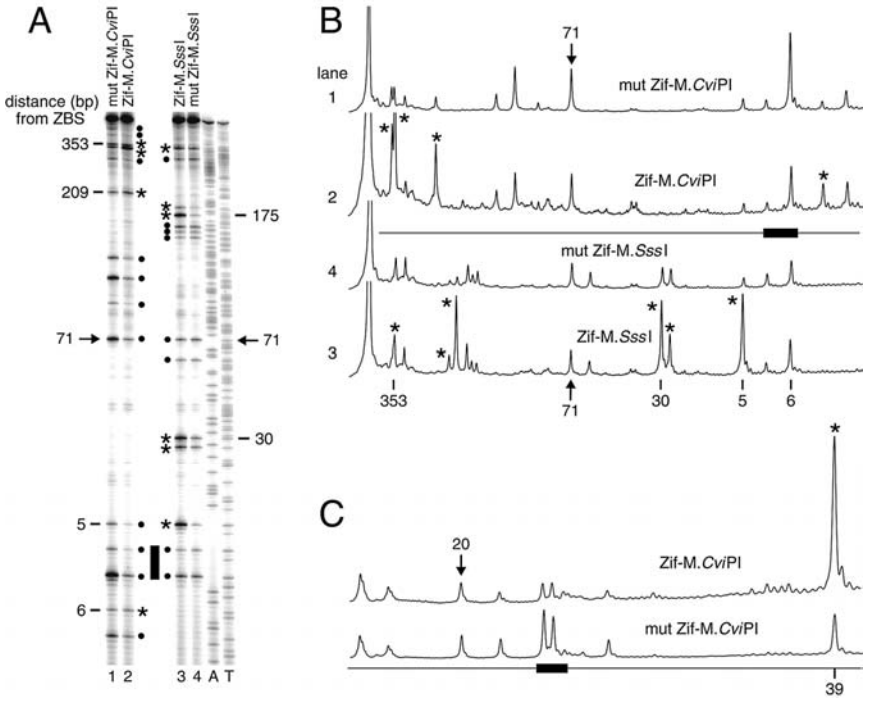

Figure 5-2. Zif268 targets M.CviPI and M.SssI to additional ZBS.

(A) Determination of m^5C levels. A region of *YBR108W* (+1564 to +2163) spanning a single Zif268 site (+2067 to +2075; filled bar) was PCR amplified from the same bisulfite-treated samples analyzed in Figure 5-1A. Sequencing ladders (A, T) are at the right. Symbols are defined in the caption to Figure 5-1.
(B) Scans of the phosphoimage in (A). The scanned lanes are indicated at the left.
(C) Methylation targeted to a third Zif268 site (filled bar) near *YOL019W* (–397 to –389). Only scans of the phosphoimage resulting from the bisulfite genomic sequencing of the region from –509 to +254 are shown.

Figure 5-3. Absence of site-selective methylation at unlinked loci.

The *PHO5* promoter (–1009 to –205), lacking Zif268 sites, was PCR amplified from the same bisulfite-treated samples analyzed in Figures 5-1 and 5-2A to determine levels of m^5C. The positions of the two known Pho4 transactivator binding sites, UASp1 and UASp2 (open bars), localized to a histone-free, DNase I-hypersensitive region and positioned nucleosome –2 (partial ellipse), respectively, are indicated. GC (lanes 1 and 2) and CG (lanes 3 and 4) sites (filled circles). Note that, relative to the mut Zif–M.CviPI control (lane 1), the lower methylation frequencies in the Zif–M.CviPI strain (lane 2) at each GC site is consistent with the conclusion that it has reduced overall methylation activity. However, the similar ratios of site intensities within lanes 1 and 2 (M.CviPI) as well as within lanes 3 and 4 (M.SssI) demonstrate that m^5C accumulates independent of the Zif (or mut Zif) fusion moiety.

Targeting M.CviPI via phage display-selected Zip53

The enginereed zinc-finger protein Zip53, which specifically binds to a p53 consensus site (5'-GGGACATGT-3'; hereafter Zip53 binding site) (Greisman and Pabo, 1997), was previously fused to M.SssI and used *in vitro* to target methylation next to a Zip53 binding site in an oligonucleotide substrate (Xu and Bestor, 1997). To corroborate further our initial methylation targeting studies

using Zif268, we tested if Zip53 could direct methylation by M.CviPI to regions containing a single Zip53 site *in vivo*. The Zip53-M.CviPI fusion protein was integrated as a single copy at *LYS2* and expressed from the *GAL1* promoter. First, we analyzed C5 methylation levels near the consensus Zip53 binding site located in the *DED1* coding sequence (Fig. 5-4; +284 to +276). As expected, since yeast do not have endogenous cytosine DMTases, no modified cytosines are evident in a strain that does not contain a functional copy of M.CviPI (Fig. 5-4A, lane 4). Normalizing to site 141, relative to the "free" DMTase control (mut Zif-M.CviPI), on expression of Zip53-M.CviPI, targeted methylation is detected 30 bp from the *DED1* consensus Zip53 site (Fig. 5-4A; compare lanes 2 and 3 to lane 1). Further, long-range methylation at sites 162 and 178 bp from the ZBS is substantially enhanced. Lastly, there is reproducible low-level protection of a GC site located 3 bp from the ZBS, indicative of Zip53 binding (Fig. 5-4A-B).

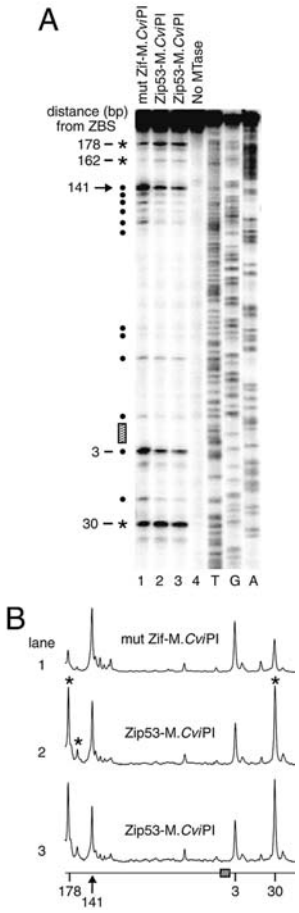

Figure 5-4. The engineered zinc-finger protein, Zip53, targets a DMTase to *DED1*.

(A) Determination of m^5C levels at *DED1* (+475 to +67). Targeted methylation (asterisks), normalized to site 141 (arrow), is detected at GC sites 30, 162 and 178 bp away from the Zip53 binding site (hatched bar) in two Zip53–M.CviPI strains (lanes 2 and 3) that are representative of four independent strains containing the integrated Zip53–M.CviPI fusion gene. Filled circles indicate remaining CG and GC sites of non-targeted methylation (not selectively methylated) on expression of Zip53–M.CviPI. Lanes 1 and 4 contain bisulfite genomic sequencing results from the mut Zif–M.CviPI strain and a Zip53–M.CviPI transformant that contains a non-functional DMTase, respectively. Sequencing ladders (T, G, A) are at the right.
(B) Quantitative scans of the phosphoimage in (A). Symbols are defined as in (A).

We also observed long-range targeting of m^5C at a second consensus Zip53 site located in the *YLR016C* coding sequence (+298 to +306; Fig. 55-B). Methylation was enhanced 5.5-fold at site 184, and somewhat less but significantly (~2.2-fold), at sites 157 and 190 in strains expressing Zip53-

M.CviPI relative to mut Zif-M.CviPI. Protection against DNA methylation could not be observed because no GC sites are adjacent to or within the Zip53 binding site. To examine the specificity of the Zip53-DMTase fusion protein, we analyzed m^5C levels at the *CAR1* locus (*cf.* Fig. 5-1), which contains a Zif268 site, but no Zip53 site (Fig. 5-5C). In each lane of the gel in Figure 5C, little to no change exists in the relative methylation levels of 13 GC sites at *CAR1*. In particular, methylation at site 19 of the *CAR1* region, which shows >20-fold enrichment following expression of Zif-M.CviPI (Fig. 5-1), is not increased in the presence of Zip53-M.CviPI. This result demonstrates that Zip53 specifically binds its site, but not that of Zif268 (the two binding sites have 22% identity). We conclude that, as for Zif268, Zip53 is able to target M.CviPI and thereby significantly increase cytosine methylation at select GC sites near and distal to a cognate ZBS. The use of Zip53 to deliver m^5C selectively further demonstrates that zinc-finger proteins engineered to recognize pre-determined sequences can be used to introduce *de novo* methylation essentially to any region of interest.

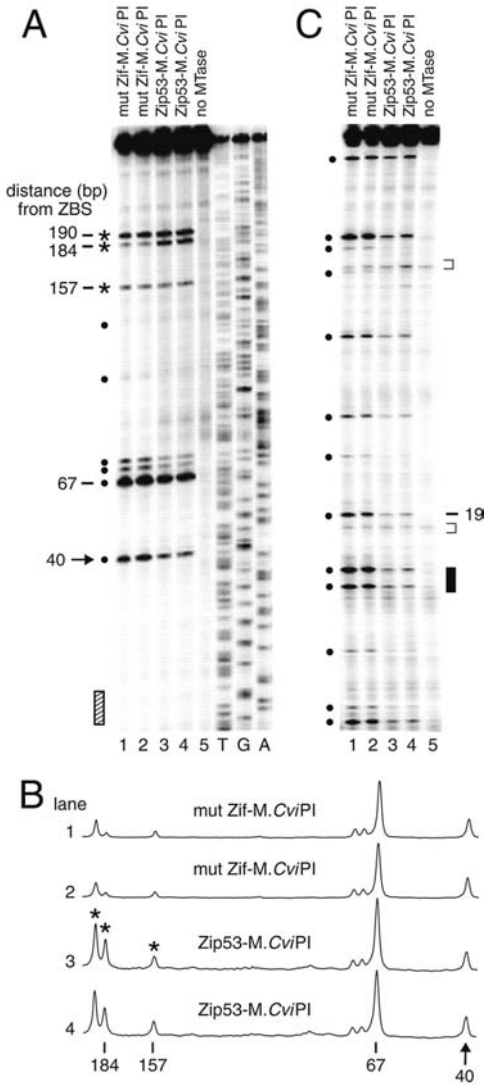

Figure 5-5. Zip53-mediated targeting of m^5C to *YLR016C*.

(A) Determination of m^5C levels at *YLR016C* (+418 to +28). Targeted methylation (asterisks), as normalized to site 40 (arrow), is detected at GC sites 157, 184 and 190 bp away from the Zip53 site (hatched bar) in two independent transformants (lanes 3 and 4) relative to mut Zif–M.CviPI (lanes 1 and 2). T, G and A sequencing ladders are at the right. Non-targeted methylation (filled circles). The sample in lane 5 was obtained from a Zip53–M.CviPI transformant harboring a non-functional DMTase.
(B) Quantitative scans of the phosphoimage in (A).
(C) Determination of m^5C levels at the *CAR1* locus (+558 to +159) that has a Zif268 binding site (filled bar) but no consensus Zip53 binding site. The PCR products analyzed in lanes 1–4 were amplified from the bisulfite-treated genomic analyzed in lanes 1–4, respectively, in (A). Lane 5 contains a sample from a parental strain that was not transformed with M.CviPI. Non-specific primer extension pauses that do not correspond to GC sites are marked with brackets.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SET1 AS A NEGATIVE REGULATOR OF GENE EXPRESSION

Histone methylation at lysine 4 by Set1 via COMPASS is a prominent histone modification in yeast. Approximately 35% of nucleosomes contain K4 methylated histone H3. Recent evidence has demonstrated that Set1-dependent methylation requires the monoubiquitination of histone H2B and the Paf1-Rtf1 complex, which has been implicated in transcriptional elongation through the interaction of the C-terminal domain of RNA polymerase II. The fact that K4 methylation is primarily associated with euchromatic genes while K9 methylation is correlated with heterochromatin suggests that each may play an integral role in the establishment of active and inactive regions, respectively. In fact, recent evidence has shown that K9 methylation via the histone methyltransferase Suv39h leads to the recruitment of HP1 (Bannister et al., 2001; Lachner et al., 2001). Artificial targeting of histone methylation or HP1 via chimeric fusion proteins to euchromatic regions leads to local gene silencing (Snowden et al., 2002; Li et al., 2003). Though K4 methylation via Set1 is associated with active chromatin, it is not known if this modification leads to recruitment of other factors or what function it may serve. Although it is associated with transcriptional elongation and appears to be prominent throughout the promoter

regions of euchromatic genes, K4 methylation has been shown to required for the full expression of only a few genes (Nislow et al., 1997; Santos-Rosa et al., 2002). We present evidence that Set1 is involved in the repression of a subset of genes in active chromatin regions. We show that the loss of Set1 leads to higher levels of the repressible acid phosphatase *PHO5* in both repressing and activating conditions. This is evidence to demonstrate that K4 methylation may have a repressive role in gene expression as well as the previously characterized activating role. Our results go further, demonstrating that *PHO84* and *GAL1-10* are also expressed higher with the loss of Set1. We confirm that a general derepression effect by the *set1Δ* mutant is not observed since *PPH3* is down-regulated, as has been shown previously (Santos-Rosa et al., 2002).

Deletions in components of complexes which regulate Set1 methylation also show similar phenotypes as that observed with a *SET1* null strain. A strain deleted for *PAF1*, the most upstream regulator, exhibits the largest derepression on *PHO5*, suggesting that other factors are recruited in addition to Set1. It was previously reported that a *paf1* mutant caused both transcriptional defects as well as increased expression, which demonstrates a dual role in gene regulation (Shi et al., 1996). Interestingly, in that study, *GAL10* and *GAL7* were two of the genes that required Paf1 for full expression, which again suggests that Paf1-Rtf1 may recruit additional proteins that might affect gene expression positively or negatively. Similarly, a deletion of the carboxyl-terminal domain kinase (*CTK1*)

also had both a positive and negative role in transcription of various genes (Patturajan et al., 1999). More recently, the histone methyltransferase Set2, which methylates histone H3 at lysine 36, has also been associated with active chromatin, transcription elongation via the Paf1-Rtf1 complex and is required for full expression of a *GAL1-lacZ* reporter (Krogan et al., 2003b). However, when the Set2 protein is tethered to a heterologous promoter via LexA, it serves as a repressor lowering transcription greater than 20-fold (Strahl et al., 2002). Consistently, Set2 is responsible for the repression of the basal expression of *GAL4* (Landry et al., 2003). Taken together, this demonstrates that despite their association with transcription elongation, regulators may be positive in a subset of genes while negative at others. The mechanism for this remains unclear.

Histone monoubiquitination of histone H2B at lysine 123 via the Rad6-Bre1 complex also negatively regulates *PHO5*. *GAL10* is expressed at much higher levels in a *rad6Δ* null; however, the corresponding histone mutant did not show the same effect (Daniel et al., 2004). This mutant was also used in another study to show that histone ubiquitination is present at *GAL1* and *PHO5* and had less than a two-fold effect in transcriptional activation but was synergistic with *gcn5*Δ (Kao et al., 2004). It is unclear why different phenotypes are seen between a *rad6Δ* and histone H2B K123R mutant strains. This may be a result of strain differences or because the histone mutant is present on an episome, which has inherent expression differences than when integrated.

A recent study has identified a potential regulator which links histone ubiquitination of histone H2B and histone methylation of histone H3. Ezhkova and Tansey have demonstrated that the proteosomal ATPases Rpt4 and Rpt6 are required for K4 methylation (Ezhkova and Tansey, 2004). Rpt4/6 recruitment is dependent on Rad6 suggesting that histone ubiquitination is required. Further, they showed that a mutation in Rpt6 lead to increased levels of *GAL10*. The authors state that a strain lacking Set1 also showed a "similar phenomenon", however, the relative effects are not known since the data was not shown. These results are consistent with our observations at *GAL10*. Future studies should examine if similar effects are seen at *PHO5*.

Upon *PHO5* activation, histone ubiquitination increases only transiently for the first 90 minutes of induction (Kao et al., 2004), presumably due to the presence of the histone deubiquitination activity of Ubp8 of SAGA (Henry et al., 2003; Daniel et al., 2004). It is possible since K4 methylation is strictly dependent on H2B ubiquitination that its levels will also be transient as well, however, no histone demethyltransferase has been discovered. Lower amounts of K4 methylation are observed in phosphate-free media than in YPD (Reinke and Hörz, 2003), but this may be a result of histone loss.

We believe that Set1 regulates gene expression positively and negatively. The evidence that its methylation activity fully depends on the transcriptional elongation complex Paf1-Rtf1 as well as the fact that *paf1*Δ and *rtf1*Δ mutants

also derepress *PHO5* suggests that K4 methylation may serve as a negative feedback loop on basal transcription. Future studies should focus on elucidating how Set1 mediates this repression. It is likely that K4 methylation may serve as a signal to recruit additional co-repressors. HP1, a heterochromatin-associated coating factor, can bind to methylated K9 residues via its chromodomain (Nielsen et al., 2001; Bannister et al., 2001). In *Saccharomyces cerevisiae*, there is no detectable K9 methylation, however, there is at least one protein which actually contains a chromodomain, Chd1 (Woodage et al., 1997). Chd1 is a subunit of an ATP-dependent chromatin remodeling complex which has both positive and negative roles in transcription (Tran et al., 2000). Additionally, like Set1, it is associated with transcriptional elongation (Krogan et al., 2002b) and interacts directly with Rtf1 (Simic et al., 2003). Conversely, one study showed using yeast two-hybrid and *in vitro* pull-down assays, that human Chd1 interacts with the transcriptional co-repressor NCoR and co-immunoprecipitates with HDAC activity (Tai et al., 2003). Alternatively, other co-repressors such as Ssn6-Tup1 or HDACs may be targeted by Set1. *PHO5* expression was increased greater than three-fold in a strain lacking *TUP1* (Carvin and Kladde, unpublished observations), however, other experiments are needed to determine if Set1 and Tup1 are part of the same genetic pathway. Delineating what factors are involved in dictating this dual regulation of histone methylation will be important in characterizing its function.

BROMODOMAINS CAN INCREASE THE ACTIVATION ABILITY OF TRANSCRIPTION FACTORS

In this study, we have shown that fusing the bromodomain to a primary activator can increase the ability of the activator to increase transcription. In high phosphate, fusion of the Gcn5 bromodomain increases *PHO5* transcription greater than 8-fold. Mutations in essential residues of the bromodomain alleviated this effect. This control rules out the possibility that the addition of the bromodomain causes mis-regulation of Pho4 or that the bromodomain may contain a cryptic activation domain.

The fact that we see this enhancement of transcription in repressed conditions demonstrates that Pho4 must bind at least transiently in high phosphate conditions, as seen previously (Carvin et al., 2003a). The *PHO5* promoter contains significant levels of background histone acetylation that may serve as a binding surface for the Gcn5 bromodomain (Vogelauer et al., 2000). This additional binding contact could increase the overall binding affinity of the fusion factor. Ongoing studies will determine how the promoter occupancy of the *PHO5* promoter by Pho4 changes in the bromodomain fusion strain. If promoter occupancy is increased, this will demonstrate that the bromodomain functions to stabilize the recruitment of chromatin remodeling coactivators. The interaction of bromodomains with acetylated lysines, a modification correlated

with transcriptionally active regions, provides an elegant positive feedback loop for the recruitment of coactivators and the transcription machinery.

We did not detect significant enhancement of *PHO5* expression via the bromodomain fusion under fully activating conditions (Fig. 3-2). This could be either because the promoter is fully occupied or that gene expression of *PHO5* is already at its maximum. Overexpression of Pho4 by regulating *PHO4* by the *GAL1* promoter shows that *PHO5* expression saturates at very low levels of galactose, indicating that *PHO5* expression reaches a maximum expression level (Hoose and Kladde, unpublished observations). It may also be a result of loss of histone acetylation upon activation due to physical removal of the nucleosomes (Reinke and Hörz, 2003; Boeger et al., 2003).

Ongoing studies will elucidate what contributions the bromodomain makes to promoter occupancy. It is difficult to determine the contributions that the bromodomain may make to coactivators recruitment *in vivo* due to limitations of the ChIP technique. Enrichments of factors that do not bind DNA directly are very poor, usually around 2-fold, making quantification very difficult. However, proteins which bind DNA directly are immunoprecipitated rather efficiently. For instance, we achieve greater than 25-fold enrichment of Pho4 in activating conditions (Dhasarathy, Carvin, Jessen, and Kladde, manuscript in preparation). Using our novel BD fusion strategy we expect to be able to obtain quantifiable measurements of effect(s) that the bromodomain may have on

promoter binding. We will also test the effectiveness of various other bromodomains in yeast, including those from the Spt7 subunit of SAGA and Swi2 ATPase of the Swi-Snf complex. Previous work using an *in vitro* system has shown that the Spt7 bromodomain can complement loss of the Gcn5 bromodomain, but only when it is fused to Gcn5 (Hassan et al., 2002). The authors argue that bromodomain function may be determined by the subunit it is in, and our system should allow us to test this hypothesis.

In budding yeast, all of the proteins that contain bromodomains are contained in either chromatin remodeling coactivators or the general transcription machinery. However, in higher eukaryotes, there are a number of site-specific transcription factors that contain putative bromodomains. Also, in two different cancer cell lines, there is a fusion of a bromodomain with another transcription factor to form oncogenes (Lavau et al., 2000; French et al., 2003). There has very little work in determining how the bromodomain may assist the transcriptional activity of the factor. Thus, our study will be the first such study in characterizing how bromodomains may affect transcription factor binding. By constructing myriad of bromodomains fusions (*e.g.*, Swi2, Spt7) to the same factor, Pho4, we can determine the relative strength of each bromodomain. By making fusion to a site-specific DNA binding factor, we expect to obtain quantifiable measurements on increases in promoter occupancy. This work should elucidate key information into how bromodomains may affect

transcriptional activation with potential connections to temporal order of chromatin remodeling enzymes and cancer biology.

USING DNA METHYLTRANSFERASES TO SIMULTANEOUSLY DETECT DNA-PROTEIN INTERACTIONS AND PROBE CHROMATIN STRUCTURE

We have demonstrated that TAGM is a highly effective and sensitive technique for detecting DNA-protein interactions and activation-dependent changes chromatin structure *in vivo*. The method provides several distinct advantages over other available approaches (Simpson, 1999), including: 1) identification of sites of factor interaction at relatively high resolution in living cells; 2) high sensitivity, requiring only small amounts of cells and detecting factor binding even at single, native sites; and 3) the ability to monitor nucleosomal rearrangements kinetically. *In vitro*, the ability to target m^5C is primarily related to the distance between a particular DMTase site and the factor binding site, which is likely related to the length and nature of the peptide separating the targeting factor and the DMTase (Xu and Bestor, 1997; McNamara et al., 2002). In addition to these constraints, our results demonstrate that, in chromatin, the efficiency of targeting m^5C to a given site is determined by its accessibility, its rotational orientation relative to the factor binding site, and/or higher order chromosome structure.

Taken together, at *PHO5*, our data suggest that a homodimer of Pho4,

initially binding to the accessible UASp1 E box, preferentially targets M.CviPI to sites 13 and 26a (Venter et al., 1994). Subsequently, disruption of nucleosome –2, presumably mediated by the recruitment of coactivators, such as histone acetyltransferases (Gregory et al., 1998; Galarneau et al., 2000) and ATP-dependent remodelers (Santisteban et al., 1997; Sudarsanam et al., 2000; Steger et al., 2002) to *PHO5*, facilitates Pho4 binding at the high affinity UASp2 site (Svaren et al., 1994; Venter et al., 1994). Increases in the local DMTase concentration due to cooperative binding of Pho4-M.CviPI that accompanies chromatin perturbation may account for the accumulation of high levels of methylation at sites to which the DMTase is indirectly targeted (*e.g.*, sites 41 and 43). In that Pho4 targets M.CviPI at a distance, it is interesting to speculate that it can also do so with recruited coactivators and hence disrupt distal nucleosomes (Kim and Clark, 2002).

Previous studies have suggested that residual levels of Pho4 are present in the nucleus in high P_i (Han et al., 1988; Han and Grunstein, 1988; Wechser et al., 1997), despite its predominant cytoplasmic localization under these conditions (O'Neill et al., 1996). The presence of marked targeted m^5C at *PHO5*, *PHO8*, and *PHO84* provides direct evidence of a low level of Pho4 binding in the presence of P_i. This binding occurs either before phosphorylation of Pho4 by Pho80-Pho85 or after its modification and prior to subsequent nuclear export. The sensitivity of TAGM is underscored by this result because, in the presence of

P_i, Pho4 binding is not detectable by ChIP analysis (Steger et al., 2002) or genomic footprinting (Venter et al., 1994). Thus, TAGM is a powerful and complementary alternative to existing technologies.

In addition to the use of TAGM with Pho4 fused to M.CviPI presented here, we have also targeted M.CviPI as well as M.SssI, acting on CG sites, both near and several hundred nucleotides from single Zif268 as well as p53 binding sites (Carvin et al., 2003b). Thus, the successful application of TAGM for these three factors tested thus far, each at different loci, validates its efficacy in targeting C5 methylation and hence detection of factor interactions. We are currently extending TAGM to additional transcription factors and coactivators. Currently, detection of coactivators which do not bind to DNA directly is often difficult by ChIP. However, we were able to detect targeted methylation indicative of Pho4 binding in repressed conditions where we were unable to detect such binding by ChIP. I believe this is due to the fact that ChIP is technique that can only detect what is bound at the promoter at the time of cross-linking while targeted methylation is an accumulation of multiple binding events over time.

Our observation that substantially more methylation by Pho4-M.CviPI at *PHO5 vs*. *CAR1* occurs at early times following PHO activation is promising for using TAGM in genome-wide identification of targets for Pho4 and other transcription factors. McrBC is a restriction enzyme which digests Rm^5C sites

specifically. We could use this enzyme to digest genomic DNA and purify the smaller fragments (methylated) from the larger fragments (nonmethylated) by sucrose centrifugation. The enriched DNA fragments could then be fluorescently labeled with Cy3 or Cy5 dyes by random priming and hybridized to a DNA microarray containing spotted intergenic regions of DNA in order to identify other regions where Pho4 may be binding. It is important to note that this analysis must be done at early times of activation since significant background methylation is observed at later times. I believe this is due to the continual build-up of Pho4 in the nucleus when the cells are grown in phosphate limiting conditions. Basically, at later times the concentration of Pho4 leads to its maximal binding at bona fide Pho4 binding sites and then significant nontargeted methylation begins to accumulate. A previous work using the Dam DMTase fused to TetR showed that they could only achieve significant methylation targeting at TetR binding sites when their concentration of TetR-Dam fusion was very low (Lebrun et al., 2003).

Our analysis of Pho4-M.CviPI *vs.* mut Zif-M.CviPI (Fig. 4-4) showed a site of targeted methylation in nucleosome -4, some 335 bp away from the nearest UAS. When normalized to nearby site, this site showed nearly three-fold more methylation in the Pho4-M.CviPI strain than the free DMTase control. Another site that was also contained in nucleosome -4 (site 278) did not show any enhanced methylation over the free DMTase. While more analysis is needed to

confirm this, the observation of distal targeted methylation could be indicative of higher-order chromatin structure. Since chromatin remodeling enzyme recruitment is dependent on the primary activator Pho4, this could also explain why the distal nucleosomes -4 and -5 are remodeled.

Our analysis has also provided preliminary evidence regarding another question pertinent to our laboratory studies. One of the ongoing projects is to determine the temporal order of nucleosome remodeling at the *PHO5* promoter. Analysis of chromatin remodeling during a time course of phosphate starvation using the mut Zif-M.CviPI probe showed that a spreading of nucleosome remodeling occurs with nucleosome -3 being fully remodeled earlier than nucleosomes -4 and -5 (Jessen, Dhasarathy, Carvin, McKinnie, and Kladde, manuscript in preparation). This is being studied in much greater detail by another graduate student in the laboratory.

TARGETING CYTOSINE METHYLATION BY USING ENGINEERED ZINC-PROTEINS

We extend the ability to target cytosine methylation *in vivo* using two zinc-finger proteins, Zif268 and its artificially engineered derivative Zip53. First, significant targeting of cytosine methylation is observed both adjacent (5-52 bp) and distal (>150 bp) to the cognate ZBS, whereas DNA methylation is not enriched at control loci lacking the ZBS. Proximal and distal targeting of C5

methylation was also observed in our previous studies using Pho4 as the DMTase targeting factor (Carvin et al., 2003a). The reasons for selective targeting of m^5C to some sites as opposed to others in the same region are not currently understood. At least locally, presumably the length of the peptide linker separating the DMTase and the targeting factor, the helical face of a particular CG or GC site relative to the DNA-bound targeting factor, and accessibility in chromatin each contribute to the preferential targeting. In addition, the occurrence of targeted C5 methylation beyond distances of 40 nucleotides suggests that two sites well-separated in protein-free DNA are juxtaposed in the nucleosome or by higher-order chromatin structures (*e.g.*, Fig. 5-2A, 353 bp away from the ZBS). Second, since DNA-bound factors often impair access of DMTases to their target sites (Kladde et al., 1996; Xu et al., 1998b; Hsieh, 1999; Lin et al., 2000; Lin and Hsieh, 2001; Carvin et al., 2003a), the protection against methylation of CG or GC sites next to or within the ZBS provides further evidence of specific ZBS binding by each zinc-finger-DMTase fusion protein. Taken together, in addition to demonstrating selective enrichment of m^5C near ZBS, TAGM provides a highly sensitive means for detecting protein-DNA interactions (Carvin et al., 2003a). Third, accumulation of m^5C at select sites during a time course of Zif-M.CviPI induction suggests that the extent of targeted methylation parallels the cumulative amount of factor binding over time. Thus, optimizing occupancy of the targeting factor at regions

of interest will likely increase the efficacy of specific m^5C targeting *in vivo* as well as lessen nontargeted methylation. The experimental system used herein provides a useful assay for pursuing such further investigations.

The design of multiple zinc-finger modules with desired specificities is proving a versatile platform for targeting a variety of protein moieties to accessible sites *in vivo* (Urnov and Rebar, 2002). For instance, engineered zinc-finger proteins have been fused to the catalytic domain of FokI endonuclease to direct site-specific double-stranded DNA cleavage, and hence homologous recombination, of desired regions (Bibikova et al., 2001). Designed zinc-finger proteins have also been used to target the catalytic domains of the histone methyltransferases G9A and SUV39H1 (Snowden et al., 2002) as well as the VP16 activation domain (Zhang et al., 2000; Liu et al., 2001; Rebar et al., 2002), leading to repression and activation, respectively, of expression of the endogenous human erythropoietin (*EPO*), vascular endothelial growth factor A (*VEGF-A*), and other mammalian genes (Urnov and Rebar, 2002). This technology has also recently been extended to the regulation of gene expression in plants (Sanchez et al., 2002).

The targeting of DMTases by zinc-finger proteins selected to bind specific ZBS might provide an additional way to down-regulate the expression of desired genes. Moreover, since the DNA methylation state of a given promoter is maintained heritably through DNA replication by endogenous cellular

mechanisms, an initial targeting event may be sufficient to establish stable silencing of improperly expressed genes. Therefore, heritable repression could also reduce the amount of treatment necessary to establish the proper regulation of a particular gene. In addition to providing a potentially powerful therapeutic tool, methylation-mediated repression of specifically targeted genes could yield an alternative to transgenic knockouts for studying loss-of-function phenotypes. Silencing genes through DNA methylation would be particularly valuable in the case of essential genes where tissue-specific knockouts of function are needed. Finally, the ability to target m^5C specifically *in vivo* is likely to prove valuable in basic investigations of the biological roles and mechanistic consequences of DNA methylation.

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