Regulation of the DNA methylation machinery and its role in epigenetic control

By

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May 2003

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Doctor of Philosophy

Dedicated with love to Mary, Louis, and Deborah Detich,

without whom this would not have been possible.

Abstract

A growing line of evidence indicates that the proper control of DNA methyltransferases (DNMT) and DNA demethylases is critical for maintaining correct gene expression. In addition, misregulation of this machinery likely plays a role in the aberrant DNA methylation patterns and gene expression that is a hallmark of many pathologies such as cancer. A greater comprehension of the mechanisms involved in regulating the expression and activity of these proteins should provide new therapies aimed at restoring gene expression gone wrong. This thesis begins by describing the role of the 3' untranslated region (3'UTR) of the *DNMT1* mRNA in mediating both the growth-dependent mRNA regulation of *DNMT1* and cellular transformation. Our results support the hypothesis that deregulated expression of *DNMT1* with the cell cycle and not the total amount of DNMT1 is important for cellular transformation. The discovery of a DNA demethylase activity points towards a reversible DNA methylation reaction, and uncovers the possibility that deregulation of active demethylation may also contribute to pathological DNA methylation and gene expression. Studies in chapters 2-4 focus on the identification of factors that are involved in determining the activity of DNA demethylases. Chapter 2 demonstrates that the methyl-CpG-binding protein 2/DNA demethylase (MBD2/dMTase) differentially demethylates and activates some but not all promoters in a time- and concentration-dependent manner. These data suggest that promoter context is one factor shaping the complex functional role of this protein, and thus MBD2/dMTase could be involved in maintaining both the DNA hyper and hypomethylation observed in cancer. Chapter 3 identifies the first inhibitor of DNA

demethylases *in vitro* and in living cells, *S*-adenosylmethionine (AdoMet). Our data demonstrate that AdoMet inhibits active demethylation and expression of methylated DNA in living cells, and also inhibits active demethylation *in vitro*. Most importantly, we show that AdoMet can revert the transformed state of cells, suggesting that hypomethylation mediated by DNA demethylases is critical for tumorigenesis and that AdoMet might be an attractive tool in cancer therapy. In contrast, the identification of agents that can pharmacologically stimulate active demethylation might allow the modulation of DNA methylation patterns in adult non-dividing tissue. Chapter 4 describes how the anti-epileptic drug valproate (VPA) can stimulate specific active replication independent DNA demethylation, which is dependent upon histone acetylation and is mediated, at least in part, by MBD2/dMTase. Thus, VPA might be able to achieve the activation of critical genes silenced by methylation. Taken together, our data support a new hypothesis on the mechanisms and role of *DNMT1* overexpression, regional hypermethylation and global hypomethylation in cancer, and therapeutic approaches for modulating DNA methylation patterns in cancer and other pathological conditions.

Résumé

Un nombre croissant de travaux indique qu'un contrôle précis des méthyltransférases de l'ADN et des déméthylases de l'ADN est essentiel dans le maintien d'une expression génique correcte. De ce fait, une dérégulation de cette machinerie joue vraisemblablement un rôle dans la mise en place de profils de méthylation et d'expression génique aberrants, caractéristique de nombreuses pathologies comme le cancer. Ainsi, une meilleure compréhension des mécanismes impliqués dans la régulation de l'expression et de l'activité de telles protéines initierait de nouvelles thérapies ayant pour but le rétablissement d'une expression génique devenue erronée. Cette thèse débute par la description du rôle de la région non traduite (3'UTR) de l'ARNm de dnmt1, à la fois dans la transmission d'une régulation dépendante de la croissance de ce même ARNm, mais également dans l'induction de la transformation cellulaire. En effet, nos résultats supportent l'hypothèse que c'est une expression dérégulée au cours du cycle cellulaire de dnmt1, et non son niveau d'expression, qui est cruciale dans le processus de transformation. En parallèle, la découverte d'une activité déméthylase de l'ADN amène à considérer la réversibilité de la réaction de méthylation de l'ADN, et laisse entrevoir la possibilité qu'une dérégulation d'une déméthylation active puisse aussi contribuer à une méthylation de l'ADN et une expression génique inappropriées, pouvant conduire a une situation pathologique. Les études des chapitres 2-4 se concentrent sur l'identification des facteurs impliqués dans la mise en action de l'activité des déméthylases de l'ADN. En effet, le chapitre 2 démontre que la "methyl-CpG-binding protein 2"/ DNA déméthylase (MBD2/dMTase) déméthyle et active différemment certains promoteurs, et

ce d'une manière dépendante du temps et de la concentration. Ces données suggèrent que le contexte du promoteur est un élément modulant les fonctions complexes de cette protéine. Ainsi, MBD2/dMTase pourrait être impliquée dans le maintien à la fois de l'hyper- et de l'hypométhylation de l'ADN observées dans les cas de cancer. Le chapitre 3 identifie la S-adénosylméthionine (AdoMet) comme le premier inhibiteur de déméthylases de l'ADN, *in vitro* et au sein de cellules en culture. En effet, nos données démontrent que l'AdoMet inhibe la déméthylation active ainsi que l'expression d'ADN méthylé au sein de cellules en culture; elle inhibe également cette déméthylation *in vitro*. Fait plus important, nous montrons que l'AdoMet peut inverser l'état transformé de cellules, suggérant que l'hypométhylation génerée par les déméthylases de l'ADN est critique lors de la tumorigénèse. Ainsi, l'AdoMet pourrait être un outil attrayant dans le cadre d'une thérapie anti-cancéreuse. D'autre part, l'identification d'agents pharmacologiques pouvant stimuler la déméthylation active pourrait permettre la modulation des profils de méthylation de l'ADN au coeur de tissus adultes programmés pour ne pas se diviser. Enfin, le chapitre 4 décrit comment le valproate (VPA), médicament anti-épileptique, stimule spécifiquement la déméthylation de l'ADN indépendante de la réplication, laquelle stimulation est dépendante d'une acétylation des histones et, est egendrée, au moins en partie, par MBD2/dMTase. Ainsi, le VPA pourrait permettre l'activation de gènes d'importance inactivés par méthylation. En résumé, nos données supportent une nouvelle hypothèse sur les mécanismes et le rôle de la surexpression des DNMTs, l'hyperméthylation regionale et l'hypométhylation globale dans le cancer, ainsi que sur les approches thérapeutiques pouvant moduler les profils de méthylation dans les cancers et autres situations pathologiques.

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Moshe Szyf, for his careful guidance, exceptional ideas, and especially for inspiring me to persevere in the face of adversity. I am also thankful for his ability to maintain an intense learning and work environment, interspersed with periods of amusement, which has allowed my Ph.D. to be such a rewarding experience.

A sincere thank you goes to Johanne Theberge for her unsurpassed technical and organizational skills, calm and pleasant demeanor, and most of all for her extreme tolerance to the chaotic environment we surround her with everyday.

I am very grateful to the past lab members, Shyam Ramchandani, Drew Slack, Jing Deng, David Knox, Nadia Cervoni, Paul Campbell, Felipe Araujo, and Huiping Ren for their instruction and guidance that helped me reach this point. Many thanks also go to my present lab mates, Snezana Milutinovic, Ian Weaver, Veronica Bovenzi, Ana Catalina d'Alessio, Shelley Brown, Jing-Ni Ou, Stefan Hamm, Jerome Torrisani, and Samir Patra for their friendships, collaborations, and advice.

Thanks very much to Jerome Torrisani and Anne De Cian for taking the utmost care in translating my abstract into French.

I would like to thank to Martin Gagnon for coming to the rescue of the computers in the lab, and especially for stetting up my own laptop, which enabled me to write this thesis in peace and quiet.

I would like to acknowledge the FRSQ-FCAR Santé (1997-1999), the Canadian Institute of Health Research (1999-2002), and the McGill Faculty of Medicine (2002-2003) for their financial support.

My final and most heartfelt thank you goes to my family, Mom, Dad, Debby, and Jean. I would never have been able to complete this degree if it were not for a lifetime of your boundless love, encouragement, and selflessness, especially over the past several years. Beyond a doubt, this thesis belongs to all of you as much as it does to me.

Statement of Contributions

This thesis is written in manuscript format as permitted by the McGill Faculty of Graduate Studies and is composed of four manuscripts, plus appendix. The contribution of each author is described below.

Chapter 1: A conserved 3' untranslated element mediates growth regulation of DNA methyltransferase 1 and inhibits its transforming activity. *Journal of Biological Chemistry* (2001). Nancy Detich, Shyam Ramchandani and Moshe Szyf.

Figure 1: Northern blot was performed by Nancy Detich. FACS analysis was provided by the McGill flow cytometry facility.

Figure 2: Alignment was done by Nancy Detich.

Figure 3: pRBG constructs and stable lines were generated by Shyam Ramchandani. Nancy Detich was responsible for the DNMT1 Adenovirus constructs, transfections, and Northern blots.

Figure 4: pSK and pCRII DNMT13'UTR constructs for generating probes were made by Shyam Ramchandani. Nancy Detich performed all the remaining experiments.

Figure 5: All experiments were done by Nancy Detich.

Figure 6: Nancy Detich generated the hDNMT1 stable lines and conducted all experiments.

Manuscript was prepared by Nancy Detich and Moshe Szyf.

Chapter 2: Promoter Specific Activation and Demethylation by MBD2/Demethylase. *Journal of Biological Chemistry* (2002). Nancy Detich, Johanne Theberge and Moshe Szyf.

Figures 1 and 2: Nancy Detich performed all transfections and experiments.

Figure 3: Transfections, purification of MBD2/dMTase, demethylase assay, and MBD2 Western blot were performed by Johanne Theberge. Analysis was done by Nancy Detich.

Figure 4: Nancy Detich carried out the transfections and experiments.

Manuscript was prepared by Nancy Detich and Moshe Szyf.

Chapter 3: The methyl donor S-adenosylmethionine inhibits active demethylation of DNA; a candidate novel mechanism for the pharmacological effects of S-adenosylmethionine. *Journal of Biological Chemistry* (2003). Nancy Detich, Stefan Hamm, George Just, J. David Knox, and Moshe Szyf.

Figure 1: Diagram was prepared by Nancy Detich.

Figures 2, 3, and 4: Nancy Detich performed all transfections and experiments.

Figures 5 and 6: David Knox established the MBD2/dMTase baculovirus expression system. Johanne Theberge carried out the infections. All experiments were done by Stefan Hamm.

Figure 7: Model was constructed by Nancy Detich.

Manuscript was prepared by Nancy Detich, Stefan Hamm, and Moshe Szyf, and edited by George Just.

Chapter 4: Valproate induces replication independent active DNA demethylation. *Journal of Biological Chemistry* (2003). Nancy Detich, Veronica Bovenzi, and Moshe Szyf.

Figures 1, 2, 3, 4: Nancy Detich was responsible for all transfections and experiments. Figure 5: MBD2 Western blots were performed by Veronica Bovenzi. Remaining experiments (transfections, GFP Southern and Western blots) were done by Nancy Detich.

Manuscript was prepared by Nancy Detich and Moshe Szyf, and edited by Veronica Bovenzi.

Contributions to original knowledge

In this thesis, I have presented the following original results:

1) A 54-nucleotide highly conserved element within the *DNMT1* 3'UTR is necessary and sufficient to mediate the growth-dependent regulation of *DNMT1*.

2) This 54-nucleotide element increases mRNA turnover rates, and does so to a greater extent in the presence of extracts prepared from growth-arrested cells.

3) UV crosslinking analysis reveals that the binding of a 40 kDa protein (p40) is dramatically increased in growth-arrested cells; this binding is inversely correlated with *dnmt1* mRNA levels as cells are induced into the cell cycle.

4) Inclusion of the 3'UTR within a *DNMT1* construct prevents the cellular transformation previously shown to occur following ectopic expression of *DNMT1* lacking the 3'UTR .

5) Transient transfection of an MBD2/dMTase expression construct differentially activates two methylated reporters, pSV40-CAT and pGL2T+I4xTBRE, but does not activate the p19-ARF promoter.

6) The activation by MBD2/dMTase is both time and concentration dependent.

7) Exogenous expression of MBD2/dMTase induces demethylation at 8 of the 10 CpG sites within the SV40 promoter.

8) In living cells, AdoMet inhibits the TSA induced active demethylation and gene expression of exogenously introduced methylated CMV-GFP in a dose dependent manner.

9) AdoMet treatment does not result in the *de novo* methylation of unmethylated CMV-GFP.

10) *In vitro,* AdoMet inhibits the demethylase activity of both MBD2/dMTase and endogenous demethylase extracted from HEK 293 cells.

11) AdoHcy does not inhibit active demethylation *in vitro*, nor does it affect the TSA induced demethylation and expression of methylated CMV-GFP in living cells.

12) AdoMet, but not AdoHcy, treatment reverses the transformed state of both HEK 293 and A549 cell lines.

13) Histone acetylation within ectopically introduced methylated CMV-GFP is induced following treatment with VPA.

14) VPA induces active demethylation and gene expression of ectopically introduced methylated CMV-GFP in a dose dependent manner.

15) Demethylation by VPA appears to be selective since VPA does not induce genome wide demethylation.

16) VPM, an analogue of VPA previously shown to be incapable of inducing histone acetylation, does not induce demethylation or expression of CMV-GFP.

17) VPA induces the phosphorylation of MBD2/dMTase in a concentration dependent manner.

18) Antisense knockdown of MBD2/dMTase attenuates VPA induced demethylation.

List of abbreviations:

Ac-H3 - Acetylated histone H3

AdoHcy - S-adenosylhomocysteine

AdoMet - S-adenosylmethionine

APC - Adenomatus polyposis coli

ARE - AU-rich element

AU - Adenine and uridine

5-azaC - 5-azacytidine

5-aza-CdR - 5-aza-2'-deoxycytidine

CAT- Chloramphenicol acetyltransferase

CG/CpG - Cytosine and guanine

ChIP - Chromatin immunoprecipitation

CMV - Cytomegalovirus

MBD2/dMTase - Methyl-CpG-binding domain protein 2/DNA demethylase

DNMT - DNA methyltransferase

DNMT - Human DNA methyltransferase gene/mRNA (default)

dnmt - Mouse DNA methyltransferase gene/mRNA

EBV - Epstein-Barr virus

GM-CSF - Granulocyte macrophage colony-stimulating factor

GFP - Green fluorescent protein

HAT - Histone acetyltransferase

HDAC - Histone deacetylase

H3-K4 - Histone H3 lysine 4

H3-K9 - Histone H3 lysine 9

HP1 - Heterochromatin protein 1

IL-6 - Interleukin 6

INHAT - Inhibitor of histone acetyltransferases

MBD - Methyl-CpG-binding domain protein

5mC - 5-methylcytosine

MTase - Methyltransferase

PCNA - Proliferating cell nuclear antigen

PCR - Polymerase chain reaction

PRMT1 - Protein-arginine methyltransferase I

Rb - Retinoblastoma protein

SAHA - Suberoylanilide hydroxamic acid

TSA - Trichostatin A

UTR - Untranslated region

VPA - Valproate/valproic acid

VPM- Valpromide

Table of Contents

I. Goals of the thesis and literature review

The epigenome is the layer of information that dictates which parts of the genome are expressed in a specific place and at a specific time. While the genome is fixed and identical in all tissues of multicellular organisms, the epigenome is modeled during development in order to enable multiple gene expression programs, and is continuously modified throughout life in response to physiological, environmental and pathological cues. It is now clear that the epigenome consists of multiple layers that act in a combinatorial fashion to either activate or suppress a genomic region. An active region of the DNA is distinguished by an open chromatin structure that allows access to enzymatic machineries that interact with the genome. Most of the components of the epigenome such as histones, which form the core of chromatin, and histone modifying enzymes, which determine chromatin structure, are independent chemical entities that interact with the genome but are not part of it. The association of these molecules with the DNA is therefore reversible by its nature and could be modified at any time. In addition, the epigenome consists of an additional covalent component, a coating of methyl groups, which is established by a family of DNA methyltransferase (DNMT) enzymes. In general, regions of the genome that are active are less methylated than other regions, and are also associated with an open chromatin structure. It has long been believed that once the DNA methylation pattern is set during development it is irreversible and maintained during subsequent cell divisions. However, the recent identification of an active DNA demethylase enzyme raises the possibility that the

methylation pattern is actually a reversible process in both dividing and postmitotic somatic cells.

Many studies have established that abnormal regulation of the DNA methylation machinery, as well as aberrant methylation patterns, are critical for the development of pathogenic conditions such as cancer and other diseases. Thus, if we can achieve a better understanding of how this machinery is regulated, new and more effective opportunities for therapeutic intervention may be revealed. In addition, a greater comprehension of the functions of the DNA demethylase is needed due to the prominent occurrence of global hypomethylation in cancer. Moreover, if we can uncover ways of modulating DNA demethylase activity, we may have an additional method with which to restore proper methylation patterns and gene expression in cells gone wrong. This is especially critical for postmitotic cells in which active demethylation is the only means of changing methylation patterns.

This thesis is focused on understanding 1) the regulation of the DNMT1 enzyme during the cell cycle and how it might be involved in tumorigenesis, and 2) the functions of the protein MBD2/demethylase and its modulation by different agents as potential therapies for cancer and other conditions. The literature review does not discuss all the processes in which DNA methylation is involved, but rather focuses on topics that will provide a better understanding of both the rationale behind the studies of the thesis and the results obtained. Briefly, the review will cover the fundamental relationship between DNA

methylation, chromatin structure, and gene expression, the proteins involved and their regulation, and how these may play a role in certain diseases, most notably cancer.

II. Fundamentals of methylation and demethylation

Methylation reactions are alkylation reactions which are fundamentally important in many cellular processes such as metal detoxification (Bentley and Chasteen, 2002), phospholipid and neurotransmitter biosynthesis (Vance and Walkey, 1998; Weinshilboum et al., 1999), and of course gene expression via DNA methylation. These reactions are catalyzed by methyltransferase enzymes (MTases), the majority of which use S-adenosylmethionine (AdoMet) as the methyl group source (Cheng and Roberts, 2001; Roberts and Macelis, 2000). Twenty AdoMet-dependent MTases have be structurally characterized, and include seven DNA MTases (DNMTs), four protein MTases, five RNA MTases, and four small molecule MTases, which all act on either the carbon, nitrogen, or oxygen atoms of their corresponding substrates (Cheng and Roberts, 2001). The only common characteristic between these enzymes (with the exception of the protein arginine MTase) is a core structure containing a mixed seven stranded antiparallel β-sheet referred to as the 'AdoMet dependent fold'; this structure has been used as a criteria to identify other potential MTases using structural proteomics (Cheng and Roberts, 2001). This section will give a brief overview of the various types of methylation reactions, including DNA methylation/demethylation.

(i) DNA methylation

In 1948, Hotchkiss discovered the presence of the methylated base 5-methylcytosine (5mC) in calf thymus DNA (Hotchkiss, 1948). Since then many studies have sought out the functions of this modification and how it is generated and maintained. DNA methylation is catalyzed by various DNMT enzymes, which will be described in further detail in section IV. In different species, DNA methylation serves diverse biological roles. For example, in bacteria, the principal task of DNA methylation is in restriction modification, a bacterial immune system that protects them against invading foreign DNA (Hotchkiss, 1948). In addition, bacterial DNA methylation has several other functions such as DNA replication, (Bakker and Smith, 1989; Landoulsi et al., 1990), mismatch repair (Stambuk and Radman, 1998), and control of gene expression (Oshima et al., 2002). While in bacteria, methylation can occur to yield N6-methyladenine, N4-methylcytosine, and 5mC (Jeltsch, 2002), 5mC is the only modified base in vertebrates (Razin and Riggs, 1980). The levels of 5mC and its sequence location also vary within different eukaryotic species. For example, only 70-80 % of cytosines are methylated in vertebrates, and although the majority of methylated cytosines occurs in the dinucleotide CG (Razin and Szyf, 1984), low levels of CA, CT, and CC methylation have been documented in early embryonic development (Ramsahoye et al., 2000). The genomes of fungi such as *Neurospora crassa* and *Aspergillus flavus* display less than 2% overall methylation, mainly in transposons and other repeats, while up to 40% of cytosine residues in plants can be methylated at CG or CNG sites (Jeltsch, 2002).

The hallmark of the vertebrate genome is that the pattern of methylation is both gene and tissue specific (Razin and Szyf, 1984). Dense regions of CG sequences are usually found

close to promoters, the majority of which are non-tissue specific promoters and are usually free of methylation (Bird, 1986; Cooper et al., 1983). In contrast, within other areas of the genome CGs are under represented, sparsely distributed, and usually heavily methylated. These CGs are more often found in promoters of tissue specific genes, which are methylated in non-expressing tissues and unmethylated in tissues that express these genes (Razin and Szyf, 1984). Moreover, when two alleles of the same genes are differentially expressed in the same cell, as is the case in X inactivation (Singer-Sam and Riggs, 1993) and parental imprinting (Polychronakos, 1993; Tilghman et al., 1993), the inactive allele is believed to be marked by methylation of some CGs. Thus, the general conclusion is that regions of the genome that are active are less methylated than other regions (Razin and Riggs, 1980). In fact, a long list of data has established a correlation between the state of activity of genes, DNA methylation, and also chromatin structure (Razin, 1998). Several mammalian DNMTs have been identified, which transfer the methyl group from AdoMet onto the CG dinucleotide, forming S-adenosylhomocysteine (AdoHcy) in the process (**Figure 1**). A review of their characteristics, functions, and regulation will follow in the parts IV and V of the introduction.

(ii) DNA demethylation

During the process of development a sequence of global and site-specific demethylation events are involved in shaping the DNA methylation pattern (Kafri et al., 1992; Kafri et al., 1993; Razin and Shemer, 1995). It is also well established that global changes in DNA methylation occur during cellular differentiation (Lucarelli et al., 2000; Paroush et

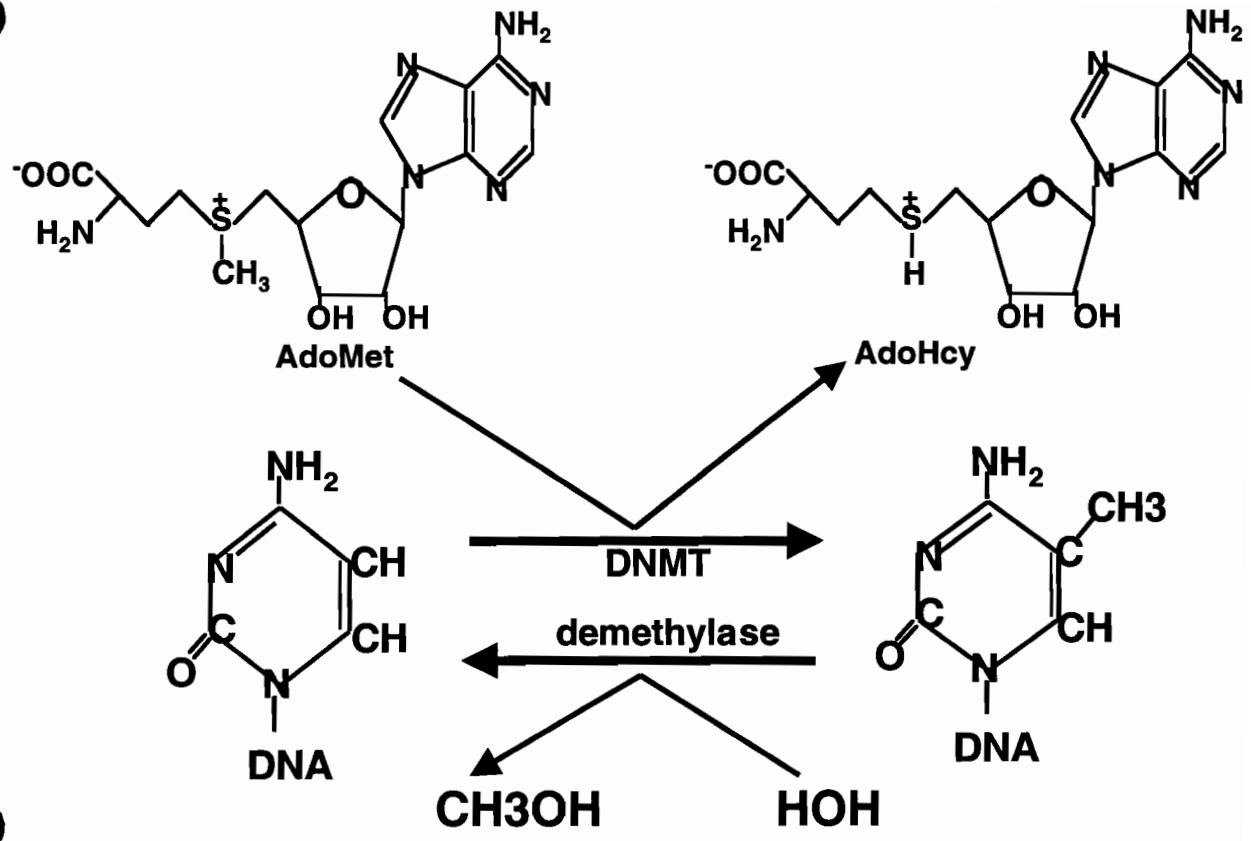

Figure 1 -The DNA methylation and demethylation reactions.

DNMT catalyzes the transfer of a methyl group from AdoMet onto the 5th position of the cytosine residue in mammalian DNA, forming AdoHcy in the process. DNA demethylase catalyzes the hydrolytic cleavage of the bond between the methyl group and the cytosine ring releasing the methyl group in the form of methanol.

al., 1990; Razin et al., 1984). One mechanism that would seem simple and obvious is the active removal of the methyl moiety from the DNA. The main theoretical problem with active demethylation is the strength of the covalent bond between the methyl residue and the cytosine ring (Cedar and Verdine, 1999). Since cleavage of this bond has been considered impossible, it was suggested that demethylation could be accomplished by replication in the absence of DNMT activity, whereby critical sites might be masked during replication and thus protected from maintenance DNA methylation (Razin and Riggs, 1980). In accordance with this hypothesis, experiments have demonstrated that high affinity-site specific binding of an ectopically expressed bacterial protein to an engineered recognition element can result in site-specific demethylation (Hsieh, 1999; Lin et al., 2000). This is possibly accomplished by inhibiting access of the DNMT to the protein bound site. It is not yet clear whether this resembles actual demethylation *in vivo*.

However, numerous studies indicate that demethylation can be an active process occurring in the absence of cell division. For instance, induction of a lytic Epstein-Barr virus (EBV) cycle in some B-cell lymphomas is associated with replication independent global demethylation (Szyf et al., 1985a); global demethylation of the paternal genome occurs within hours of cell fertilization before the start of the first cell division (Oswald et al., 2000); the first exon of *myogenin* is demethylated within minutes after initiation of differentiation of C2C12 cells into myotubes in a process that cannot be explained by passive demethylation (Lucarelli et al., 2000), and a promoter enhancer region of *interleukin-2* becomes rapidly demethylated following T-cell activation and this is not prevented by inhibitors that block DNA replication (Bruniquel and Schwartz, 2003). It is

interesting to note that a study in the plant *Zea mays* showed that active demethylation can even occur in response to an environmental stress such as cold (Steward et al., 2002). Several enzymatic processes have been proposed to resolve the existence of active demethylation, such as DNA repair or the direct removal of the methyl group from the cytosine residue (**Figure 1**). However, the discussion of these mechanisms is reserved for section IV.

DNA methylation patterns are formed during development through a series of methylation and demethylation events (Reik et al., 2001). It is believed that the pattern is then fixed and accurately inherited through subsequent cell divisions through the action of DNMT1, which has a preference for hemi-methylated DNA (Gruenbaum et al., 1982) and localizes to the replication fork (Leonhardt et al., 1992). The mechanisms involved in shaping and maintaining methylation patterns will be explored in section VI.

(iii) Other methylation reactions

In 1887, Wilhelm His discovered that dogs excreted N-methylpyridine following administration of pyridine, and was the first description of methylation of an exogenous compound (His, 1887). Today, methylation and demethylation are known to be essential pathways in the metabolism of many drugs, xenobiotics, neurotransmitters and hormones (Weinshilboum et al., 1999). Several small molecule MTases, most notably the prototypical catechol-O-methyltransferases (COMT), thiopurine methyltransferase (TPMT), and histamine-N-methyltransferase (HNMT) have been characterized

(Weinshilboum et al., 1999). COMT methylates the hydroxy group at the third position of the catechol ring (Kopin, 1985), and in 1958, it was one of the first MTases to be biochemically characterized (Axelrod, 1958). Among many substrates, COMT methylates the catecholamine neurotransmitters dopamine and (nor)epinephrine, which is important for termination of their synaptic action (Kopin, 1985). The important catechol drug L-dopa, used to treat Parkinson's disease, is also methylated and inactivated by COMT. S-methylation is important for the biotransformation of numerous sulphur containing compounds, including many sulfhydryl drugs such as various antineoplastic and immune suppressant thiopurine compounds, the antihypertensive agent captopril, and the anti-inflammatory drug D-penicillamine. These reactions are catalyzed by at least three different enzymes, TPMT, thiol methyltransferase (TMT), and thioether methyltransferase (TEMT). There are also several N-methyltransferases, such as HNMT, nicotinamide-N-methyltransferase (NNMT), and phenylethanolamine-N-methyltransferase (PNMT), which are involved in the metabolism of various endogenous neurotransmitters and hormones (Weinshilboum et al., 1999). In addition, 0- and N-demethylation of a number of important compounds such as caffeine, morphine, and codeine also occurs, and is mediated by various cytochrome P450 enzymes, and in contrast to methylation, is mediated in a relatively non-specific manner (Kirkwood et al., 1997; Rasmussen et al., 1998).

Numerous proteins are also subject to methylation, which can occur within the amino acids lysine, arginine, or histidine, on either the carboxyl group or side chain nitrogen residues (Aletta et al., 1998). This modification is involved in a number of processes

such as aging/repair of proteins (Najbauer et al., 1996), cellular stress responses (Desrosiers and Tanguay, 1988), and chromatin remodeling and gene expression (Kouzarides, 2002). Several classes of protein arginine methyltransferases (PRMTs) are known to modify a number of substrates, including RNA-binding and transporting proteins (Najbauer et al., 1993), transcription factors (Gary and Clarke, 1998), growth factors (Sommer et al., 1989), and histones (Kouzarides, 2002). Recently, the important role of histone methylation by these enzymes in regulating chromatin structure and gene expression has come to light, and an in depth discussion will follow in subsequent sections. Research has also indicated that protein methylation might be a dynamic process involved in ligand-mediated signal transduction, similar to phosphorylation (Aletta et al., 1998). Studies have identified novel protein MTases that act on Ras, protein phosphatase 2A, and other signaling molecules, although a direct demonstration of ligand-stimulated regulation of specific MTases has yet to be shown (Aletta et al., 1998).

Methylation of mammalian ribosomal RNA can take place on either the 2' hydroxyl of the sugar moiety or on adenine or guanine residues, and is important for various processes such as transport, transcription, and protection from RNases (Maden, 1990). This methylation is mediated by 'snorps', snoRNA-protein complexes in which the snoRNA serves as the guide providing site specificity and the protein catalyzes the reaction (Decatur and Fournier, 2003). Eukaryotic mRNAs are modified by a 'cap' at the 5' end by the addition of a 7-methylguanosine residue. This is accomplished by the sequential action of three enzymatic activities including an RNA MTase that methylates the G cap at the seventh position (Wen and Shatkin, 2000). The cap structure enhances

the binding of the elongation initiation factor eIF-4E, which functions to facilitate translation elongation (Sonenberg et al., 1978). In addition, other RNAs such as tRNA and splicing RNAs are also modified by methylation (Decatur and Fournier, 2003).

III. Chromatin and gene expression

(i) Chromatin structure and function

The basic unit of chromatin is the nucleosome, which is composed of 146 bp of DNA wrapped around an octamer of histone proteins, H2A, H2B, H3, H4. Higher eukaryotes also posses H1 linker histones, which bind at the entry and exits points of the DNA as it winds around the histones, and facilitates packaging of the chromatin in to a helical structure known as the 30 nm fiber. Higher order folding leads to the 240 nm fiber, which is the fundamental unit of the metaphase chromosome (Dillon and Festenstein, 2002; Lusser, 2002).

It was originally believed that the sole function of chromatin was to bring about the compact packaging of the tremendous amount of genetic information in the nucleus. However, in 1930 Hermann Muller discovered that *Drosophila* mutants generated following X-ray irradiation displayed a mosaic expression of the 'white' gene. This silencing was associated with a translocation that placed the 'white' gene next to heterochromatin (compact chromatin). Remarkably, this silencing effect was stable throughout subsequent cell divisions and was the first indication that chromatin might be

involved in stable gene regulation (Muller, 1930). Many subsequent studies have now determined that chromatin is critical for a number of cellular processes such as centromere function, nuclear organization, and recombination and repair. In addition, it forms the non-covalent module of the epigenome, thereby regulating gene expression. (Dillon and Festenstein, 2002; Strahl and Allis, 2000)

Chromatin structure determines the state of activity of genes by either promoting or hindering the access of the transcription machinery to regulatory regions. Families of ATP-dependent chromatin remodeling enzymes have been identified, which disrupt the nucleosome so as to expose regulatory sequences for transcriptional repressors or activators (Spotswood and Turner, 2002). Furthermore, the amino-terminal domains of the histones, which protrude from the nucleosome core, are subject to a number of enzyme catalyzed posttranslational modifications, including acetylation, phosphorylation methylation, ubiquitination, and ADP-ribosylation (Strahl and Allis, 2000). These modifications, which are established and maintained by families of modifying and demodifying enzymes, play important roles in either determining the positioning of nucleosomes on DNA and the compactness of chromatin, or in acting as 'receptors' to recruit specific complexes, both with the purpose of regulating gene transcription (Strahl and Allis, 2000). In addition, specific transcriptional activators or repressors can either directly modulate chromatin or recruit chromatin modifying/demodifying complexes to distinct genes (Dillon and Festenstein, 2002). The number of possible combinations of histone modifications is quite incredible. For example, the 50 different acetylated forms of the four core histones can be further modified by phosphorylation of serine on H2B,

H3, and H4, methylation of certain lysines or arginines on H3 or H4 (which can involve the attachment of either one, two, or three methyl groups), and also ubiquitination and ADP-ribosylation (Spotswood and Turner, 2002). Recent efforts have focused on determining the exact code that connects histone modifications to gene expression, and although this 'histone code' is still unclear, general principles are beginning to surface (Jenuwein and Allis, 2001; Richards and Elgin, 2002). The following section will describe the major histone modifications, the enzymes involved, and their relation to gene expression and DNA methylation.

(ii) Histone modifications and gene expression

The findings that transcriptionally active chromatin is enriched in acetylated histones, while transcriptionally silent constitutive and facultative heterochromatin contain mainly underacetylated histones, were the first links between a histone tail modification and chromatin functionality (Spotswood and Turner, 2002). Acetylation is now by far the most widely studied and understood histone modification, although the field of histone methylation appears to be rapidly catching up.

The core histones, with the exception of H2A, can be acetylated at several specific lysines within the N-terminal domains, which results in the neutralization of their positive charge. This is thought to weaken the interactions between the histones and the DNA backbone, as well as between neighboring nucleosomes, leading to a more open and transcriptionally active chromatin state (Spotswood and Turner, 2002). Conversely,

deacetylation of the histone tails re-establishes their positive charge and thus favors a 'closed' and repressed chromatin structure (Lusser, 2002). Since most histone acetates have very short half-lives (a few minutes to several hours), the level of acetylation at any particular time is likely to be in a dynamic steady-state that is dependent upon the activities of both histone acetylases (HAT) and deacetylases (HDAC). Such a mechanism is quite advantageous for genes whose expression must be rapidly altered, such as those regulated by growth factors or by hormones (Spotswood and Turner, 2002).

Many families of HATs and HDACs have been identified in organisms from yeast to humans, and both are found together with transcriptional activators (for HATs) and repressors (for HDACs) in multiprotein complexes (Ng and Bird, 2000; Roth et al., 2001). Recruitment of these complexes to particular regions of the genome is important for determining the specific pattern of acetylation and gene expression in a certain cell type. For example, the HAT p300/CBP is recruited to particular promoters through interactions with various transcription factors such as c-Jun, MyoD, nuclear hormone receptors, and many others (Roth et al., 2001). The mammalian Sin3 deacetylase complex, which is comprised of at least seven subunits including HDACs 1 and 2, can repress transcription via an extensive list of proteins that includes NCoR, p53, Ikaros, MeCP2, and more (Ng and Bird, 2000).

Several inhibitors of HATs and HDACs have been identified, including both endogenous proteins and pharmacological agents, which may be important for the dynamic and reversible potential of chromatin structure, as well as for treating diseases where the

underlying problem is aberrant gene expression. For instance, a multiprotein complex termed INHAT was found to potently inhibit the activity of the HATs PCAF and p300/CBP by binding to histones and masking the acetylatable sites (Seo et al., 2001). Subunits of this complex, including the oncoprotein Set/TAF-1β, associate with chromatin *in vivo* and can inhibit coactivator mediated transcriptional activation (Seo et al., 2002). Notably, Set/TAF-1β is upregulated in a number of tumor types and thus could potentially be involved in the abnormal gene regulation observed in cancer (Cervoni et al., 2002 Jul 12). In addition, several pharmacological inhibitors of HDACs have been characterized, including sodium butyrate (Candido et al., 1978; Vidali et al., 1978), trapoxin, and trichostatin A (TSA) (Yoshida and Horinouchi, 1999). Interestingly, the anti-epileptic drug valproic acid (valproate, VPA) was recently discovered to also possess the ability to inhibit HDACs (Gottlicher et al., 2001; Phiel et al., 2001). TSA can rapidly activate certain genes by increasing the acetylation of histones, which results in the opening of chromatin structure in the region of the genes (Strahl and Allis, 2000). In accordance with the observation that changes in chromatin structure play an important role in the silencing of certain genes in cancer, HDAC inhibitors have demonstrated anticancer effects (Weidle and Grossmann, 2000).

Although the existence of methylated histones has been known since 1964 (Murray, 1964), the first histone lysine MTase, SUV39, was not discovered until 3 years ago (Rea et al., 2000). Our knowledge of histone methylation, which occurs on either lysine or arginine residues of histones H3 and H4, has been rapidly expanding ever since, and several histone MTases as well as proteins that interpret the methyl-histone signal have

now been identified (Kouzarides, 2002). Both lysine and arginine methylation can occur in a mono and dimethylated manner, and lysines can be further converted to the trimethylated form (Bannister et al., 2002). Besides SUV39, several other histone MTases have been discovered, including G9A (Tachibana et al., 2001), SET2 (Strahl et al., 2002), ESET (Yang et al., 2002) and Dot1 (Ng et al., 2002), which are specific for lysines, and PRMT1 (Wang et al., 2001b) and CARM1/PRMT4 (Chen et al., 1999a), which only methylate arginine residues. It has been known for quite a while that different mammalian tissues or cell types possess different levels of the various methyl lysine and arginine species (Byvoet, 1972; Byvoet et al., 1972), however it was only recently discovered that the specific methylation pattern of histones, either within themselves or within a gene, is modified during transcriptional activation (Kouzarides, 2002; Zhang and Reinberg, 2001). Similar to DNA methylation, the long accepted belief has been that histone methylation is an irreversible epigenetic mark. Although there are several studies that suggest a necessity for the swift reversal of histone methylation if it is involved in gene expression (Bauer et al., 2002; Nielsen et al., 2001; Strahl et al., 2001), no active histone demethylases have been identified as of yet.

In general, histone H3 lysine 9 (H3-K9) methylation is primarily associated with inactive heterochromatin (Lachner et al., 2001; Noma et al., 2001). The mammalian H3-K9 histone MTase, SUV39, can act as a repressor when expressed as a GAL4 fusion protein (Firestein et al., 2000) and localizes to silent heterochromatin (Aagaard et al., 1999). In addition, the protein HP1 is recruited by the methylase activity of SUV39 and was found to bind specifically to H3-K9 (Bannister et al., 2001), where it plays an as of yet

unknown role in the formation of heterochromatin. As with HAT and HDAC complexes, some studies have shown that histone MTase-HP1 complexes may be targeted to certain genes via interactions with other factors, such as the retinoblastoma protein (Rb) (Nielsen et al., 2001) and myocyte enhancer factor 2 (MEF2) (Zhang et al., 2002). In contrast, histone H3 lysine 4 (H3-K4) methylation is found in transcriptionally active regions that are generally free of H3-K9 methylation (Litt et al., 2001; Noma et al., 2001; Strahl et al., 1999). H3-K4 methylation was found to disrupt the binding of the NuRD repressor and deacetylase complex resulting in an open chromatin structure (Zegerman et al., 2002). Arginine methylation is also correlated with gene activity, which was first indicated by the discovery that CARM1/PRMT4 histone MTase functions to enhance steroid hormone-dependent activation and is dependent on its ability to methylate H3 (Chen et al., 1999a). Other studies also support this conclusion (Bauer et al., 2002; Ma et al., 2001).

The functions of histone phosphorylation and ubiquitination are less understood. H3 phosphorylation is required for correct segregation and condensation of chromosomes during mitosis, and other studies suggest that phosphorylation, specifically H3 serine 10, is directly correlated with the induction of immediate-early genes *c-fos*, *c-myc*, and *c-jun* (Strahl and Allis, 2000). A strong functional correlation between ubiquitination and meiosis and spermiogenesis has been well documented (Jason et al., 2002). Ubiquitination may also play a role in transcriptional regulation as it was recently determined that ubiquitination of histone H2B is required for histone H3 methylation (Dover et al., 2002; Sun and Allis, 2002).

(iii) Epigenetic crosstalk

To complicate matters even further, many studies have demonstrated that different modifications within or between histone tails can mutually affect each other, and could potentially contribute to the fine-tuning of gene expression. The following are but a few examples: Methylation of H3-K9 interferes with the phosphorylation of H3 serine 10 (Rea et al., 2000); methylation of arginine 3 of H4 facilitates the subsequent acetylation of H4 (Wang et al., 2001b); methylation of H3-K4 and H3-K9 inhibit each other, and H3-K4 methylation facilitates subsequent H3 acetylation while methylation on H3-K9 inhibits H3 acetylation (Wang et al., 2001a).

In addition, a relationship between histone modifications and DNA methylation is also well established. Early observations by Cedar and Razin have demonstrated a tight correlation between chromatin structure and DNA methylation (Razin and Cedar, 1977), such that DNA packaged in tightly configured chromatin was shown to be hypermethylated while DNA that was associated with open chromatin was hypomethylated **(Figure 2).** Furthermore, there is a complicated connection between the targeting of both HDAC complexes and histone MTases, and DNA methylation. DNMT1 and DNMT3a were found to associate with various HDACs (Fuks et al., 2001; Robertson et al., 2000a; Rountree et al., 2000), and the methyl-CpG-binding proteins MeCP2, MBD2, and MBD3 have also been found in various repressor complexes containing HDACs (Feng and Zhang, 2001; Jones et al., 1998; Zhang et al., 1999). MeCP2 associates with a H3-K9 histone MTase activity *in vivo* (Fuks et al., 2003), and

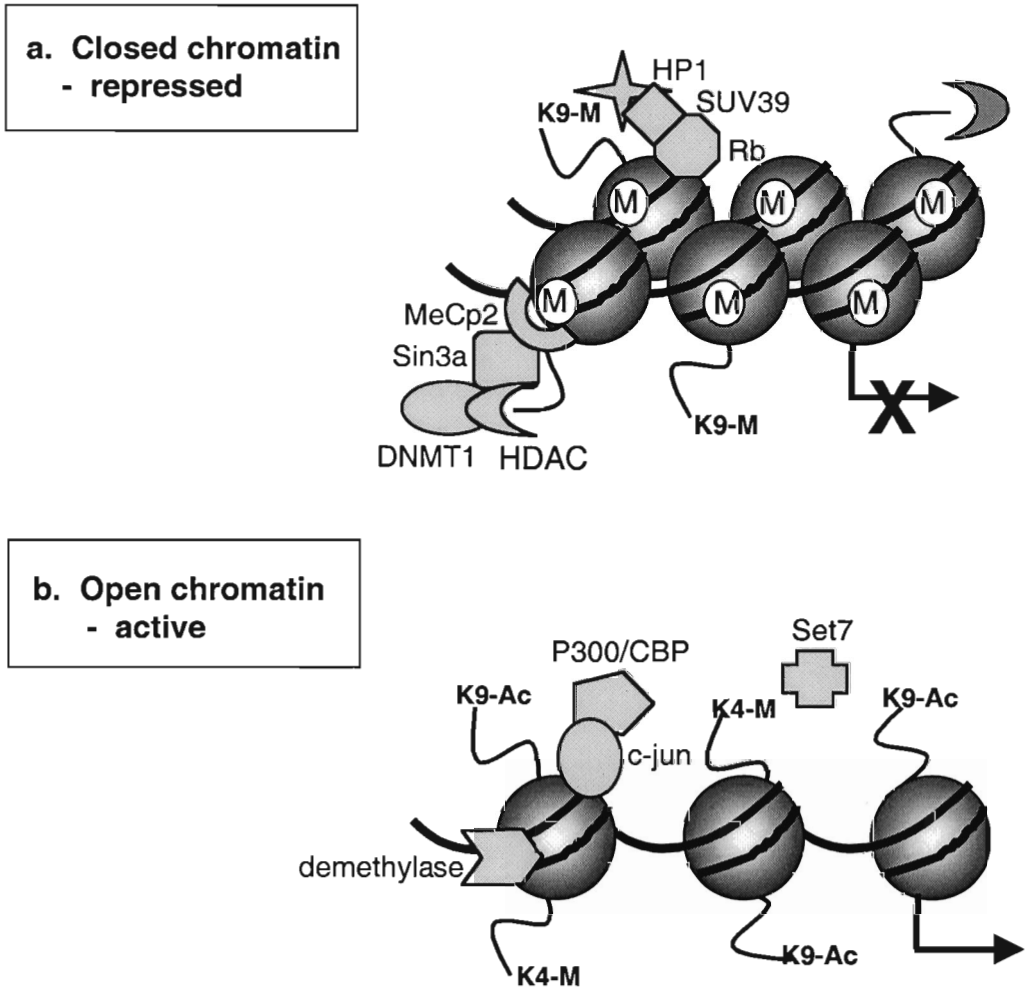

Figure 2 -Epigenetic modifications determining gene expression.

Nucleosomes consist of DNA (black line) wrapped around histone octamers (grey ball); bent arrow indicates transcription. (a) DNA methylation, histone deacetylation, and histone H3-K9 methylation induce a condensed chromatin configuration leading to transcriptional repression. Proteins involved in this process include DNMTs (e.g. DNMT1), HDACs, corepressors (e.g. Sin3a), MBDs (e.g. MeCP2), sequence specific repressors (e.g. Rb), histone H3-K9 MTases (e.g. SUV39), and the methyllysine binding protein HP1. (b) Unmethylated DNA, histone acetylation, and histone H3-K4 methylation lead to a more open chromatin structure that allows for transcriptional activation. Proteins involved include HATs (e.g. CBP/300), which are recruited by sequence specific activators (e.g. c-jun), as well as histone H3-K4 MTases (e.g. Set7) and DNA demethylases.

DNMT3a was found to co-localize with HP1 and heterochromatin (Bachman et al., 2001). These studies have led to the suggestion that DNA methylation targets these histone-modifying proteins to specific genes to bring about chromatin remodeling and repression. However, recent findings in both *Neurospora* and *Arabidopsis* have demonstrated histone MTases are required for DNA methylation (Jackson et al., 2002 Apr 4; Tamaru and Selker, 2001), implying that chromatin modifications determine DNA methylation. So which comes first, DNA methylation or chromatin structure? Discussion of this topic is reserved for section VII, where the causal relationship between DNA methylation, chromatin structure, and gene expression will be examined.

IV. The DNA methylation machinery

(i) Overview of DNA methyltransferases

DNA methyltransferases (DNMT) are ubiquitous enzymes, found in multiple organisms ranging from bacteria to plants, which catalyze the transfer of a methyl group from AdoMet onto cytosine or adenine residues in DNA (Cheng and Roberts, 2001; Wu and Santi, 1985). Three different classes of enzymes exist, which catalyze the formation of either N6-methyladenine, N4-methylcytosine, or 5mC, however the latter type is the sole one found in both prokaryotes and eukaryotes (Jeltsch, 2002). Remarkably, 700 different DNMTs that recognize almost 300 different sequences have been characterized in prokaryotes. In contrast, only three catalytically active DNMTs have been identified in mammals (Bestor, 2000) and two in fungi (Martienssen and Colot, 2001). Although the

Drosophila genome possesses low levels of methylation (Gowher et al., 2000; Lyko et al., 2000), only putative 5mC DNMTs have been described so far (Hung et al., 1999). Plants contain, in addition to several homologs of the mammalian DNMTs, a unique family of DNMTs termed Chromomethylases that methylate the sequence CNG (Martienssen and Colot, 2001). As described in section II, the addition of methyl groups by these enzymes results in a host of different functions.

The structure of the 5mC DNMTs includes one small and one large domain, with the DNA binding region positioned at the interface between the two domains. The large domain contains a common mixed seven stranded β-sheet called the 'AdoMet dependent fold', as well as several conserved motifs (I-VIII and most of X) including a domain required for cofactor binding and a catalytic region containing a Pro-Cys dipeptide. The small domain of most 5mC DNMTs displays much more variability (with the exception of conserved motif IX) and encodes unique features and regulatory functions. The variable region between motifs VIII and IX is responsible for target recognition, which can vary widely (Cheng and Roberts, 2001; Kumar et al., 1994). For example, while type II bacterial DNMTs display a high sequence specificity for 4 and 6 base pair sites, which are in most cases palindromic, the only requirement for the vertebrate DNMTs is simply the dinucleotide sequence CG (Razin and Szyf, 1984).

Our basic understanding of 5mC DNMTs stems from structural and functional analysis of the bacterial enzymes, most notably HhaI. The target cytosine is buried within the double helix and therefore not easily accessible, but DNMTs solve this problem by rotating the

sugar-phosphate bond and flipping out the cytosine so that it projects unto the catalytic domain (Cheng et al., 1993). The sulfhydryl group of the enzyme's catalytic cysteine residue first makes a nucleophilic attack onto the 6th position of the cytosine, resulting in a 5,6-dihydropyrimidine intermediate. This activates position 5 of cytosine, which attacks the methyl group of AdoMet, followed by the release AdoHcy. The subsequent deprotonation at position 5 leads to the dissociation of the enzyme-DNA complex and reforms the aromatic ring (Wu et al., 1996).

(ii) Mammalian DNA methyltransferases: multifunctional proteins

DNMT1 was the first DNMT to be cloned from vertebrates (Bestor, 1988), and has been extensively studied for many years. It is a very large protein of 1620 amino acids with both alternative start sites (Rouleau et al., 1992; Yoder et al., 1996) and splicing isoforms (Bonfils et al., 2000; Deng and Szyf, 1998; Mertineit et al., 1998). DNMT1 contains a long N-terminal regulatory domain that is responsible for such functions as the ability to discriminate between hemimethylated and unmethylated DNA substrates (Araujo et al., 2000), allosteric activation by methylated DNA (Bacolla et al., 2001), targeting the protein to the replication fork (Leonhardt et al., 1992), and interactions with various proteins such as PCNA (Chuang et al., 1997), HDACs (Fuks et al., 2000), Rb, and E2F1 (Robertson et al., 2000a).

DNMT1 is believed to be the enzyme responsible for replicating the DNA methylation pattern during cell division due to its preference for hemimethylated DNA (Gruenbaum et

al., 1982) and as demonstrated by mouse knockout experiments (Li et al., 1992). The roles of DNMT1 in DNA replication (Knox et al., 2000; Milutinovic et al., 2003; Vertino et al., 2002) and cellular transformation (Szyf, 2001b) are also well documented and will be reviewed in following sections.

During the ten years following its cloning, DNMT1 was the only mammalian DNMT known. Since embryonic stem cells derived from DNMT1 knockouts retained *de novo* methylating ability, it was obvious that there were other methylating enzymes waiting to be discovered. However it wasn't until 1998 that the DNMT3 family was identified (Okano et al., 1998a). Both DNMT3a and DNMT3b are similar in structure to DNMT1, and although their N-terminal region is much smaller, they also form multiple protein-protein interactions as will be discussed below. They have been defined as *de novo* DNMTs since they show no preference for hemi- versus unmethylated DNA *in vitro* (Okano et al., 1998a). During development new patterns of methylation are laid out and must be catalyzed by enzymes that are not guided by the methylation of the parental stand. DNMT3a and DNMT3b are believed to be partly responsible for *de novo* methylation during embryogenesis, as well as *de novo* methylation of proviral sequences (Liang et al., 2002 Jan; Okano et al., 1999). DNMT3a is also capable of methylating DNA at non-CG sites (Gowher and Jeltsch, 2001; Ramsahoye et al., 2000), however the biological relevance of this form of methylation is not yet known. DNMT3a and 3b cannot explain all the *de novo* methylation occurring during embryogenesis since *dnmt3a-/-* and *dnmt3b-/-* are not completely devoid of methylation. DNMT3a and 3b are probably involved in methylation of specific satellite sequences. Interestingly, mutations

in DNMT3b lead to the development of ICF syndrome, a disease characterized by immunodeficiency, centromeric instability, and facial anomalies, in which hypomethylation of certain satellite sequences is observed (Hansen et al., 1999). The rest of *de novo* DNA methylation must be carried out by either DNMT1 or other DNMTs that have not yet been identified. In 1998, the DNMT2 protein was also discovered, which contains all the sequence motifs characteristic of DNMTs but lacks the large N-terminal regulatory domain (Yoder and Bestor, 1998). Although DNMT2 was found to be incapable of methylating DNA *in vitro* and was not required for methylation of viral DNA in ES cells (Okano et al., 1998b), a recent study suggests that it may be active *in vivo* (Liu et al., 2003). The DNMT3L protein also does not possess any methyltransferase activity, however it may be involved in establishing maternal methylation imprints (Hata et al., 2002).

It must be noted that this simple differentiation of DNMT activities into maintenance methylation, which replicates the DNA methylation pattern during cell division, and *de novo* methylation, which generates new methylation patterns, might not be accurate. Studies illustrate that the difference between maintenance and *de novo* DNMTs is blurred in some instances. For example, overexpression of DNMT1 can lead to *de novo* methylation (Mikovits et al., 1998; Vertino et al., 1996) and recent studies suggest that DNMT3a and DNMT3b are required for maintenance methylation of repetitive sequences (Liang et al., 2002). Thus the maintenance of DNA methylation patterns in living cells must involve other mechanisms in addition to the biochemical property of substrate discrimination identified *in vitro*. A number of data suggest that chromatin structure

plays an important role in targeting both DNMT and DNA demethylase. Recent findings also uncover a functional cooperation between DNMT1 and the DNMT3 family members (Chedin et al., 2002; Fatemi et al., 2002; Liang et al., 2002), which may also involve physical interactions. The roles of protein interactions and chromatin structure in mediating methylation patterns will be explored in sections V and VII.

In addition to their role as methylating enzymes, DNMTs have also been shown to interact with other chromatin modulating proteins such as MeCP2, the corepressors RP58 and DMAP, and HDACs 1 and 2 (Fuks et al., 2001; Kimura and Shiota, 2002; Robertson et al., 2000a; Rountree et al., 2000) **(Figure 3).** It was therefore proposed that DNMTs silence gene expression through their protein-protein interactions by recruiting factors that modify and inactivate chromatin. For example, DNMT3a was found to bind a sequence- specific DNA binding repressor, RP58, and repress transcription by recruiting HDAC activity (Fuks et al., 2001). DNMT1 forms a complex with Rb, E2F, and HDAC1 to repress transcription from E2F-responsive promoters (Robertson et al., 2000a), and DNMT1 was shown to repress the expression of the tumor suppressor p21 (Milutinovic et al., 2000). In all three cases, repression was found to occur in the absence of methylation. Even DNMT3L, which has no methylation activity, has the ability to repress transcription by recruiting HDACs (Deplus et al., 2002), providing further evidence that the DNMTs are more than just methyltransferases.

Other data also provide evidence that DNMT1 plays a role in DNA replication and cell cycle control, and will be covered in section VI and chapter I.

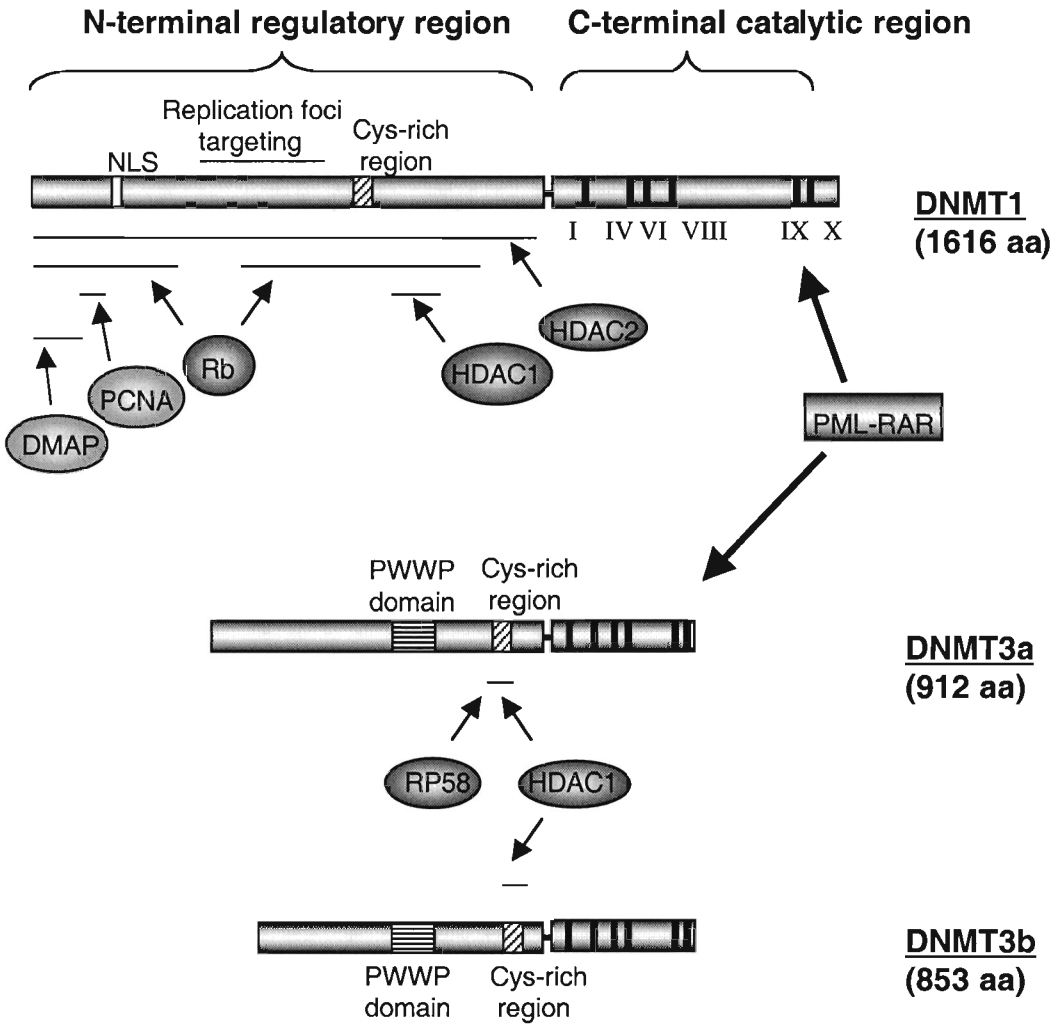

Figure 3 - Schematic structure of the catalytically active DNMTs and their interacting proteins.

Specific motifs indicated include: DNMT1: Nuclear localization signal (NLS), replication fork targeting region, HRX-like cys-rich region (Zn binding). DNMT3a and DNMT3b: Cys-rich region (similar to ATRX Zn finger), PWWP domain (involved in cell growth and differentiation). Black bars in all DNMTs represent the six most conserved methyltransferase motifs (I, IV, VI,VIII, IX and X). A horizontal line indicates the interacting region of each DNMT protein. Note, in the case of PML-RAR the interacting regions on DNMT1 and DNMT3a have not been mapped. Number of amino acids in each DNMT is indicated (aa).

(iii) Active DNA demethylases

Section II provided evidence to indicate that DNA demethylation can be an active process occurring in the absence of cell division. Since the direct removal of the methyl group, by breaking a C-C bond, has long been considered an energetically unfeasible reaction, alternative demethylation mechanisms were sought. Two different repair mechanisms have previously been proposed to cause site-specific active demethylation. The first mechanism involves a glycosylase, which recognizes methylated CGs and cleaves the bond between the methylated cytosine base and the deoxyribose. The apyrimidinic base is removed and replaced with an unmethylated cytosine nucleotide by a repair activity (Jost et al., 1995; Razin et al., 1986). 5mCG glycosylase activity was demonstrated in a cloned glycosylase that was also found to be a G/T mismatch DNA glycosylase, as well as in MBD4 (Zhu et al., 2000a; Zhu et al., 2000b). Such a mechanism was suggested to play a role in chicken embryos (Jost et al., 1995) and during the differentiation of both erythroleukemia cells in culture (Razin et al., 1986) and mouse myoblasts (Jost et al., 2001). An alternative repair mechanism involves nucleotide excision of the methylated CG dinucleotide and its replacement with an unmethylated CG (Weiss et al., 1996).

However, the global repair of DNA to achieve hypomethylation, especially during development, might create a serious threat to the integrity of the genome. A true active demethylase should catalyze the flip side of the methylation reaction, i.e., the direct removal of the methyl group from DNA. And in 1999, five years following the

demonstration that P19 cells that ectopically express Ras also express high demethylating activity (Szyf et al., 1995), a *bona fide* demethylase was purified from human lung cancer A549 (Ramchandani et al., 1999). The demethylase enzyme from A549 cells catalyzes the hydrolytic cleavage of the bond between the methyl group and the cytosine ring releasing the methyl group in the form of methanol. The demethylase activity is processive (Cervoni et al., 1999), which is probably critical for the rapid global hypomethylation observed during early development. It is also consistent with the regional differences observed in methylation of CG islands, suggesting that a whole region rather than one specific site is demethylated in active genes.

In addition, the methyl-CpG-binding protein MBD2 was found to possess a demethylase activity (Bhattacharya et al., 1999). MBD2/dMTase was found to actively demethylate DNA both *in vitro* (Bhattacharya et al., 1999) and *in vivo* (Cervoni and Szyf, 2001; Detich et al., 2002). Furthermore, its expression is associated with demethylation of endogenous genes (Hattori et al., 2001), and a recent report demonstrated that the *Drosophila* homolog of MBD2, dMBD2/3, formed foci that associated with DNA at the cellular blastoderm stage, concurrent with the activation of the embryonic genome, and also associated with the active Y chromosome (Marhold et al., 2002).

Then again, MBD2 was also independently cloned and identified as a methylated DNA binding repressor (Hendrich and Bird, 1998; Ng et al., 1999) (discussed in section VII). As a result, its ability to demethylate DNA has triggered obvious controversy in the field, and other groups (Hendrich et al., 2001; Ng et al., 1999) have disputed the demethylase

activity of MBD2/dMTase. In addition, *Mbd2-/-* knockouts did not exhibit global differences in DNA methylation (Hendrich et al., 2001), however this study did not examine the demethylation of defined genes, therefore it is possible that MBD2/dMTase is involved in specific demethylation events. Data presented in chapter 2 suggests a bi-functional role for MBD2/dMTase, depending on several critical parameters (Detich et al., 2002). It is possible that both functions reside in one protein to coordinate a program of gene expression that requires suppression of some methylated genes and activation of others.

An important point to note is that the discovery of a demethylase activity raises the possibility that DNA methylation is a reversible biological modification (Ramchandani et al., 1999), and that the observed pattern of methylation results from an equilibrium of methylation and demethylation activities. In addition, the fact that a demethylase is found in somatic cells, and especially in cancer cells, has obvious implications on our understanding of how the DNA methylation pattern is inherited and maintained in normal cells as well as in cellular transformation (Szyf et al., 2000). The roles of demethylase in determining methylation patterns and in cancer will be discussed in upcoming sections.

V. Regulation of the DNA methyltransferases

Considering the importance of the DNMTs in maintaining proper epigenetic information, the cells must undoubtedly possess mechanisms to regulate their expression and activity. The existence of multiple layers of regulation, such as transcriptional, posttranscriptional,

posttranslational, and protein interactions, is an indication that the proper control of the DNMTs is critical. By maintaining the correct levels and activity of the DNMTs during different events such as cell signaling, cell cycle progression, and differentiation, the proper gene expression for these events can be established. If DNMT regulation is compromised, it might promote improper gene expression and thus lead to pathogenic conditions. The following sections will describe in detail various mechanisms implicated in regulating the expression and activity of the DNMTs.

(i) Transcriptional regulation

Several years ago, an analysis of the *DNMT1* gene was performed by Bigey *et al.* (2000). This study demonstrated that *DNMT1* contains four transcription start sites, each regulated by independent, TATA-less, promoter and enhancer elements. The first promoter lies in a CpG island 5' to the first exon, possesses the highest basal activity, and can be repressed by the retinoblastoma protein Rb. In contrast, the three downstream promoters, which are located 5' to the second, third and fourth exons, are within CpG poor regions and have a low basal activity, but can be induced by the transcription factor c-Jun. In addition, three enhancer elements were identified, and were found to be either dependent or induced on the ectopic expression of c-Jun (Bigey et al., 2000). These enhancers may thus be able to activate transcription from all four promoters. The biological significance of these AP-1 recognition regulatory elements will be discussed in further detail below.

Recently, the promoters of *DNMT 3a* and *3b* have also been characterized (Yanagisawa et al., 2002), and were found to bear a striking resemblance in structure to that of *DNMT1*. This study determined that *DNMT3a* is composed of at least four transcription start sites controlled by three different promoters. Two of these promoters contain CpG islands and all three of them lack TATA sequences. The *DNMT3b* gene possesses at least two start sites that are controlled by different TATA-less promoters, one of which also contains a CpG rich region. All the *DNMT3a* and *3b* promoters also contain AP-1 sites, although regulation by these sites was not examined. However, it was shown that many binding sites for the transcription factor Sp1 are located within the *DNMT3b* upstream promoter, and progressive deletion of these sites results in a corresponding decrease in promoter activity (Yanagisawa et al., 2002). Similarly, the upstream CpG rich promoter of the mouse *dnmt1* gene was also found to contain an element that is required for *dnmt1* expression and is activated by both Sp1 and Sp3 (Kishikawa et al., 2002).

The promoter similarities between the three *DNMT*s suggest that similar cellular signals might coordinately regulate the three enzymes through transcription. It is tempting to speculate that the regulatory mechanisms described below, which have only been shown for *DNMT1*, may play a role in the regulation of *DNMT3a* and *3b* as well.

AP-1 regulatory elements can promote transcription upon the binding of dimeric complexes of the Fos and Jun oncoproteins, both of which are activated by the mitogenic Ras signaling pathway. The initial molecular link between DNMT1 and oncogenic

signaling was the discovery that the protooncogenic Ras signaling pathway regulates *DNMT1* (Bigey et al., 2000; MacLeod et al., 1995; Rouleau et al., 1995). It was first demonstrated that the murine *dnmt1* promoter is induced by Jun or Ha-Ras but not by a dominant negative mutant of Jun in P19 cells (Rouleau et al., 1995). The human *DNMT1* promoter is similarly induced by the Ras signaling pathway (Bigey et al., 2000). In addition, over expression of *Ha-ras* in P19 cells induced transcription and steady-state levels of endogenous *dnmt1* mRNA (Rouleau et al., 1995). It was then shown that the levels of cellular *dnmt1* mRNA could be reduced by downregulating the Ras signaling pathway by ectopic expression of either a human GAP or a dominant negative c-Jun in Y1 cells (MacLeod et al., 1995). This reduction was accompanied by a reversion of the transformed morphology of Y1 cells. If Ha-Ras was then introduced into the GAP transfectants, DNMT1 levels increased and cells regained a transformed phenotype (MacLeod et al., 1995). These data demonstrating that *dnmt1* is downstream to the AP-1 signaling pathway is further supported by a study showing that *dnmt1* is one of the genes induced by forced expression of *c-fos,* and that inhibition of *dnmt1* by antisense expression reverses cellular transformation induced by *c-fos* (Bakin and Curran, 1999). Since it has been shown that overexpression of *DNMT1* can lead to hypermethylation (Vertino et al., 1996; Wu et al., 1993), the hypermethylation observed in certain cancer cells with Ras mutations may be a result of increased DNMT1 levels. This data is consistent with fact that tumor suppressor hypermethylation is correlated with activating mutations of Ras in some human colon tumors (Guan et al., 1999; Toyota et al., 1999).

DNMT1 regulation by the Ras pathway has also been found to occur in human lymphoid

(T) cells. This was initially demonstrated by the finding that mitogenic T-cell stimulation increased *DNMT1* mRNA and enzyme activity (Yang et al., 1997). Overexpression of Ha-Ras in T-cells was then found to increase DNMT1 levels, while inhibiting signaling through the Ras-MAPK pathway decreased DNMT1 (Deng et al., 1998). It is interesting to note that inhibiting T-cell DNMT1 leads to a lupus-like disease by altering DNA methylation and gene expression (Cornacchia et al., 1988; Richardson et al., 1994), and T cells from patients with lupus possess reduced DNMT1 enzyme activity, hypomethylated DNA and modified gene expression (Yung and Richardson, 1994). It is therefore not surprising to learn that signaling through the Ras pathway was reduced in lupus patients (Deng et al., 2001). Taken together, these data indicate that misregulation of DNMT1 by the Ras pathway may lead to cellular transformation, lupus-like conditions, and perhaps other as of yet unknown pathogenic states.

Another possible link between *DNMT1* and critical cellular control pathways is the APC-β-Catenin-Tcf pathway (Clevers and van de Wetering, 1997; Korinek et al., 1997; Rubinfeld et al., 1997). When β-Catenin associates with nuclear Tcf factors, they form a transcriptional activator. The tumor-suppressor protein APC, which binds to β-Catenin and causes its destruction, negatively regulates this factor. The *APC* (adenomatus polyposis coli) gene is mutated in many cases of familial colon cancer. In APC-deficient colon carcinoma cells, β-catenin accumulates and is constitutively complexed with Tcf, resulting in transcriptional activation of Tcf protooncogenic target genes such as *C-MYC* (He et al., 1998a). In addition, *Min* mice bearing a mutation in the mouse homologue of the *APC* gene spontaneously develop colonic adenomatous polyps. However, when *Min*

mice are genetically crossed with heterozygous *dnmt1* knockouts, they show a reduction in polyp formation (Laird et al., 1995), suggesting that *dnmt1* is a downstream target of APC signaling.

Support for the link between APC and DNMT1 has recently been demonstrated (Campbell and Szyf, 2003). It was found that ectopic expression of the wild type APC in HT-29 *APC-/-* colon carcinoma cells resulted in downregulation of both a *DNMT1* promoter driven reporter construct, as well as the endogenous *DNMT1* mRNA. This was further confirmed though the use of a dominant negative mutant of Tcf, which was also found to suppress the *DNMT1* mRNA. These results suggest that a mutated APC protein, through its inability to degrade β-Catenin, leads to a Tcf dependent transcriptional upregulation of *DNMT1*. The causal role of DNMT1 in cellular transformation was also demonstrated by the finding that DNMT1 knockdown by antisense treatment resulted in the inhibition of anchorage independent growth of HT-29 cells (Campbell and Szyf, 2003). Although the *DNMT1* promoter does not contain any consensus Tcf binding sites, it is possible that Tcf either binds non-consensus sites, or that it functions through an intermediary transcription factor. Further studies are required to determine the exact mechanism involved. A summary of the promoter structure and transcriptional regulation of the DNMTs is shown in **Figure 4.**

(ii) Cell growth

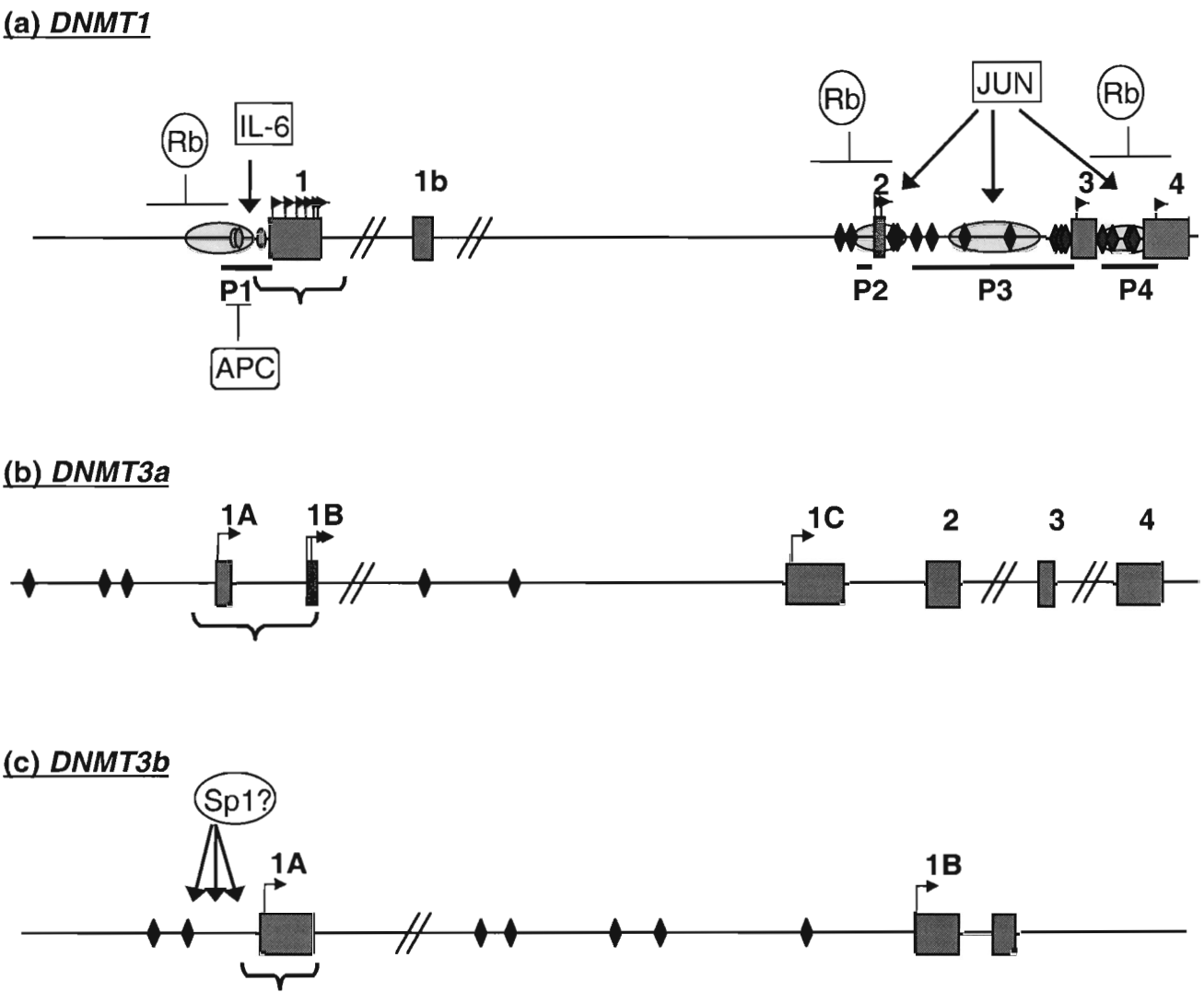

Figure 4 - DNMT promoter structure and regulation.

Schematic diagrams of the *DNMT1* (a) *DNMT3a* (b) and *DNMT3b* (c) genes are shown. Boxes (numbered above) designate exons and bent arrows indicate transcription initiation sites. DNMT1 promoter boundaries (P1-P4) and enhancers (horizontal ovals) are shown. An open bracket denotes CG rich regions. Potential binding sites for AP-1 (diamonds) and Fli-1 (vertical ovals) are also indicated. The different regulatory pathways that are postulated to regulate the *DNMT1* and *DNMT3b* genes are noted above and below the map of the gene. A vertical arrow specifies gene activation, while repression is indicated by a blunted line.

Deregulated expression of *DNMT1* was previously suggested to play a causal role in cellular transformation (el-Deiry et al., 1991; Szyf, 1994). Several lines of evidence are in accordance with this hypothesis; these will be discussed in detail in section VIII of the introduction. However, deregulated expression of *DNMT1* during the cell cycle is one mechanism that that has been proposed for DNMT1's effects on cell growth (Szyf, 2001a). This hypothesis is supported by the findings that the coordinated cell cycle regulation of DNMT1 is disrupted in colorectal cancer cells *in vivo* (De Marzo et al., 1999), as well as in estrogen receptor negative breast cancer cells (Nass et al., 1999).

The primary studies involving the regulation of DNMT with the cell cycle were performed in the mid 80's to early 90's. Initially, Szyf *et al.* determined that DNMT activity is regulated with the cell cycle (Szyf et al., 1985b), and subsequently, that murine *dnmt1* mRNA is not present in arrested Balb/c-3T3 cells but is highly induced at the G1-S boundary. *dnmt*1 levels remain high during the S-phase, and are then reduced as cells enter G2/M (Szyf et al., 1991). Subsequent runoff transcription experiments demonstrated that *dnmt1* is transcribed throughout the cell cycle (Szyf et al., 1991), suggesting that the levels of *dnmt1* mRNA are regulated with the cell cycle at the posttranscriptional level.

The mechanism involved in this posttranscriptional regulation was not unraveled until many years later, and is the topic of chapter 3 of the thesis. Data presented in chapter 3 demonstrate that the 3' untranslated region (3'UTR) of the *DNMT1* mRNA plays a role in regulating its levels with the cell cycle, and that deregulation at this level has an effect on

cellular transformation by DNMT1 (Detich et al., 2001). These results support the hypothesis stated above, that deregulated expression of *DNMT1* with the cell cycle and not the total amount of DNMT1 is important for cellular transformation.

In addition to the abovementioned posttranscriptional mechanism, it appears that DNMT1 may also be regulated in S phase at the translational and posttranslational levels. It was found that upon S phase arrest of MEL 11A2 cells (using an aphidicolin block), both an increase in the synthesis of DNMT1 protein, as well as an increase in protein half-life, takes place (Suetake et al., 1998). The finding that DNMT1 is controlled during the cell cycle by more than one mechanism further emphasizes the importance of this regulation, so that DNA replication does not proceed in the absence of DNMT1 and ensuring that the replicating DNA is properly methylated.

The growth regulation of DNMTs 3a and 3b has not been studied as extensively. It has been shown that *DNMT3b* mRNA is also regulated with the cell cycle, and its profile is similar as to that of *DNMT1*. The mRNA levels of *DNMT3a*, on the other hand, display less of a down regulation upon cell arrest (Robertson et al., 2000b). Whether the *DNMT3a* and *DNMT3b* mRNAs are controlled transcriptionally, or at a posttranscriptional level as for *DNMT1*, is still unresolved.

(iii) Viral infection

It has been shown that the DNA of endogenous and exogenous retroviruses can be highly methylated in the host genome (Gunthert et al., 1976; Masucci et al., 1989; Youssoufian et al., 1982). In addition, infected cells can exhibit increased DNA methylation in other regions of the genome as well (de Bustros et al., 1988; Jahner and Jaenisch, 1985). It is therefore fitting that the expression of the DNMTs is regulated by certain viruses or viral proteins at both the transcriptional and posttranscriptional levels.

T antigen is a protein product of the SV40 virus that can immortalize primary lines (Tevethia, 1984), transform immortalized lines (Aaronson and Todaro, 1968), and induce tumors in mice. The transformation induced by T antigen results, at least in part, from its interaction with the tumor suppressor Rb (DeCaprio et al., 1988). Following the observation that two SV40 transformed cell lines displayed increased DNMT1 levels (Chuang et al., 1997), the regulation of *dnmt1* by SV40 T antigen was examined (Slack et al., 1999). This study demonstrated that ectopic expression of SV40 T antigen induces *dnmt1* expression, protein levels, and global DNA methylation, and was found to depend on T antigen's interaction with Rb. Furthermore, inhibiting *dnmt1* expression by antisense oligonucleotides could reverse cellular transformation by T antigen (Slack et al., 1999). These data demonstrate that a viral oncoprotein can upregulate *dnmt1*, and that this upregulation plays a causal role in T antigen induced transformation. In contrast to the transcriptional upregulation of *dnmt1* by the Ras pathway, T-antigen was found to influence *dnmt1* at the posttranscriptional level, by increasing the stability of the *dnmt1* mRNA (Slack et al., 1999).

Recently, the latent membrane protein 1 (LMP1), which is an oncogenic product of EBV, was shown to upregulate DNMTs 1, 3a, and 3b (Tsai et al., 2002). EBV is a human herpes virus that has been implicated in several cancers (Klein et al., 1974; Shibata and Weiss, 1992). In line with its ability to promote transformation (Wang et al., 1985), it was found that LMP1 can induce the mRNA, protein levels, and activity of DNMT1, DNMT3a, and DNMT3b, and that this results in hypermethylation and downregulation of E-cadherin, a gene that is often hypermethylated in cancer (Tsai et al., 2002). Although a steady-state increase in the *DNMT* mRNA was demonstrated, the mechanism by which this occurs was not determined (Tsai et al., 2002).

Results from another study demonstrate that DNMT1 regulation is not limited to cancer promoting viruses. The role of the HIV-1 virus in regulating DNMT1 levels was examined, and it was found that this virus is able to upregulate *DNMT1* mRNA and DNMT1 activity, which is accompanied by an increase in overall DNA methylation. Additionally, *de novo* methylation of a CpG within the gamma interferon (IFN-γ) promoter and its subsequent downregulation was also observed (Mikovits et al., 1998). Thus, DNMT1 may be involved in the loss of the type 1 immune response (which involves IFN-γ) observed in AIDS patients (Mikovits et al., 1998; Shearer and Clerici, 1991). This study did not determine the mode, transcriptional or posttranscriptional, by which HIV-1 increases *DNMT1* mRNA.

(iv) Cellular differentiation

A number of studies have shown that DNMT1 is upregulated during cellular differentiation (Hodge et al., 2001; Slack et al., 2001; Soultanas et al., 1993). On the other hand, DNMT1 is also downregulated in certain differentiation processes (Deng and Szyf, 1999; Liu et al., 1996; Soultanas et al., 1993; Teubner and Schulz, 1995). Thus, it appears that the role of DNMT1 in determining cell fate is a complex one, and may depend on cell type. It stands to reason that several mechanisms, (post)transcriptional, posttranslational, and regulation of enzyme activity, have evolved to precisely coordinate DNMT1 levels with a particular differentiated phenotype.

For instance, when K562 cells are treated with Interleukin 6 (IL-6), they enter a megakaryocytic differentiation pathway. Coincident with this process is an upregulation of *DNMT1* mRNA and enzyme activity (Hodge et al., 2001). This upregulation was found to occur at the transcriptional level, since the *DNMT1* promoter is activated following transfection into K562 cells and treatment with IL-6. In addition, this activation appears to involve several Fli-1 binding sites within the *DNMT1* promoter, since the loss of these sites greatly diminishes *DNMT1* promoter induction. Another example where DNMT1 is upregulated during differentiation occurs when U937 cells are induced to differentiate to a more monocyte-like phenotype by the phorbol ester TPA (Soultanas et al., 1993). Treatment of U937 cells with TPA leads to both an increase in DNMT1 enzyme activity and *DNMT1* mRNA expression, the latter of which occurs at the posttranscriptional level. As with the previous example, the functions of DNMT1 in this differentiation process, and whether changes in genomic methylation occur, are undefined.

There are also several well-documented examples where DNMT1 is downregulated during the differentiation process (Deng and Szyf, 1999; Liu et al., 1996; Soultanas et al., 1993; Teubner and Schulz, 1995). At the start of mouse myoblast differentiation, a decrease in both DNMT1 activity and global methylation is observed (Jost and Jost, 1994). It was then shown that posttranscriptional and posttranslational mechanisms are involved (Liu et al., 1996), as both the mRNA and protein stabilities are reduced in differentiated cells. Due to the fact that the global methylation in these cells occurs in the absence of replication (Jost and Jost, 1994), it cannot be directly attributed to the decrease in DNMT1; however, the possibility of an indirect role has been suggested (Liu et al., 1996). During the differentiation process of F9 mouse embryonal carcinoma cells, a similar post transcriptional decrease in *dnmt1* mRNA is observed, accompanied by a decrease in DNMT1 activity (Teubner and Schulz, 1995). However, in contrast to myoblast differentiation, global demethylation in F9 cells is found to occur gradually during differentiation after several rounds of replication (Razin et al., 1984). DNA replication in the presence of reduced levels of DNMT1 could directly explain this process.

Contrary to myoblast differentiation, where downregulation of DNMT1 is an early event, a reduction in DNMT1 levels occurs late in PC12 differentiation (Deng and Szyf, 1999). PC12 cells are induced to differentiate into sympathetic neuron like cells upon treatment with NGF. When the levels of DNMT1 during the progression of differentiation were studied, it was found that *dnmt1* mRNA, as well as DNMT1 protein and activity, are

decreased 4 days post induction (Deng and Szyf, 1999). Since neurite outgrowth is already present after 2 days, it implies that downregulation of DNMT1 is not required for PC12 differentiation but is rather an adaptation to the postmitotic state of the cell, as has been proposed (Deng and Szyf, 1999). It is not known at what level DNMT1 is regulated in this process nor was it determined if PC12 differentiation involves changes in DNA methylation.

(v) Protein-protein interactions

This section discusses several recent studies demonstrating that the activity of the DNMTs can be modulated through their interactions with other DNMT family members, as well as with proteins that are not part of the methylation machinery (Hata et al., 2002; Kim et al., 2002; Pradhan and Kim, 2002). DNMTs also interact with several other factors to either coordinate DNA replication and methylation, regulate gene expression, or possibly target site specific methylation; these topics will be covered in following sections of the introduction.

Previously, the involvement of Rb in the posttranscriptional regulation of DNMT1 was discussed. In addition, another mode of DNMT1 regulation by Rb has recently been elucidated (Pradhan and Kim, 2002). Rb is able to physically associate with DNMT1 and can inhibit its methyltransferase activity by disrupting the formation of the DNA-DNMT1 complex. This interaction was found to involve part of the N-terminal region of DNMT1 and the B and C pocket regions of Rb. Moreover, overexpression of Rb led to

genomic hypomethylation as well as hypomethylation and activation of a transfected reporter plasmid (Pradhan and Kim, 2002). Since Rb is inactivated in several cancers (Ohtani-Fujita et al., 1997; Weinberg, 1991), this finding may partly explain the phenomenon of region specific hypermethylation in cancer.

Several studies have demonstrated that DNMT1, DNMT3a, and DNMT3b functionally cooperate to generate methylation patterns. Both DNMT1 together with DNMT 3a or DNMT3b are required for the methylation of a certain class of sequences including LINE-1 elements (Liang et al., 2002). Disruption of both DNMT1 and DNMT3b in a colorectal cancer cell line resulted in a > 95% reduction of genomic DNA methylation, concomitant with the re-expression of the *p16* and growth suppression (Rhee et al., 2002). In addition, functional cooperation has also been observed between DNMT1 and DNMT3a (Fatemi et al., 2002), and between DNMT 3L and DNMT3a (Chedin et al., 2002).

Two recent studies may provide an explanation as to how this cooperation is established. Kim *et al.* demonstrated that DNMT1, DNMT3a, and DNMT3b physically interact with each other (Kim et al., 2002). Through a combination of immunoprecipitation and GST pull down assays, it was shown that DNMT1 is able to bind DNMT3a, DNMT3b, or both at the same time. Furthermore, DNMT3a and DNMT3b can also interact with each other in the absence of DNMT1. These interactions all occur within the N-terminal domains of the proteins. Functional cooperation was also demonstrated, since combinations of the three enzymes showed increased methylation rates over the individual proteins. An

especially notable increase was observed when DNMT1 was added to DNMT3a + DNMT3b. These data point to a model that can perhaps explain how the methylation pattern is established during development. In pre-implantation embryos, when DNMT1 is restricted to the cytosol (Carlson et al., 1992), DNMT3a and DNMT3b are active and can interact to establish the initial wave of *de novo* methylation. At later stages, these enzymes are joined by DNMT1, which then leads to methylation spreading followed by maintenance of the methylation pattern (Kim et al., 2002).

Recent data indicate that protein interactions are not limited to the active DNMT enzymes. Hata *et al.* demonstrated that DNMT3a and DNMT3b also physically interact with DNMT3L, and that DNMT3L is required for the establishment of maternal methylation imprints and the proper expression of the corresponding genes (Hata et al., 2002). Since DNMT3L does not possess methyltransferase activity, it could not be directly responsible. Moreover, *dnmt3a*$^{-/-}$ *dnmt3b*$^{+/-}$ knockout mice are also defective in forming proper maternal imprints. Thus it is possible that DNMT3L might be responsible for targeting the *de novo* DNMTs to imprinting regions (Hata et al., 2002). Since a study by Chedin *et al.* also illustrated that DNMT3L can stimulate *de novo* methylation by DNMT3a at both imprinted and non-imprinted sequences, it is possible that DNMT3L may also act as a general activator of DNMT3a (Chedin et al., 2002).

VI. Formation and maintenance of DNA methylation patterns

(i) Establishment of DNA methylation during development

During mammalian development, at least two stages in which genome wide reprogramming of methylation pattern are known (Reik et al., 2001). The first takes place in the primordial gems cells of both the male and female, where rapid demethylation of both imprinted and single copy genes occurs, followed by *de novo* methylation several days later. Only hours after fertilization, before the onset of the first cleavage divisions, the entire paternal genome is rapidly demethylated by an active process (Oswald et al., 2000). In contrast, the maternal genome only becomes demethylated during the first cleavage divisions by a passive mechanism, likely caused by the exclusion of the oocyte specific DNMT1o from the nucleus (Doherty et al., 2002). Following implantation, *de novo* methylation is believed to occur, which is consistent with the high levels of DNMT3a and DNMT3b observed at this stage (Okano et al., 1998a). A summary of the different methylation processes is depicted in **Figure 5**.

Targeted inactivation of the various components of the DNA methylation machinery have determined their importance in the establishment and maintenance of DNA methylation patterns. While DNMT1 knockouts display an embryonic lethal phenotype, homozygous ES cells are viable despite a 70% reduction in total 5mC (Li et al., 1992). Knockouts of DNMT3b also die very early in embryonic development, and centromeric minor satellite DNA repeats of their ES cells are substantially demethylated (Okano et al., 1999), similar as to the previously mentioned condition ICF syndrome. While both DNMT3a and DNMT3b knockout ES cells show normal methylating activity on proviral DNA, the DNMT3a and 3b double knockouts completely lack such *de novo* methylating activity,

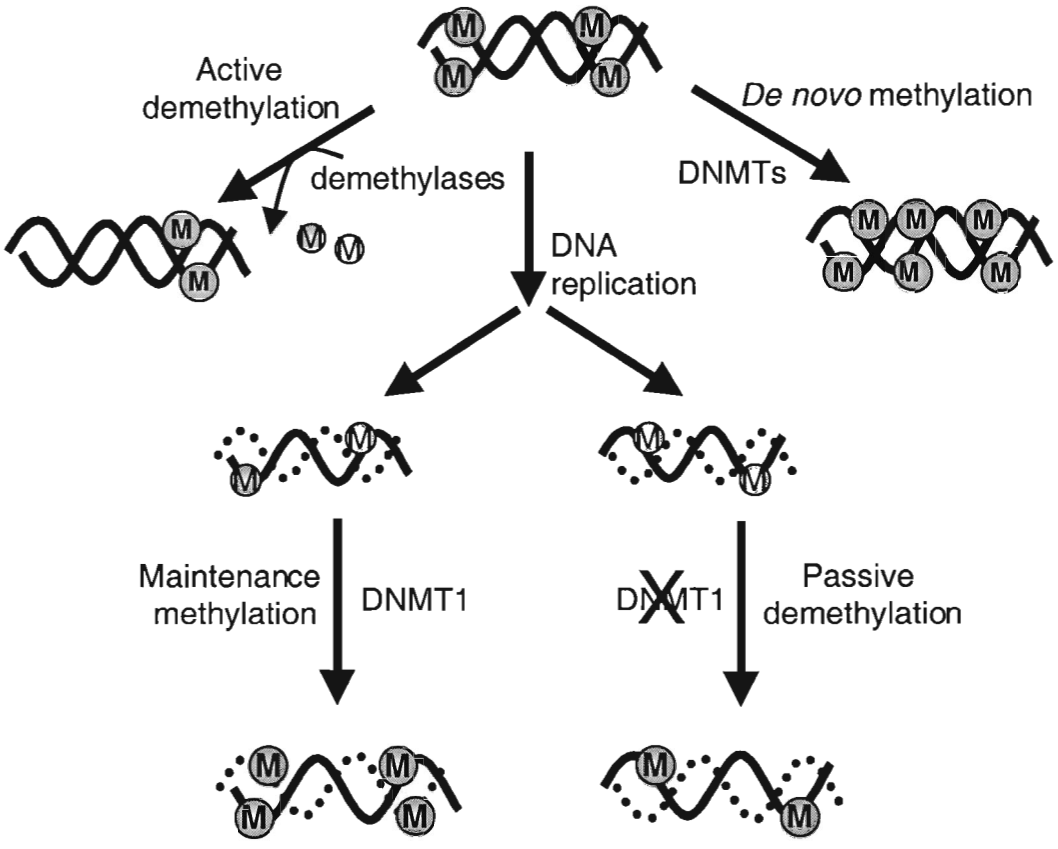

Figure 5 -Reactions involved in reprogramming the DNA methylation pattern.

De novo methylation is the process by which methyl groups are added to CG sites on the DNA that were previously unmethylated, thereby generating a new pattern. Active demethylation occurs when methyl groups are removed independently of DNA replication, either by repair mechanisms or by the direct removal of the methyl group by DNA demethylases. Maintenance methylation is the process whereby DNMT1 copies the DNA methylation pattern of the parental strand onto the nascent strand during cell division. However, if DNMT1 activity is inhibited or if proteins mask critical CG sites, then passive demethylation occurs upon DNA replication because the newly synthesized strand cannot be methylated.

suggesting some redundancy between the two (Okano et al., 1999). Mice with targeted disruption of MBD2/dMTase are viable and display no changes in global methylation levels but are defective in maternal care behavior. As previously mentioned, it is possible that MBD2's function is specific to certain genes, or alternatively, it might be redundant in development where other unknown demethylases may be involved.

One question that still remains elusive is how *de novo* methylation and demethylation events are able to generate the proper DNA methylation pattern? What determines the methylation of certain regions of the genome and not others? Although it is possible that DNMTs and demethylases could possess an intrinsic specificity for certain target regions, no such evidence in support of this has been demonstrated. A more substantiated explanation is that the following mechanisms are involved: First, DNMTs and demethylases may be directed to specific regions through their interactions with other proteins (see sections IV and VII). Second, there is evidence to suggest that the binding of other proteins to specific regions of the genome may protect them from *de novo* methylation or demethylation (Brandeis et al., 1994; Han et al., 2001; Lin and Hsieh, 2001; Macleod et al., 1994). Last, local changes in chromatin structure may play a role in determining the accessibility of DNMTs and demethylases. The latter mechanism will be covered in a subsequent section.

(ii) Maintenance of DNA methylation in somatic cells

A simple mechanism to explain the inheritance of the DNA methylation pattern in somatic cells during cell division, the 'semiconservative DNA methylation model' was proposed by Razin and Riggs almost two decades ago (Razin and Riggs, 1980). According to this model, the maintenance DNMT, now believed to be DNMT1, is responsible for copying the DNA methylation pattern of the parental strand onto the nascent strand during cell division since it has a preference for hemimethylated DNA (Gruenbaum et al., 1982). This faithful replication and accurate maintenance of the DNA methylation pattern is obviously critical for the integrity of genome function. One mode of coordinating DNA replication and methylation is by regulating expression of DNMT1 mRNA and protein levels with the initiation of S-phase, as discussed in section V. Since DNMT1 levels are induced upon entrance to S, DNA replication does not proceed in the absence of DNMT1, ensuring that the replicating DNA is properly methylated.

The positioning of DNMT1 in the replication fork is another important means of ensuring the correct propagation of methylation patterns. DNMT1 is targeted to the replication fork by a specific domain in the protein (Leonhardt et al., 1992), which binds the proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997). A recent study determined the functional significance of this interaction. Iida *et al.* demonstrated that DNMT1 has a higher affinity for DNA when it is first bound by PCNA. In addition, PCNA-bound DNA is also methylated more efficiently that the corresponding free DNA (Iida et al., 2002). It is of interest to note that DNMT1 binds PCNA at the same position as p21, a tumor suppressor that inhibits DNA replication by forming a complex with PCNA (Chuang et al., 1997). Since DNMT1 cannot bind PCNA in the presence of p21,

whose levels are highest when cells are in Go/G1, inappropriate DNA methylation is prevented during this phase of the cell cycle. In addition, the region of DNMT1 that binds PCNA is the same one that binds Rb (see section V), which could provide another mechanism by which the coordination of replication and methylation is achieved: In Go and G1, Rb binds DNMT1 and precludes PCNA binding, thus preventing methylation when cells are not dividing. The findings that DNMT1 has a preference for PCNA-bound DNA, and that this DNA is a better substrate for methylation, explains another mechanism of ensuring that DNA is not methylated in the absence of replication. In accordance with the above, it has been shown that nascent DNA is immediately fully methylated following its synthesis (Araujo et al., 1998).

The close interrelationship between DNMT1, DNA replication, and growth regulatory circuits raises the possibility that DNMT1 is critical for replication, at least under some conditions. In accordance with this hypothesis, the expression of *DNMT1* antisense mRNA, or treatment of A549 human lung cancer cell line with either antisense oligonucleotides or modified hairpin inhibitors, results in the inhibition of DNA synthesis and halts the progression of the cells through the cell cycle (Knox et al., 2000; Milutinovic et al., 2003). A recent study determined that antisense knockdown of DNMT1 triggers an intra-S-phase arrest of DNA, which could possibly serve to protect the genome from the extensive demethylation that could come about by replication in the absence of DNMT1 (Milutinovic et al., 2003).

The semiconservative DNA methylation model suggests that once a DNA methylation pattern is generated during development, it is fixed and accurately inherited (as determined by the state of methylation of the parental strand) through multiple cell divisions. It is important to note that results from several studies provide challenges to this model. Most importantly are the facts that 1) exogenous sequences can become *de novo* methylated in somatic cells (Szyf et al., 1989), 2) DNMT1 overexpression can lead to hypermethylation in somatic cells (Vertino et al., 1996), and 3) clonal populations of cells exhibit the presence of differentially methylated sites (Park and Chapman, 1994; Silva et al., 1993). If DNA methylation is exclusively determined by the methylation pattern of the parental strand, all the progeny of a single cell should have identical patterns of methylation. Thus, the question of whether the DNA methylation pattern is dynamic in somatic mature tissues still remains an unresolved issue. Since DNA methylation patterns can change under pathological stress such as tumorigenesis (Ehrlich, 2002) and aging (Ahuja et al., 1998; Issa, 1999), the potential for change might be present in normal cells as well.

VII. Role of DNA methylation in gene expression

As mentioned in previous sections, there is a tight correlation between DNA methylation, inactive chromatin structure, and silencing of gene expression. This observation led to the hypothesis proposed almost two decades ago that DNA methylation silences gene expression (Razin and Riggs, 1980). A long line of data has established that *in vitro* methylation can suppress genes when ectopically introduced into vertebrate cells (Cedar

et al., 1983; Stein et al., 1982; Vardimon et al., 1982). Numerous *in vivo* studies also support this hypothesis: Both the nucleoside analog 5-azacytidine (5-azaC), a potent inhibitor of DNA methylation (Christman, 2002; Jones, 1985), as well as antisense knockdown of *DNMT1* (Ramchandani et al., 1997; Szyf et al., 1992), have been shown to induce the demethylation and expression of methylated and silenced genes. Furthermore, primary fibroblasts derived from conditional DNMT1 knockouts, which exhibited genomic DNA demethylation, also displayed an induction in 10% of genes as determined by a gene expression profile (Jackson-Grusby et al., 2001). Curiously, while these experiments support the hypothesis that DNA methylation silences gene expression, there are several studies indicating that methylation follows, rather than precedes, gene silencing (Bachman et al., 2003; Lock et al., 1987; Szyf et al., 1990). The following sections will discuss the two currently proposed mechanisms by which methylation is believed to cause transcriptional repression as well as examine the perplexing relationship between methylation, chromatin, and gene expression.

(i) DNA methylation and direct repression

The first mechanism suggests that methylation inhibits gene expression by interfering with the binding of transcription factors to recognition sites that include a CG dinucleotide. A number of documented examples of this type of inhibition include c-Myc (Prendergast and Ziff, 1991), AP-2 (Comb and Goodman, 1990), cAMP response element CRE (Moens et al., 1993), and the interaction of CTCF with the parentally imprinted *Igf2* insulator (Kanduri et al., 2000). This mechanism provides a simple explanation for the inhibition of gene expression by methylation. However, a number of

studies have established that many transcription factors either do not contain a CG in their recognition element, or are not inhibited by methylation even when they do contain a CG sequence. A classical example of a transcription factor that does not discriminate between methylated and unmethylated CGs in its consensus sequence is the ubiquitous Sp1, which controls the expression of many genes that bear CG rich islands within their promoter region (Harrington et al., 1988; Holler et al., 1988). It is clear that an additional mechanism must exist to explain the suppression of CG island promoters by methylation.

(iii) Methyl-CpG-binding proteins and indirect repression of methylated genes

A second mechanism that has attracted significant interest is an indirect mechanism, whereby methylated CG dinucleotides are recognized by a conserved family of methyl-CpG-binding proteins (MBD) such as MeCP2 (Lewis et al., 1992; Meehan et al., 1992), MBD1 (Cross et al., 1997), MBD2, and MBD3 (Hendrich and Bird, 1998) **(Figure 6).** The MBDs share a highly conserved domain for binding methylated DNA, and associate with large protein complexes containing corepressors and HDACs, and thus function to target a 'closed' and inactive chromatin structure to methylated genes (Boeke et al., 2000; Jones et al., 1998; Nan et al., 1998; Ng et al., 1999). A number of studies using either a co-immunoprecipitation approach or chromatographic fractionation, show that different MBDs are found in different multiprotein repressor complexes such as the chromatin remodeling Sin3A (Boeke et al., 2000; Nan et al., 1998) and NuRD complexes (Zhang et al., 1999). Some of the repression mediated by MBDs is relieved by treating cells with the HDAC inhibitor TSA, which demonstrates the critical role of histone deacetylation in

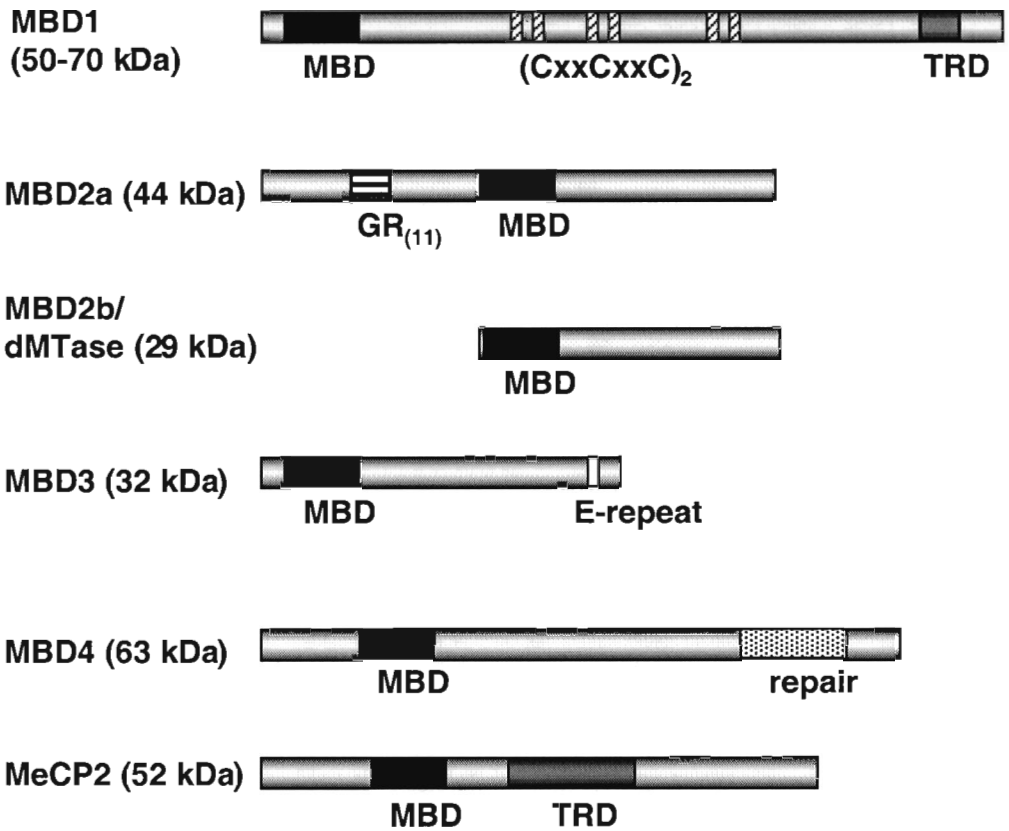

Figure 6 -Schematic structure of the mammalian MBD family members.

MBD1, MBD2, MBD3, and MeCP2 function as transcriptional repressors; MBD2b, which starts at a downstream initiation codon, is also capable of actively demethylating DNA; MBD4 acts as a 5mCG glycosylase. Specific motifs indicated include: Conserved methyl-CpG-binding domain (MBD), transcriptional repression domain (TRD), cysteine-rich repeats (CxxCxxC) in MBD1, glycine-arginine repeat (GR) in MBD2a, glutamic acid repeat (E-repeat) in MBD3, repair domain in MBD4. Note, the molecular weight of MBD1 varies due to alternative splicing.

mediating gene suppression by DNA methylation (Nan et al., 1998). However, since not all repression by MBDs can be removed by inhibiting histone deacetylation (Yu et al., 2000), there must be additional mechanisms through which MBDs inhibit the expression of methylated genes, although none have been defined as of yet.

The first member of the MBD family to be characterized was MeCP2, a multidomain protein that contains, in addition to a region required to bind methylated DNA, a transcriptional repression domain (TRD) and a nuclear localization signal (Lewis et al., 1992; Meehan et al., 1992). Although MeCP2 only requires a single methylated CG irrespective of sequence context to bind to DNA, it preferentially localizes to densely methylated regions such as satellite DNA (Lewis et al., 1992). MeCP2 was found to associate with the Sin3A histone deacetylase complex (Nan et al., 1998), and can repress transcription from various promoters through both HDAC dependent (Drewell et al., 2002; Yu et al., 2001) and independent manners (Yu et al., 2000). Since mutations in MeCP2 have been found in the majority of cases of RETT syndrome (Amir et al., 1999), one of the leading causes of mental retardation in females, the functional importance of this protein cannot be disputed.

In 1997, Cross *et al.* identified MBD1 (formerly PCM1), and a year later, Hendrich *et al.* discovered MBD2 to MBD4. Both of these studies took advantage of the MBD motif of MeCP2 to search for EST clones with sequence similarity (Cross et al., 1997; Hendrich and Bird, 1998). Out of these four, MBD4 is the only one which is not a transcriptional repressor, and but rather possesses 5mCG glycosylase activity. MBD3 is associated with

the Mi2/NuRD chromatin-remodeling complex, which also contains HDACs and is known to be involved in establishing inactive chromatin structures (Zhang et al., 1999). However, the role of MBD3 in silencing methylated genes is still unclear since MBD3 does not preferentially bind methylated DNA (Saito and Ishikawa, 2002). Because it was found that MBD2 associates with NuRD in certain cases, collectively forming the MeCP1 complex, it was proposed that MBD2 might be partly responsible for targeting NuRD to methylated DNA (Feng and Zhang, 2001; Ng et al., 1999; Zhang et al., 1999).

Different splice forms of MBD1 (Fujita et al., 2000; Fujita et al., 1999) and MBD2 (Hendrich and Bird, 1998) were identified, however, it is not yet clear what is the differential role of the various MBDs as well as their different splice forms in mediating gene suppression. One interesting point is that the interaction between DNA and some MBDs, such as MeCP2 (Lewis et al., 1992) and some splice forms of MBD1 (Fujita et al., 2000), is independent of the density of the methylated CGs, whereas other MBDs such as other splice forms of MBD1 (Fujita et al., 2000) and MeCP1 (possibly containing MBD2 as the methylated DNA recognition component (Ng et al., 1999)) require a high density of methylated CGs for binding (Meehan et al., 1989). It was also recently determined that MBD2a promoted the activation of unmethylated cAMP-responsive genes (Fujita et al., 2003). Thus, it is possible that the different MBDs are responsible for regulating different classes of both methylated and unmethylated genes.

One other perplexing issue, mentioned earlier in section IV, is the designation of MBD2b (the shorter form lacking the N-terminal region) as both a transcriptional repressor and as

a DNA demethylase and activator **(Figure 6)**. One possible explanation for the dual role of MBD2/dMTase is that its function depends on the interaction with other proteins. This is supported by the demonstration that different MeCP1 complexes, some of which do not contain MBD2, are formed in diverse cell types (Hendrich et al., 2001). Furthermore, a recent report identified a novel protein, MBDin, which relieves the repression by MBD2, although no demethylation was observed in this study (Lembo et al., 2003). This topic will be further examined in chapter 2, where data illustrating the effects of several parameters on MBD2 function will be presented.

(iii) DNA methylation, chromatin structure, and gene expression

The abovementioned data provide strong support for the hypothesis that DNA methylation is causal in mediating gene repression by first precipitating an inactive chromatin structure **(Figure 7)**. Experiments performed with pharmacological inhibitors of both DNA methylation and histone deacetylation have also led to the conclusion that DNA methylation is primary. While it was shown that inhibitors of DNA methylation (5-azaC, 5-aza-CdR) could activate a number of methylated tumor suppressor genes, TSA treatment was insufficient to induce these genes (Cameron et al., 1999; Ghoshal et al., 2002). However, it is important to note that TSA would only directly affect histone acetylation and not the other modifications that affect chromatin structure. It was recently demonstrated that MeCP2 can recruit a histone MTase to methylated genes (Fuks et al., 2003); therefore the silencing of these genes would likely involve both histone H3-K9 methylation and deacetylation. In accordance, a detailed mapping of the

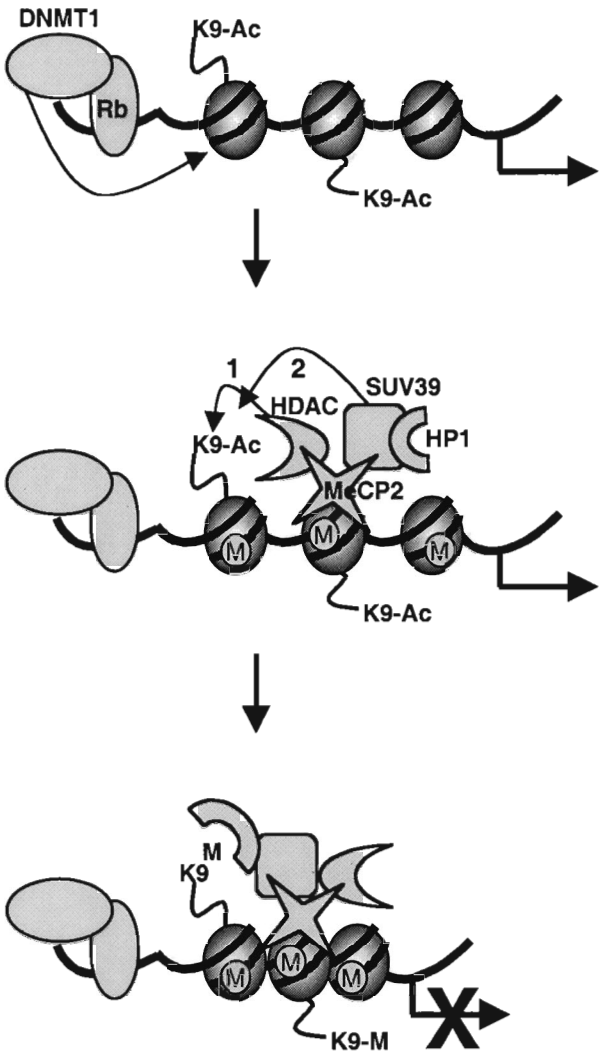

Figure 7 - DNA methylation determines chromatin structure - a model.

Nucleosomes consist of DNA (black line) wrapped around histone octamers (grey ball); bent arrow indicates transcription and 'X' marks repression. Transcriptional repressors, such as Rb, recruit DNMTs to methylate the DNA. MBDs (e.g. MeCP2) can then bind and recruit HDACs and histone H3-K9 MTases (e.g. SUV39) to deacetylate (1) and methylate (2) the histones. The protein HP1 can further bind the methylated histones. The resulting effect is the formation of a condensed chromatin structure leading to gene inactivation.

chromatin modifications associated with the methylated tumor suppressor p16 demonstrated that this gene was associated with MeCP2, methylated H3-K9, deacetylated histones, and was silenced (Nguyen et al., 2002). As expected, treatment with 5-aza-CdR led to a reduction in both MeCP2 binding and H3-K9 methylation, and also promoted an increase in acetylation (Nguyen et al., 2002). Since TSA does not affect histone methylation (Kondo et al., 2003), the activation of these methylated genes, in the absence of 5-aza-CdR, would require inhibitors of both HDACs and histone MTases. Unfortunately, no inhibitors specific for histone MTases have yet been found. In addition, the interaction of other proteins, such as INHATs, with certain promoters might prevent histone acetylation even in the presence of an HDAC inhibitor (Cervoni et al., 2002; Seo et al., 2001). Since chromatin structure might not be altered sufficiently by TSA to enable a change in gene expression, it is premature to draw the conclusion that DNA methylation, and not chromatin structure, is the dominant factor in gene silencing.

Additional clues suggesting that DNA methylation may not be primary stem from several studies demonstrating that chromatin modifications and gene silencing can lead to changes in DNA methylation. Experiments delineating the temporal relationship of DNA methylation and the silencing of the *hprt* gene on the inactive X chromosome demonstrated that methylation follows rather than precedes gene silencing (Lock et al., 1987). Similarly, exogenously introduced *steroid 21-hydroxylase* is silenced in the adrenocortical cell line Y1 a few weeks prior to selective *de novo* methylation (Szyf et al., 1990). Moreover, the HDAC inhibitor sodium butyrate was found to trigger a genome wide loss of methylation in EBV producing cells (Szyf et al., 1985a), while a

study by Selker has shown that treating *Neurospora crassa* with TSA results in selective DNA demethylation (Selker, 1998).

Over the last several years, a number of genetic mutants with defects in DNA methylation have been found to harbor mutations in various genes involved in regulating chromatin structure. For instance, in the plant *Arabidopsis*, a gene required for maintenance of DNA methylation, *DDM1*, is similar to the SWI/SNF family of ATP-dependent chromatin remodeling genes (Jeddeloh et al., 1999). Similar situations were also found in the mouse and human, where genes belonging to chromatin remodeling families were required for DNA methylation (Dennis et al., 2001; Gibbons et al., 2000). In addition, DIM-5 is a histone MTase that is required for DNA methylation in *Neurospora crassa* (Tamaru and Selker, 2001), while CNG methylation by the *Arabidopsis* Chromomethylase 3 requires *Kryptonite*, a gene encoding a histone MTase specific to H3-K9 (Jackson et al., 2002). Along similar lines, a mouse ES cell knockout of the histone MTase G9a was found to impair both H3-K9 methylation and DNA methylation at the Prader-Willi syndrome imprinting center (PWS-IC), resulting in loss of imprinting. Remarkably, although *dnmt1-/-* ES cells also lacked methylation at PWS-IC, both H3-K9 methylation and imprinting were normal, suggesting that, at least in this case, methylation is not the primary factor determining expression (Xin et al., 2003). Taken together, these studies provide a strong indication that chromatin structure is a critical factor in determining gene expression and methylation patterns.

One obvious question is: what is the mechanism involved by which chromatin can affect methylation? Several studies, most fairly recently, provide us with some possibilities. As previously mentioned, both DNMT1 and DNMT3a were shown to physically interact with HDAC1 and HDAC2 (Fuks et al., 2000; Fuks et al., 2001; Rountree et al., 2000). In addition, the leukemia-promoting PML-RAR fusion protein was found to induce gene hypermethylation and silencing by recruiting an HDAC complex as well as DNMT1 and DNMT3a to retinoic acid target promoters (Di Croce et al., 2002; Grignani et al., 1998). The general belief stemming from these results is that DNMTs recruit HDACs to bring about an inactive chromatin and repression. However one cannot ignore the possibility that the opposite mechanism might be true as well, in which HDACs recruit DNMTs to methylate genes following their silencing by histone deacetylation **(Figure 8a)**. This model could explain both the abovementioned observations that methylation sometimes follows gene silencing and that HDAC inhibitors such as butyrate and TSA can cause demethylation in certain instances. In the case of PML-RAR, treatment with retinoic acid results in dissociation of the HDAC-PML-RAR complex (Grignani et al., 1998), as well as activation and demethylation of the promoter (Di Croce et al., 2002), supporting the hypothesis that both DNA methylation and chromatin structure are reversible and dynamic

Along a similar line, there are several examples where proteins involved in histone methylation might be responsible for targeting DNMTs to certain genes. This could occur either directly, as in the case of *Arabidopsis* Chromomethylase 3, which interacts with a homologue of the H3-K9 binding protein HP1 (Jackson et al., 2002), or through

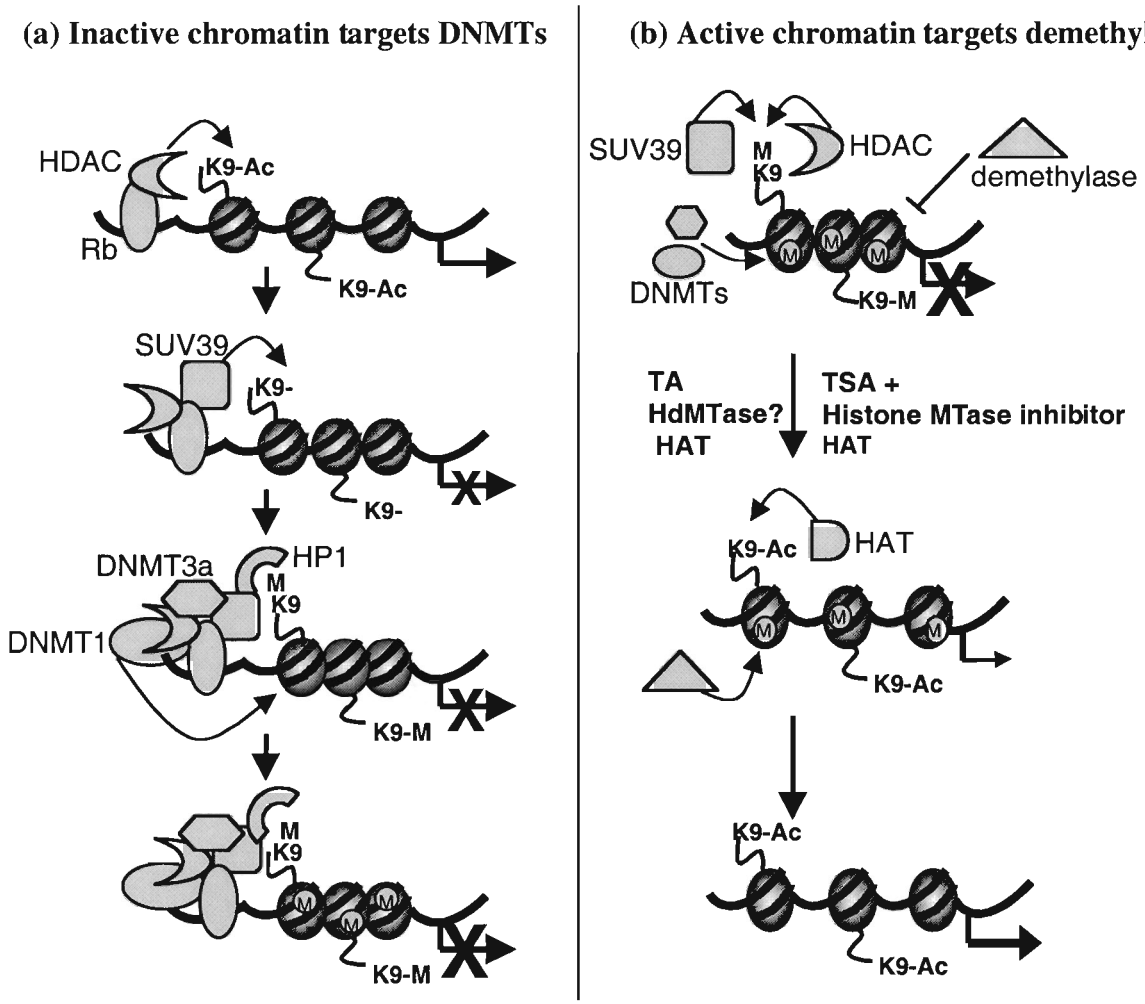

Figure 8 - Chromatin structure determines DNA methylation - a model.

Nucleosomes consist of DNA (black line) wrapped around histone octamers (grey ball); size of bent arrow indicates degree of transcriptional activation and size of 'X' reflects degree of repression. (a) Transcriptional repressors, such as Rb, recruit HDACs to DNA, which deacetylate the histones leading to a tighter chromatin structure and gene repression. The deacetylated tails are then free undergo methylation on H3-K9 by histone MTases (e.g. SUV39), which further stabilizes the inactive state by recruiting HP1, a protein involved in supra-nucleosomal chromatin structure. DNMTs can then be recruited by either HDACs, transcriptional repressors such as Rb, or by histone MTases and HP1. The resulting methylated DNA then serves to guard the inactive state. (b) A silenced gene is maintained by the actions of HDACs, SUV39, and DNMTs, and is inaccessible to DNA demethylase. Activation of such a gene requires either the recruitment of a strong transcriptional activator (TA), which brings putative histone demethylases (HdMTases) and then HATs to the gene, or pharmacological inhibitors of HDACs (e.g.TSA) and of histone MTases. The result of either combination is the acetylation of histone tails at K9 and transient activation of the gene. Once the histone tails are acetylated, the chromatin is more open and therefore accessible to DNA demethylase, which demethylates the DNA resulting in stable activation of the gene.

intermediary proteins such as Rb and MeCP2, which bind both DNMTs and histone MTases (Fuks et al., 2003; Kimura and Shiota, 2002; Nielsen et al., 2001; Robertson et al., 2000a). Although no studies have demonstrated a direct interaction between a histone MTase and a DNMT, it was recently discovered that Dnmt3a co-purified with a H3-K9 histone MTase activity (Datta et al., 2003).

It was recently demonstrated that the interaction of HP1β preferentially occurs within the H3-K9 enriched maternal genome immediately after fertilization in the mouse zygote. In contrast, the paternal genome has neither HP1β binding nor methylated H3-K9 at these early stages (Arney et al., 2002). Since the paternal genome is globally demethylated shortly after fertilization by an active mechanism (Oswald et al., 2000), this suggests that histone methylation might also modulate DNA methylation by protecting genes from active demethylation.

The possibility that chromatin structure can influence DNA demethylation is also supported by several studies using an assay that specifically measures active demethylation (Cervoni et al., 2002; Cervoni and Szyf, 2001). It was demonstrated that following transient transfection into HEK 293 cells, an active gene was demethylated whereas an inactive gene remained methylated. This demethylation was found to be dependent on the state of histone acetylation associated with the DNA *per se*, and not on the presence of other cis-acting sequences in active promoters, because upon TSA treatment even a promoterless vector was demethylated. Using a ChIP assay it was then shown that the physical association of DNA with acetylated histones was required for

demethylation (Cervoni and Szyf, 2001). These studies illustrate that the unmethylated state of active genes is maintained by a demethylase activity; which probably has a greater accessibility to DNA due to the open chromatin configuration brought about by histone acetylation **(Figure 8b)**. However, because only selective demethylation was observed with TSA (Selker, 1998), and since in many instances TSA treatment alone is insufficient to induce demethylation and activation of many genes (Cameron et al., 1999), then other factors must exist that determine the accessibility of transcription factors and demethylase to DNA. One of these factors might be INHATs, which were shown to block histone acetylation (Seo et al., 2001) and active demethylation (Cervoni et al., 2002) even when HDACs are inhibited with TSA. As previously mentioned, histone methylation might be another factor regulating active demethylation, although it is not yet known whether histone methylation directly or indirectly affects DNA demethylase accessibility.

Taken together, the above data illustrate how proteins that modulate chromatin structure can also determine the state of DNA methylation, most probably by recruiting DNMTs or gating access to demethylases. The resulting DNA methylation might serve to reinforce or stabilize the repressed state originally determined by signals acting on chromatin structure. Since both histone acetylation and histone methylation have been shown to be dynamic and reversible (Cervoni et al., 2002; Kondo et al., 2003; Kouzarides, 2002; Kuo and Allis, 1998; Nguyen et al., 2002; Yoshida et al., 1990), this raises the possibility that the same is true for the DNA methylation pattern. This is in contrast to the semiconservative model of maintenance methylation, and has important consequences in

somatic non-dividing cells. Further importance of such a 'reversible epigenome' will be discussed in chapter 4, in which results are presented demonstrating that a commonly prescribed drug can induce active DNA demethylation.

VIII. Aberrant DNA methylation in cancer

(i) Regional hypermethylation versus global hypomethylation

It has been a longstanding observation that aberrations in DNA methylation patterns are a hallmark of many tumor cells (Baylin et al., 1998; Ehrlich, 2002b), leading to the abnormal regulation of a wide range of genes (Jones, 1996). However the nature of these changes has been confusing since both global hypomethylation (Bedford and van Helden, 1987; Cravo et al., 1996; Feinberg et al., 1988; Jurgens et al., 1996; Lu et al., 1983; Soares et al., 1999) and regional hypermethylation (Baylin, 1992; Baylin et al., 1986; Belinsky et al., 1998; Esteller et al., 1999; Esteller et al., 2000; Herman et al., 1994) of tumor suppressors and other specific genes have been observed. Initial studies investigating the relationship between DNA methylation and cancer focused on the hypomethylation of protooncogenes as a mechanism for their activation (Feinberg and Vogelstein, 1983a; Feinberg and Vogelstein, 1983b). However, later studies discovered that cancer related hypomethylation is not restricted to oncogenes, but occurs globally throughout the genome (Ehrlich, 2002b).

Over the last two decades, a multitude of studies involving various types of cancer have

led to the conclusion that DNA derived from tumors has a lower level of methylation that the corresponding normal tissue DNA (Ehrlich, 2002b). Many of these experiments identified the types of sequences that are most frequently associated with cancer hypomethylation. For example, hypomethylation of repeat sequences, such as satellite DNA, LINE-1 elements, and endogenous retroviruses is often detected in various cancers and may contribute to genomic instability (Ehrlich, 2002a). In addition, single copy genes have also been found to be hypomethylated in tumors, including genes which could directly contribute to cancer progression such as oncogenes (Feinberg and Vogelstein, 1983b; Nambu et al., 1987) and genes involved in tumor invasion, motility, and metastasis (Guo et al., 2002). In some cases, the degree of hypomethylation has been found to correlate with tumor progression (De Capoa et al., 2003; Kim et al., 1994; Sharrard et al., 1992; Soares et al., 1999), suggesting a causal role.

Paradoxically, in the midst of cancer associated global hypomethylation, the presence of regional hypermethylation is also observed. The results from an extensive amount of research indicate that hypermethylation most often occurs within dense CG island regions (Baylin, 1992) and varies depending on specific tumor types (Adorjan et al., 2002). Since promoter CG islands are a common characteristic of many tumor suppressor genes, it is not surprising that many tumor suppressors such as *p16*, *p15*, *BRCA1*, *Rb*, and many others have been found to be hypermethylated and consequently silenced in cancer (Esteller, 2002). This would obviously be a favorable scenario for tumor progression. Recently, studies using novel genomic approaches have led to the establishment of CG methylation profiles for various tumor classes (Adorjan et al., 2002; Shi et al., 2002;

Smiraglia and Plass, 2002), which could ultimately lead to important diagnostic applications (Adorjan et al., 2002).

One obvious question that is of great interest is how global hypomethylation and regional hypermethylation can co-exist in the same cell? Since global hypomethylation tends to occur in sparsely distributed CG sequences while hypermethylation targets CG islands, this suggests that different factors are responsible for each. Furthermore, recent experiments have shown that CG island hypermethylation and global hypomethylation are independent processes, supporting the hypothesis that multiple factors determine DNA methylation patterns in cancer cells (Ehrlich et al., 2002).

(ii) DNMT1 as an anticancer target

A separate line of evidence linking DNA methylation and cancer is the interrelationship between the overexpression of DNMT1 and cellular transformation. First, high levels of *DNMT1* mRNA and DNMT1 activity were reported in tumor samples and in cancer cells (Belinsky et al., 1996; Issa et al., 1993; Kautiainen and Jones, 1986), and *DNMT1* expression is regulated by nodal protooncogenic signaling pathways as discussed earlier. Second, ectopic expression of *Dnmt1* results in cellular transformation (Bakin and Curran, 1999; Slack et al., 1999; Wu et al., 1993). Third, knockdown of *Dnmt1* leads to demethylation of tumor suppressor genes and reverses tumorigenesis both *in vitro* and *in vivo* (Fournel et al., 1999; Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997). Although there is evidence suggesting that DNMT3a and DNMT3b also play

a role in cancer (Beaulieu et al., 2002; Choi et al., 2003; Mizuno et al., 2001; Robertson et al., 1999), this review will focus mainly on DNMT1. The obvious question is whether there is link between the induction of *DNMT1* expression by oncogenic pathways, the requirement for high levels of DNMT1 in cellular transformation, and the hypermethylation of tumor suppressor genes.

The generally accepted paradigm is that *DNMT1* is overexpressed in tumors, leading to the methylation of tumor suppressors and consequently tumor growth. Inhibition of DNMT1 results in demethylation of tumor suppressor genes, their activation, and suppression of tumor growth. According to this model, the therapeutic goal is to inhibit DNA methylation. Several widely used agents that inhibit the catalytic activity of the DNMTs are nucleoside analogs such as 5-azacytidine (5-azaC), 5-aza-2'-deoxycytidine (5-aza-CdR), and the non-aza nucleoside zebularine (Cheng et al., 2003). In the cell, these nucleosides are converted to trinucleotides followed by incorporation into DNA during DNA replication. Once incorporated into DNA, they bind to DNMTs and form a covalent bond with the active site cysteine residue. However, methyl transfer is impossible due to the presence of the aza group at the 5' position, trapping the enzyme onto the DNA and resulting in its depletion from the replication fork. Subsequently, as replication proceeds in the absence of DNMT, the nascent DNA is not methylated and results in passive demethylation. 5-azaC and 5-aza-CdR were shown to cause global demethylation (Jones and Taylor, 1980) and reactivation of numerous tumor suppressors such as *p15* (Daskalakis et al., 2002), *BRCA1* (Magdinier et al., 2000), *E-cadherin* (Yoshiura et al., 1995), and *p16* (Otterson et al., 1995). Notably, although 5-azaC and 5-

aza-CdR are both being tested in international clinical trials, especially for acute myeloid leukemia and myelodysplastic syndrome, their use is limited by the fact that they are chemically unstable, toxic, and cannot be given orally (Cheng et al., 2003).

An important point to note is that the previously discussed effects of chromatin structure on DNA methylation are inconsistent with the above model. Since inactive chromatin and gene silencing often precede DNA methylation, it is possible that the methylation of tumor suppressors is not the primary reason for their inactivation. For example, whereas ectopic expression of *DNMT1* leads to methylation of tumor suppressor genes (Vertino et al., 1996), this process is often slow and cannot explain the relatively rapid transformation observed (Bakin and Curran, 1999; Slack et al., 1999). Moreover, a recent study demonstrated that the silencing of *p16* was associated with histone H3-K9 methylation but was independent of DNA methylation, which only occurred many cell passages later (Bachman et al., 2003). The potential role of chromatin in the silencing of tumor suppressors is evident from the dramatic antitumor effects of HDAC inhibitors (Weidle and Grossmann, 2000). Methylation, on the other hand, might be responsible for stabilizing the silent chromatin structure of certain tumor suppressors. This concept is consistent with data showing that TSA cannot induce the expression of methylated genes on its own, but these genes are induced with 5-azaC, and in many instances this induction is synergized with TSA (Cameron et al., 1999). Therefore, such inhibitors of DNMT catalytic activity might be required for activation of certain methylated tumor suppressor genes. On the other hand, if it is possible to alter other histone modifications, such as H3-K9 methylation, around such a tumor suppressor gene, it might sufficiently activate

the chromatin structure to allow access to active demethylases. In this case, catalytic inhibition of DNMTs would not be necessary. This might have important consequences considering the fact that catalytic inhibitors of DNMTs bring about global hypomethylation, which may also contribute to cancer progression as mentioned above. In support of this hypothesis, 5-azaC/5-aza-CdR has been shown by several studies to promote metastasis (Takenaga, 1986), probably by inducing genes that promote tumor invasion (Guo et al., 2002; Moisan et al., 2003; Simizu et al., 2003; Tulchinsky et al., 1995). For instance, in one study 5-azaC was shown to induce the invasive and metastatic potential of BW5147 T-lymphoma cells, similar to the activated human *c-Ha-Ras* oncogene (Habets et al., 1990). And recently, Sato *et al.* demonstrated that treatment with 5-aza-CdR increased the invasive potential of four out of five pancreatic cancer cell lines. This enhanced invasiveness was associated with the induction of mRNAs for one or more matrix metalloproteinases critical for tumor invasion (Sato et al., 2003).

So what are other potential mechanisms by which DNMT1 might promote tumorigenesis? Seeing as DNMT1 is a multifunctional protein that interacts with factors involved in DNA replication (Chuang et al., 1997) and in chromatin modification (Fuks et al., 2000; Kimura and Shiota, 2002; Robertson et al., 2000a; Rountree et al., 2000), it is possible that DNMT1 possesses methylation-independent mechanisms that are critical for its role in tumorigenesis. Accordingly, co-expression of DNMT1 and Rb/E2F led to the suppression of an unmethylated *p14* tumor suppressor gene (Robertson et al., 2000a), while antisense knockdown of DNMT1 promoted the activation of the unmethylated *p21* tumor suppressor (Milutinovic et al., 2000). This suggests that DNMT1 controls the

expression of at least some tumor suppressor genes by a mechanism that does not involve DNA methylation.

Section VI described studies illustrating that knockdown of DNMT1 inhibits DNA replication, which is another potential mechanism by which tumorigenesis might be inhibited. It must be noted that there is an important difference between catalytic inhibitors of DNMT1 (5-azaC, 5-aza-CdR) and DNMT1 knockdown agents (antisense oligonucleotides, hairpin inhibitors). The mechanism of action of 5-azaC involves its incorporation into DNA during replication. Since 5-azaC does not inhibit initiation of replication, it traps the DNMT during movement of the replication fork resulting in passive demethylation of DNA. In contrast, passive demethylation by agents that knock down DNMT1 is limited and delayed since replication is inhibited (Bigey et al., 1999). Since knockdown inhibitors are effective in reversing tumorigenesis, it implies that demethylation of tumor suppressor genes is not necessarily required. However, it is interesting to note that treatment of the bladder carcinoma cell line T24 with DNMT1 antisense triggers re-expression and demethylation of *p16*, but only well after cell arrest (Fournel et al., 1999). This supports the possibility that demethylation of *p16* by knockdown of DNMT1 is achieved by active demethylation, possibly by inducing an active chromatin configuration. Another potential mechanism by which DNMT1 might promote tumorigenesis will be described in chapter 1, in which data is presented suggesting that the inappropriate expression of DNMT1 at the wrong phase of the cell cycle could promote aberrant cell growth and lead to cellular transformation. This mechanism likely involves DNMT1's relationship with proteins involved in cell cycle

regulation, such as PCNA and p21, and will be further discussed in chapter 1. The potential roles of DNMT1 in tumorigenesis are summarized in **Figure 9**.

Taken together, these data have important implications on the therapeutic application of DNMT inhibitors. It would appear that knockdown inhibitors might be the agents of choice since they can achieve an antitumorigenic effect, possibly through the various mechanisms stated above, while avoiding the risk of global hypomethylation and its potential consequences. One such inhibitor, MG98, is currently in phase II clinical trials (Reid et al., 2002).

(iii) Role of DNA demethylase in cancer

Several important questions for which recent studies may provide an answer are: What is the cause of global hypomethylation in cancer, how does it contribute to tumor progression, and how are hypermethylated regions maintained in the presence of such hypomethylation? One possibility is that a high activity of a general demethylase is present in cancer cells. This is supported by several findings: First, MBD2/dMTase is expressed at high levels in breast cancer cells and tumors (Billard et al., 2002; Vilain et al., 1999), human gliomas (Schlegel et al., 2002), and ovarian cancer (Hattori et al., 2001). Second, MBD2 was identified as a colon cancer antigen that reacted exclusively with sera from colon cancer patients and not with sera from normal blood donors (Scanlan et al., 2002). Third, expression of the *Ras* oncogene in P19 cells was found to induce a general demethylating activity (Szyf et al., 1995). And last, both the purification

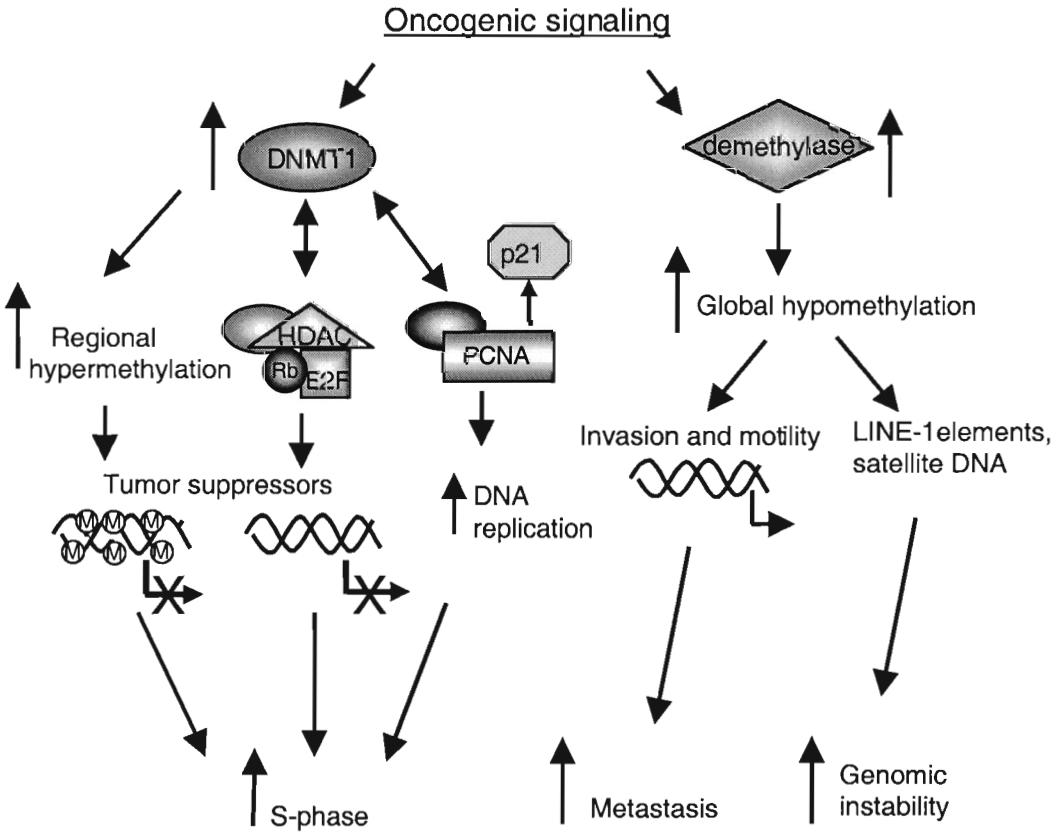

Figure 9 -The roles of DNMT1 and DNA demethylase in tumorigenesis.

Oncogenic signals induce both DNMT1 and DNA demethylase activities. DNMT1 methylates tumor suppressor genes leading to their silencing. DNMT1 also silences tumor suppressors in a methylation independent manner through its interactions with chromatin modifiers (e.g. HDACs) and transcriptional repressors (e.g. Rb/E2F). In addition, the interaction of DNMT1 with the replication factor PCNA during G1 phase disrupts the p21-PCNA growth inhibitory complex, thus stimulating the initiation of DNA replication. The end result is an overriding of growth arrest signals and inappropriate entry into the S phase of the cell cycle. Induction of DNA demethylase results in the demethylation of repetitive elements (LINE-1, satellite DNA), thus promoting genomic instability, as well as demethylation and activation of a class of genes involved in tumor invasion and migration. Tumor suppressors are masked from this effect because of regional chromatin modifications, such as histone deacetylation and H3-K9 methylation. The combined action of demethylase and DNMT1 leads to tumor growth and metastasis.

of a demethylase activity and the cloning of MBD2/dMTase were carried out using cancer cell lines (Bhattacharya et al., 1999; Ramchandani et al., 1999). High levels of demethylases could thus provide a simple explanation as to how global hypomethylation is generated. In addition, the observation that active demethylation is dependent on chromatin structure can explain the presence of regional hypermethylation in cancer even amongst high levels of demethylase activity. If the acetylation of tumor suppressor genes is prevented by INHATs and other histone modification such as H3-K9 methylation, then these genes will also protected from demethylation.

Although it is not yet known which demethylases are responsible for maintaining the hypomethylated state of tumor invasion and metastasis genes, recent studies indicate that MBD2/dMTase is critical for tumorigenesis. Adenovirus mediated antisense knockdown of MBD2/dMTase resulted in a dramatic reduction in the ability of a wide range of human cancer cells to grow in an anchorage independent manner, but had no effect on the capacity of the cells to grow normally, and there was no effect on cell cycle kinetics (Slack et al., 2002). This is consistent with the finding that *Mbd2-/-* knockouts are viable (Hendrich et al., 2001), and indicates that MBD2/dMTase controls genes that are required for tumorigenic growth but not normal growth. Further studies *in vivo* using nude mice harboring lung or colon xenoplast tumors demonstrated that inhibition of MBD2/dMTase by both adenovirus delivered MBD2/dMTase antisense and second-generation antisense oligonucleotides inhibited tumor growth (Slack et al., 2002)(Campbell et al., unpublished data). Importantly, the antisense oligonucleotides had no effect on the cell cycle kinetics of normal skin fibroblasts and no toxic side effects. These data suggest that

MBD2/dMTase might be an ideal anticancer target since its inhibition would not result in arrest of normal growth, which is a problem with many cancer therapies currently in use.

Earlier it was mentioned that global hypomethylation has been found in various repeat sequences and in single copy genes such as oncogenes and genes involved in tumor invasion, motility, and metastasis. Several mechanisms have been proposed to explain how these elements could be involved in the mechanism by which hypomethylation contributes to cancer (**Figure 9**). For example, one hypothesis is that global hypomethylation of repeat sequences promotes genomic instability, and is supported by a number of genetic and pharmacological data (Chen et al., 1998; Ehrlich, 2002a; Ji et al., 1997; Schuffenhauer et al., 1995). Another model is that oncogene hypomethylation contributes to their activation, but although the hypomethylation of genes such as *Ha-Ras* (Feinberg and Vogelstein, 1983b), *c-myc* (Nambu et al., 1987), *EGFR* (Kaneko et al., 1985), and *erb-A1* (Lipsanen et al., 1988) has been observed in various cancers, there is no direct evidence that it plays a role in the expression of these genes. A third hypothesis that is strongly substantiated is that hypomethylation promotes tumor invasion and metastasis. Numerous studies have determined a correlation between the degree of hypomethylation and the tumor metastatic capacity (Bedford and van Helden, 1987; Liteplo and Kerbel, 1987; Shen et al., 1998; Soares et al., 1999). And, as previously mentioned, treatment with the demethylating agent 5-azaC has also been found to promote metastasis in several instances.

More direct evidence comes from a study of the metastasis-associated protein Mts1/S110A4, which encodes a calcium binding protein that is involved in cell motility. This protein is overexpressed in metastatic variants relative to non-metastatic variants of tumors (Grigorian et al., 1993), and transfection of this gene into the nonmetastatic human breast cancer cell line MCF-7 increases invasiveness *in vitro* and *in vivo* (Grigorian et al., 1996). It was then shown that *Mts1* expression in human colon carcinoma correlates with its state of methylation (Nakamura and Takenaga, 1998), and that overexpression of Mts1 in pancreatic ductal carcinoma correlates with hypomethylation and is associated with poor differentiation (Rosty et al., 2002). In addition, the protease Urokinase-Type Plasminogen Activator (uPA), which is required for tumor invasion, is hypermethylated and not expressed in MCF-7, a nonmetastatic cell line, whereas it is hypomethylated and expressed in MDA-MB-231, a highly metastatic line. Upon treatment of MCF-7 cells with 5-azaC, *uPA* is demethylated and expressed and the cells become invasive and metastatic. It is interesting to note that this study observed a higher global demethylase activity in MDA-MB-231 cells than in MCF7 cells (Guo et al., 2002). A possibility is that MBD2/dMTase is involved in controlling genes involved in metastasis; however, at the present time there is no direct evidence to support this hypothesis.

Taken together, the above examples suggest that hypomethylation is a critical factor in mediating tumorigenesis. Moreover, since knockdown experiments indicate that MBD2/dMTase is a key player in tumorigenesis, inhibition of this enzyme might prove to be a successful cancer therapy, especially since normal cell growth is unaffected Further

support will be presented in chapter 3, which describes the inhibition of MBD2/dMTase by a novel mechanism that involves the ubiquitous methyl donor AdoMet, and results in the phenotypic reversion of transformed cells.

IX. Summary

The preceding review provides overwhelming evidence indicating that proper control of DNMTs and DNA demethylases is critical for maintaining correct gene expression. In addition, misregulation of this machinery likely plays a role in the aberrant methylation patterns and gene expression that is a hallmark of may pathologies such as cancer. Therefore, a greater comprehension of the mechanisms involved in regulating the expression and activity of these proteins will undoubtedly provide new therapies aimed at restoring gene expression gone wrong. For this reason, this thesis addresses the following previously unresolved questions. First, how is DNMT1 regulated with cell growth and is this control involved in cellular transformation? Since previous studies have indicated that the DNMT1 protein, rather than DNA hypermethylation, might play a causal role in cancer, and since the cell cycle regulation of DNMT1 was found to be disrupted in certain cancers, we tested the hypothesis that deregulation of DNMT1 with the cell cycle plays a critical role in cancer. Chapter 1 demonstrates that regulation of *DNMT1* expression with cell growth occurs at a posttranscriptional level involving the 3' untranslated region of the *DNMT1* mRNA and plays an important role in cellular transformation. Second, what is the mechanism by which regional hypermethylation and global hypomethylation co-exist in cancer? Since the protein MBD2 has been shown to

be both a repressor of methylated DNA and a DNA demethylase, the possibility exists that global hypomethylation and local repression are catalyzed by the same multifunctional protein. In chapter 2, we hypothesize that different promoter interactions can direct the opposite activities of MBD2, and thus even its overexpression will not result in the activation of all genes. Our results indicate that MBD2/dMTase can induce demethylation and transcriptional activation in a promoter dependent manner, suggesting that it could be involved in maintaining both the DNA hyper and hypomethylation observed in cancer. Third, since hypomethylation plays such a prominent role in cancer, is it possible to inhibit demethylase activity by pharmacological agents and inhibit tumorigenesis? One potential agent, AdoMet, was shown to cause hypermethylation of DNA and to protect against cancer in rodent models, however the current model is that AdoMet does so by stimulating DNMT reactions. In contrast, we hypothesized that AdoMet might exert its effects through inhibition of demethylase activities. Chapter 3 investigates the inhibition of demethylase activity by AdoMet and its effects on cellular transformation. Lastly, because of the existence of an active DNA demethylase, does the potential exist to alter DNA methylation patterns even in somatic tissues in the absence of DNA replication? This could have important implications on DNA methylation therapeutics in postmitotic tissues such as the brain. Chapter 4 examines the effects of the anti-epileptic drug valproate on the induction of active DNA demethylation. In summary, our data support a new hypothesis on the mechanisms and role of *DNMT1* overexpression, regional hypermethylation and global hypomethylation in cancer, and therapeutic approaches for modulating DNA methylation patterns in cancer and other pathological conditions. Our data show how one might take advantage of the dynamic

epigenome to manipulate DNA methylation patterns to cause either hypo or hypermethylation in different contexts. The therapeutic implications of this data will be discussed in the general discussion section.

Chapter 1

A conserved 3' untranslated element mediates growth regulation of DNA methyltransferase 1 and inhibits its transforming activity

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This chapter has been published in the Journal of Biological Chemistry (2001) 276, 24881-24890.

Ectopic expression of DNA methyltransferase 1 (DNMT1) has been proposed to play an important role in cancer. *Dnmt1* mRNA is undetectable in growth-arrested cells but is induced upon entrance into the S phase of the cell cycle, and until now, the mechanisms responsible for this regulation were unknown. In this manuscript, we demonstrate that the 3' untranslated region (3' UTR) of the *DNMT1* mRNA can confer a growth-dependent regulation on its own message as well as a heterologous β-globin mRNA. Our results indicate that a 54-nucleotide highly conserved element within the 3'UTR is necessary and sufficient to mediate this regulation. Cell-free mRNA decay experiments demonstrate that this element increases mRNA turnover rates, and does so to a greater extent in the presence of extracts prepared from arrested cells. A specific RNA-protein complex is formed within the 3'UTR only in growth-arrested cells, and a UV crosslinking analysis reveals a 40 kDa protein (p40), whose binding is dramatically increased in growth arrested cells and is inversely correlated with *dnmt1* mRNA levels as cells are induced into the cell cycle. While ectopic expression of human *DNMT1* lacking the 3'UTR can transform NIH-3T3 cells, inclusion of the 3'UTR prevents transformation. These results support the hypothesis that deregulated expression of *DNMT1* with the cell cycle is important for cellular transformation.

INTRODUCTION

DNA methylation is a covalent modification of DNA that can modulate gene expression, and is now recognized as a major component of the epigenome (Razin, 1998; Razin and Riggs, 1980). The methylation of cytosines in vertebrates occurs when they are found 5' to deoxyguanosine in the sequence CpG. 80 % of CpGs are methylated (Szyf et al., 1984), and are distributed in a pattern which is unique in each tissue, and is inversely correlated with gene expression (Szyf et al., 1984).

The pattern of methylation is faithfully maintained during cell division by the enzyme DNMT1, the maintenance DNA methyltransferase (Li et al., 1992), which catalyzes the transfer of a methyl group from S-adenosylmethionine to the 5th position of the cytosine ring (Kumar et al., 1994; Wu and Santi, 1985). DNMT1 has a bilateral relationship with DNA replication and cell growth (Szyf, 2001c). DNA methylation occurs concurrently with DNA replication (Araujo et al., 1998) and the expression of *dnmt1* is tightly coordinated with the cell cycle (Szyf et al., 1991), while inhibition of DNMT1 inhibits DNA replication (Knox et al., 2000) leading to senescence and inhibition of cell growth (Jackson-Grusby et al., 2001; Young and Smith, 2001). Deregulated expression of *DNMT1* was previously suggested to play a causal role in cellular transformation (el-Deiry et al., 1991; Szyf, 1994). Several lines of evidence are in accordance with this hypothesis. First, elevated levels of *DNMT1* mRNA and activity were reported in tumors and cancer cell lines (Belinsky et al., 1996; Issa et al., 1993; Kautiainen and Jones, 1986). Second, ectopic expression of *DNMT1* results in cellular transformation (Vertino et al.,

1996; Wu et al., 1993). Third, *DNMT1* expression is regulated by nodal protooncogenic signaling pathways (MacLeod et al., 1995; Rouleau et al., 1995; Slack et al., 1999). And fourth, inhibition of DNMT1 by 5-azacytidine (a pharmacological inhibitor of DNMT1), or a reduction in DNMT1 levels by either antisense mRNA or antisense oligonucleotides, reverses tumorigenesis (Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997).

The mechanism by which DNMT1 influences cellular transformation is unclear. It has been proposed that high levels of DNMT1 can lead to ectopic methylation and silencing of tumor suppressor genes (Baylin et al., 1998). However, a clear correlation between general *DNMT1* overexpression and tumor suppressor hypermethylation has not been established (Eads et al., 1999; Jurgens et al., 1996). It has recently been suggested that deregulated expression of *DNMT1* during the cell cycle might be critical for DNMT1's effects on cell growth (Robertson et al., 2000b; Szyf et al., 2000). Previous nuclear run on experiments have demonstrated that while both growth-arrested and cycling cells transcribe *dnmt1* mRNA at a similar rate, the mRNA is only detected in cycling cells (Szyf et al., 1991). This suggests that the cell cycle regulation of *dnmt1* occurs at the posttranscriptional level. It stands to reason that the mechanisms involved are linked to basic control points of the cell cycle and potentially linked to cellular transformation. To test this hypothesis, the factors responsible for regulation of *DNMT1* expression with the cell cycle must be identified. One potential element is the AU-rich 3' untranslated region (3'UTR) of the *DNMT1* mRNA, since it is well documented that this type of element can regulate mRNA levels (Wilson and Brewer, 1999).

Regulation of mRNA stability by AU-rich elements (AREs) is an important mechanism involved in orchestrating the expression of critical genes in development (Surdej et al., 1994; Zhou et al., 1998) and cell cycle (Joseph et al., 1998; Maity et al., 1997), early response genes involved in cellular growth such as *c-myc* (Jones and Cole, 1987) and *c-fos* (Treisman, 1985), as well as cytokines such as granulocyte macrophage colony-stimulating factor *GM-CSF* (Shaw and Kamen, 1986) and *IL-3* (Ming et al., 1998). These regions often modulate mRNA levels in response to a change in cell environment by growth factors (Jang et al., 2000; Scheper et al., 1996), developmental factors (Buzby et al., 1999), and hormones (Staton et al., 2000). Although the mechanism by which AREs act has not been fully elucidated, it appears to involve the binding of various protein factors, several of which have been well characterized, such as AUF1 (DeMaria and Brewer, 1996; Zhang et al., 1993) and the ELAV family members (Atasoy et al., 1998; Ford et al., 1999).

In this study, we test the hypothesis that the 3'UTR of the *DNMT1* mRNA plays a role in regulating its levels with the cell cycle, and that deregulation at this level has an effect on cellular transformation by DNMT1. We show that a 54-nucleotide highly conserved element within the 3'UTR can confer a growth-dependent regulation on both homologous and heterologous mRNAs in living cells, and that this region can destabilize mRNA *in vitro*. This element interacts with a 40 kDa protein, whose binding is inversely correlated with *dnmt1* mRNA levels throughout the cell cycle. Finally, we demonstrate that the 3'UTR influences the cellular changes observed upon overexpression of *DNMT1* in NIH-3T3 cells, thus providing a link between *DNMT1* regulation, cell cycle, and oncogenesis.

MATERIALS AND METHODS

Cell culture and flow cytometry- Balb/c-3T3 cells (ATCC) were maintained as a monolayer in Dulbecco Modified Medium (Life Technologies, Inc.) containing 10% calf serum (Colorado Serum Co). To arrest cells at Go/G1, confluent Balb/c-3T3 cells were cultured in a medium containing 0.5% calf serum for 48 h. The cells were induced to enter the cell cycle by replacing the growth medium with medium containing 10% calf serum. Cells at various stages of the cell cycle were obtained by harvesting them at varying lengths of time after serum induction. To determine the percentage of cells at different stages of the cell cycle, cells were stained with propidium iodide and the DNA content was measured by flow cytometry (Melamed, 1990).

Plasmid construction- pCRII-DNMT13'UTR: Human *DNMT1* 3'UTR (5090-5408) was amplified by RT-PCR from 1 μg of total RNA prepared from human lung cancer H446 cell line (ATCC). Reverse transcription was performed using random primers (Roche Molecular Biochemicals) and Superscript reverse transcriptase as recommended by the manufacturer (Life Technologies, Inc.). For PCR amplification with Taq polymerase (CLONTECH) 50 ng of the sense primer 5'TCTGCCCTCCCGTCACCC3' and antisense primer 5'GGTTTATAGGAGAGATTT3' were used to amplify the 3'UTR from 5 μl of reverse transcribed cDNA in the presence of 1 mM dNTPs and the manufacturer's amplification buffer supplemented to 2 mM $MgCl_2$. Cycling conditions were: 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min (30 cycles). The amplified fragment was subcloned into pCR II using TA Cloning Kit (Invitrogen) as recommended by the

manufacturer. To generate pRBG-DNMT13'UTR, the human DNMT1 3'UTR was excised from PCRII-DNMT13'UTR with *Bam*HI and *Xba*I and ligated into a *Xba*I-*Sal*I digested pRBG-GC (Shaw and Kamen, 1986). The incompatible ends were then filled using Klenow DNA polymerase (Roche Molecular Biochemicals) and the blunt ends were joined using T4 DNA ligase (MBI Fermentas).

pRBG-DNMT1Δ 5'259 and pSK-Δ5'259: sense and antisense oligonucleotides coding for bases 5349 to 5405 bearing 5' *Xba*I and 3' *Sal*I overhangs were annealed and ligated into the *Xba*I and *Sal*I sites of both pRBG-GC and pBluescript SK.

pSK-Δ3'56 and pSK-Δ3'158 were generated by PCR amplification using 10 ng of pCRII-DNMT13'UTR as a template, the 3'UTR sense primer as above, and antisense primers: 5'GTCGACTTAATTTCCACTCATACAGTGGTAG3' for pSKΔ3'56, and 5'GTCGACTTAGTTGATAAGCGAACCTCACACA3' for pSKΔ 3'158. The amplified sequences were cloned into PCRII, then removed from the vector by *Sal*I digestion and ligated into pBluescript-SK cut with *Sal*I and *Sma*I.

pUD-hDNMT1 and pUD-hDNMT1ΔUTR: The pUD1 vector (a gift from Dr. L. Chasin) was digested with *Eco*RI and *Hind*III (to remove the DHFR gene), and either blunt-ended or modified with the following linkers: 5'AATTCTAG3' and 5'AGCTCTAG3' to generate an *Xba*I site. Human *DNMT1* cDNA (360-5085) was inserted into the blunt pUD1 to give pUD-hDNMT1ΔUTR, while *DNMT1* cDNA (360-5408) was inserted into the modified pUD1 to give pUD-hDNMT1.

pAd-DNMT1, pAd-DNMT1Δ3'56, pAdDNMT1Δ3'UTR: *DNMT1* cDNA was cloned into the AdEasy shuttle vector pAdTrack cytomegalovirus in the *Xba*I site. Adenoviral recombination and preparation of the infectious particles in HEK 293 cells was performed as described previously (He et al., 1998b). The following *DNMT1* regions were used: 1- 5408 (pAd-DNMT1), 1-5350 (pAd-DNMT1Δ3'56) and 1-5085 (pAd-DNMT1Δ3'UTR).

Preparation and analysis of RNA- Total RNA was prepared by the guanidinium isothiocyanate method (Chirgwin et al., 1979) and mRNA levels were determined by Northern blot analysis. Approximately 10 μg of RNA was electrophoresed on a 1.2 % denaturing agarose gel and then transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were probed with the indicated ^{32}P-labeled cDNA probes synthesized using a random priming labeling kit (Roche Molecular Biochemicals). The membranes were hybridized at 68 °C for 4-6 h in a buffer containing 0.5 M sodium phosphate pH 6.8, 1 mM EDTA, 7% SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization the membranes were washed twice for 10 min in a 5% SDS, 0.04 M sodium phosphate pH 6.8, 1 mM EDTA solution, and then four times for 10 min in the same solution containing 1% SDS. The following probes were used: rabbit β-globin (a 500 bp *Xho*I-*Bam*HI fragment from pRBG-GC), hDNMT1 (a 1.5 kb fragment from nucleotides 601-2191 of the full length human *DNMT1* cDNA), mDNMT1 (a 1.7 kb fragment from nucleotides 844- 2544 of the full length mouse *dnmt1* cDNA), green fluorescent protein (GFP) (a 765 bp *Nhe*I-*Hind*III fragment from pEGFP-C1 (CLONTECH)). The level of expression of the different mRNAs was quantified by densitometric scanning of the

relevant autoradiogram. Each experiment was normalized for the amount of total RNA by hybridization with a ^{32}P-labeled 18s ribosomal RNA oligonucleotide probe (Szyf et al., 1990), with the exception of the adenoviral experiments, which were normalized to GFP that is expressed by the adenoviral vector.

Stable transfections- To create the pRBG stable lines, 5 μg of the following plasmids were introduced into Balb/c-3T3 cells by DNA mediated gene transfer using the calcium phosphate protocol (Ausubel, 1988): pRBG-AT (bearing the 3' UTR of human *GM-CSF* and a neomycin resistance gene as a selectable marker), pRBG-GC (control) (both plasmids were a kind gift of Dr. G Shaw) (Shaw and Kamen, 1986), pRBG-DNMT13'UTR, and pRBG-DNMT1Δ 5'259. G418-resistant colonies were cloned and propagated in selective medium containing 0.25 mg/ml Geneticin (Life Technologies Inc.).

Stable lines expressing pUD-hDNMT1 and pUD-hDNMT1Δ3'UTR (the pUD vector bears a tetracycline repressible promoter) were obtained by transfecting 6 μg of each construct together with 6 μg of a puromycin resistance expression vector pBABEpuro (Morgenstern and Land, 1990) into TetOff NIH-3T3 cells (CLONTECH) using SuperFect reagent (Qiagen) according to the manufacturer's protocol. Resistant colonies were selected in media containing 3 μg/ml puromycin (Sigma), and were then further cultured in the presence of 1 μg/ml doxycycline (Sigma) to repress transcription of the transfected *DNMT1*. Northern blot analyses were used to verify expression of the ectopic *DNMT1* for both sets of transfectants. Growth arrest was accomplished by culturing in

0.5% serum for 48 h; to induce growth, arrested cells were cultured in 10% serum-containing medium for 20 h. Doxycycline was removed 48 h before serum starvation to induce ectopic *DNMT1* transcription. Growth rates were determined following plating the cells in six-well culture dishes at a density of 25 000 cells per well. Cells were then counted on 6 subsequent occasions throughout the span of 11 days, and phase contrast photography was performed on the last day. An average of 4 counts was performed for each time point.

Adenoviral Infections- Balb/c-3T3 cells were grown to confluence in six-well plates and were then transferred to 0.5% serum medium for 48 h to arrest cell growth. Cells were then infected for 4 h in serum free media with pAd-DNMT1, pAd-DNMT1Δ3'56, and pAd-DNMT1Δ3'UTR at multiplicities of infection of 25, 50,150 respectively. 100% of cells were infected as determined by visualization of GFP, which is expressed by the adenoviral vector. Cells were then maintained in 0.5 % media for additional 24 h before the medium was replaced with one containing either 10% or 0.5% serum. RNA was harvested after 20 h and was analyzed by a Northern blot analysis as described above.

In vitro RNA synthesis- To generate RNA transcripts for the gel mobility shift, UV crosslinking, and *in vitro* degradation assays, the following plasmids were linearized at the indicated restriction sites: pCRII-DNMT13'UTR (*Bam*HI), pSK-Δ5'259 (*Sal*I), pSK-Δ3'56 (*Sal*I), pSK-Δ3'158 (*Sal*I). *In vitro* transcription was then carried out in the presence of 50 μCi of [α^{32}P]-UTP (3000 Ci/mmol, PerkinElmer Life Sciences) using either T7 polymerase (for DNMT13'UTR) or T3 polymerase (forΔ5'259, Δ3'56, Δ

3'158). The Ambion *in vitro* transcription kit was used as recommended by the manufacturer. Cold competitor RNA transcripts were synthesized using the Promega RiboMax system according to the protocol supplied by the manufacturer.

Preparation of whole cell extracts -Balb/c-3T3 cells were harvested and resuspended in extraction buffer (20 mM Tris-HCl pH 7.5, 0.4 M KCl, 20% (v/v) glycerol, 2 mM DTT, and 1X Complete™ protease inhibitor (Roche Molecular Biochemicals)). The suspension was frozen immediately at -80 °C for 1 h and the whole cell extract was isolated by centrifugation at 10, 000 x g for 15 min. The supernatant was recovered and used for RNA binding assays. Proteinase K treatment was carried out for 1 h at 37 ^{0}C at a final concentration of 1 μg/μl.

RNA mobility gel shift assays and UV crosslinking- Reaction mixtures were incubated on ice for 1 h in a 20 μl mixture containing 50 μg of whole cell extract, 1, 000, 000 cpm of probe, 10 μg tRNA (as a non specific RNA competitor), and the following buffer: 10 mM Hepes pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM DTT. Following incubation, free unbound ^{32}P -labeled RNA was removed by treating the reaction mixture with 1.5 μl RNase T1 (1 unit/ml) and 1.5 μl RNase A (10 mg/ml) for 10 min at room temperature. Heparin (5 μg/ml) was added and the reaction was incubated for additional 10 min at room temperature. The resulting complexes were resolved on a 10% non-denaturing polyacrylamide gel. For UV crosslinking assays, the same reaction mixture was subjected to incubation on ice for 1.5 h under UV light (254nm). Cold RNA competitors were used at a concentration of 1000x. RNases were added as above and the

reaction mixture was incubated for additional 30 min at 37 ^{0}C. 5 µl of 5X loading buffer (1.05 M Tris Cl, pH 6.8, 36% glycerol, 100 mg/ml SDS, 0.2% β-mercaptoethanol, 0.12 mg/ml bromophenol blue) were added and the mixture was boiled for 10 min. Protein-RNA complexes were resolved on a 10% SDS-polyacrylamide gel.

In vitro RNA degradation assay- RNA decay rates were assessed using the protocol described in (Buzby et al., 1999) with the following modifications. 10 µg of whole cell extract and 2.5 X 10^{5} cpm of in vitro synthesized RNA transcript were used, and the reactions were carried out for 0.25 to 4 h at 37 ^{0}C. The concentration of RNA at each time point was quantified by densitometry of the autoradiogram and is presented in the figures as the percentage of RNA remaining at each time point relative to the concentration of RNA at time 0. The half-life of each RNA was calculated from the logarithmic decay plots.

Quantification of DNMT1 mRNA expression by competitive RT- PCR- Total RNA (1ug) from TetOff NIH-3T3 stable lines was reverse transcribed with M-MuL-V reverse transcriptase and a random primer (MBI Fermentas) using the manufacturer's protocol in the presence of 25 µCi of ^{35}S-labeled dATP (1250 Ci/mmol) to quanify the efficiency of reverse transcription. Equal amounts of cDNA (30, 000 cpm as determined by [^{35}S]-dATP incorporation) were subjected to DNMT1 PCR amplification using Taq polymerase (MBI Fermentas) in the presence of decreasing concentrations (10^{-12} –10^{-16} nM) of a competitor DNA fragment. The following primers were used: 5'ACCGCTTCTA-CTTCCTCGAGGCCTA3' (sense, starting at 3479) and 5' GTTGCAGTCCT-CTGTGAACACTGTGG3' (antisense, starting at 3813) to amplify the

target sequence, and 5'CCTCGAGGCCTAGAAACAAAGGGAAGGGCAAG-3' (ending at 3576) to create the competitor as previously described (Tao et al., 1997). PCR conditions were as follows: 96 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min (33 cycles). To differentiate between the endogenous mouse dnmt1 and transfected human DNMT1 mRNA, the PCR was transferred to Hybond-N+ membrane and hybridized with an oligonucleotide corresponding to bases 3595-3617 of the full length DNMT1 mRNA (GenBank™ accession no. NM001379.1). The oligo was labeled at the 5' end using 20 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol, PerkinElmer Life Sciences) and T4 polynucleotide kinase (MBI Fermentas) according to the manufacturer's protocol. The membranes were incubated for 16 h at 42 °C in the following hybridization buffer: 1% SDS, 5X SSC, 5X Denhardt's solution (0.5 mg/ml Ficoll, 0.5 mg/ml polyvinylpyrrolidone, 0.5 mg/ml BSA), and 0.1 mg/ml herring sperm DNA. Membranes were then washed twice with 6X SSC + 0.1% SDS for 10 min at room temperature, twice for 10 min at 37 °C, and twice for 10 min at 42 °C.

To further confirm that equal amounts reverse transcribed cDNA were being used, we amplified β-actin using the following primers: 5'- GTTGCTATCCAGGCTGTGCTA-3' (sense, starting at 473), and 5'- GCGGATGTCCACGTCACACTT-3' (antisense, starting at 943) (GenBank™ accession no. XM_004814). Touch down PCR was used by decreasing the annealing temperature from 66 °C to 60 °C over 4 cycles, and then continuing with: 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s (21 cycles).

RESULTS

Time course of dnmt1 mRNA increase following serum stimulation of arrested Balb/c-3T3 cells- Previous studies have demonstrated that DNMT1 is regulated with cell growth (Nass et al., 1999; Robertson et al., 2000b; Szyf et al., 1991; Szyf et al., 1985b). To correlate the levels of *dnmt1* mRNA with different stages of the cell cycle in our system, Balb/c-3T3 cells were stimulated with serum for varying times following growth arrest, and concurrent FACS and Northern blot analyses were performed. As seen in Fig.1, A and C, the majority of cells are arrested at G0/G1 (70%) after serum deprivation for 48 hours (t=0). Addition of serum-containing medium induces entry of the cells into the S phase of the cell cycle, which peaks 24 hours post stimulation (S= 51%). *dnmt1* mRNA is barely detectable in arrested cells and is induced late in G1 (8-12 h), reaching its maximum levels at the G1-S boundary (20h)(Fig. 1, B and C). *dnmt1* mRNA remains elevated throughout S, and then begins to decrease as cells continue into G2-M. Since nuclear run-on transcription experiments have previously shown that *dnmt1* is transcribed at a constant rate throughout the cell cycle (Szyf et al., 1991), the variation in mRNA levels must occur at the posttranscriptional level. This mode of regulation has already been reported for *dnmt1* in several cases, such as in myoblast differentiation (Liu et al., 1996), F9 cell differentiation (Teubner and Schulz, 1995), and *dnmt1* upregulation by T-antigen (Slack et al., 1999).

The 3'UTR of the DNMT1 mRNA contains a highly conserved AU-rich element- Since the 3' untranslated region (3'UTR) of an mRNA is often implicated in mediating post transcriptional regulation, we examined this region of *DNMT1* for the presence of any potential mRNA regulating motifs. We first reasoned that an important regulatory

element should remain conserved throughout evolution. As can be seen in Fig. 2, an alignment of the 3'UTR of the *DNMT1* mRNAs from human, mouse, rat, *Xenopus*, chicken, and zebrafish (daniorerio) identifies a 54-nucleotide region which is 100% conserved between human and chicken, and a smaller region of 36 nucleotides which is conserved among all the species. Also shown are several AU-rich sequences that may act as potential RNA destabilizing motifs (Fig. 2, *boxed and underlined*) (Pende et al., 1996). Such a homology in a noncoding region is unusual (less than 30 % would be expected without selective pressure (Asson-Batres et al., 1994)), and strongly suggests an important regulatory role for this RNA element. The 54-nucleotide sequence is unique to *DNMT1* and no other example was found in the human genome by a BLAST search of the human genome data bank. Many mRNAs which are regulated posttranscriptionally contain highly conserved 3' UTRs, such as *c-fos, bcl2, fibronectin* (Spicher et al., 1998), *B-Ar* (Tholanikunnel et al., 1999), *p21* (Joseph et al., 1998), and *IgfII* (Scheper et al., 1996). As many of these regions often modulate mRNA levels in response to varying environmental and growth conditions, it is highly probable that this region of the *DNMT1* mRNA is involved in its cell cycle regulation.

The DNMT1 3'UTR can downregulate the stable B-globin mRNA in growth arrested cells- To test the hypothesis that the regulation of *DNMT1* mRNA levels involves *cis*-acting sequences within the 3'UTR, we assayed whether this region could direct growth-dependent regulation of the stable rabbit β-*globin* mRNA. Various AREs, such as those of *GM-CSF* (Shaw and Kamen, 1986) and *PAI-1* (Tillmann-Bogush et al., 1999), have been shown to destabilize the rabbit β-*globin* mRNA when inserted into its 3' UTR. We

placed either the full 3'UTR of DNMT1 (pRBG-DNMT13'UTR) or only the 54-nucleotide conserved sequence (pRBG-DNMT1Δ5'259) into the rabbit β-*globin* gene 3'UTR as indicated in Fig. 3A. We used the stable pRBG-GC construct as a negative control, and the pRBG-AT construct bearing the instability element from *GM-CSF* as a positive control (Shaw and Kamen, 1986). These were transfected into Balb/c-3T3 cells, stable transfectants expressing the different constructs were then selected, and RNA was prepared from either growth-arrested cells or from cells that had been serum-stimulated for 20 hours (time at which *dnmt1* mRNA peaks, see Fig. 1). The level of *globin* mRNA under each condition was determined by Northern blot analysis and normalized relative to 18s rRNA (Fig. 3, B and C). To take into account any possible difference in mRNA levels due to a decreased promoter activity of the RBG vector in arrested versus serum-stimulated cells, the results presented in Fig. 3C were normalized relative to that of the GC stable control. Our findings demonstrate that the *DNMT1* 3'UTR is able to downregulate the β-*globin* mRNA in arrested cells but not in serum-stimulated cells. The full 3'UTR is not required, however, since the chimeric β-*globin* construct containing the 54-nucleotide element by itself (Δ5'259) is also able to perform the same function. As expected, the stable pRBG-GC construct does not show significant variation with the growth state of the cells, while pRBG-AT is constitutively destabilized. These data indicate that the conserved 54-nucleotide region within the *DNMT1* 3'UTR is sufficient to downregulate a heterologous mRNA in growth-arrested cells.

The 54-nucleotide highly conserved region of the 3'UTR is required to downregulate the DNMT1 mRNA in growth-arrested cells- We then assessed whether the *DNMT1* 3'UTR

could regulate its own mRNA in a similar manner, by generating adenoviral vectors expressing human *DNMT1* containing deletions within the 3'UTR (Fig. 3D). The adenovirus system is advantageous since sufficient levels of transient expression of the exogenous constructs are obtained, thus avoiding the tedious process of generating stable cell lines. In addition, the adenoviral vector contains the gene for GFP, which is under control of the same promoter and poly A signal as the gene of interest, and so can serve as an ideal internal control for infection efficiency as well as for any variations in promoter activity due to changes in growth conditions.

Fig. 3E displays a representative Northern blot of RNA from Balb/c-3T3 cells infected with the different adenoviral constructs. We used a human *DNMT1* cDNA probe to differentiate between the exogenous human *DNMT1* deletions and the endogenous mouse *dnmt1*, and under our conditions of hybridization, the endogenous *dnmt1* is undetected. Once the results are quantified and normalized to GFP (Fig. 3F) they demonstrate that only the *DNMT1* construct bearing the full 3'UTR (DNMT1) is downregulated in arrested cells. By removing the 3'UTR (Δ3'UTR), or by simply deleting the conserved 54-nucleotide sequence (Δ3'56), we abolish this cell growth-dependent change in *DNMT1* mRNA levels. Taken together, these results and those from the previous experiment indicate that the *DNMT1* 3'UTR can downregulate an mRNA in either a heterologous or homologous context in a growth-dependent manner, and that the conserved 54-nucleotide sequence is necessary and sufficient for this regulation.

A specific protein (p40) binds the conserved region of the DNMT1 3'UTR and is upregulated in growth-arrested cells- The 3'UTR has been shown to mediate its

regulatory effects through interactions with various RNA binding proteins, such as AUF1 (DeMaria and Brewer, 1996; Zhang et al., 1993), and the ELAV proteins (Atasoy et al., 1998; Ford et al., 1999). Many other 3'UTR mRNA binding activities have been found (Heaton et al., 2000; Hew et al., 1999; Malter and Hong, 1991; Ostareck-Lederer et al., 1994), however most of these have not been well characterized as of yet. In many instances, the expression or binding activity of the proteins themselves is modified in response to various stimuli (Atasoy et al., 1998; Pende et al., 1996), and is correlated with either up- or down- regulation of the associated mRNAs (Buzby et al., 1996; Sirenko et al., 1997).

We therefore addressed the question of whether the growth regulation of *DNMT1* mRNA levels involves the formation of growth phase-specific RNA-protein complexes? To answer this, we performed an RNA gel mobility shift assay using a ^{32}P-labeled *DNMT1* 3'UTR RNA transcript (Fig. 4A, 3'UTR), and whole cell extracts prepared from Balb/c cells which were growth-arrested and then serum-stimulated for specific lengths of time (as in Fig. 1). The RNA-protein complexes were subjected to RNase digestion to remove free unbound RNA prior to resolving of the complex on a nondenaturing polyacrylamide gel. As observed in Fig. 4B, the profile of RNA protein complexes within the 3'UTR changes as passage into the cell cycle occurs. Most strikingly, a specific complex (indicated by an arrow) is only observed in extracts prepared from growth-arrested cells. The fact that these complexes are formed in the presence of excess of nonspecific tRNA suggests that these interactions are specific.

UV crosslinking analysis was then utilized to further study RNA-protein interactions within the 54-nucleotide element, as this region appears to be critical for *DNMT1* mRNA downregulation. The UV crosslinking method allows one to observe the binding of individual proteins to the RNA, and the molecular weight of the proteins can be approximated. As can be seen in Fig. 4C, the interaction of Δ5'259 with a protein of approximately 40 kDa (which we will from now on refer to as p40) is found to occur almost exclusively in arrested cells. A competition experiment was then carried out, with cold competitors comprising the various 3'UTR constructs (Fig. 4, A and D). As expected, both 3'UTR and Δ5'259 are effective competitors, and displace p40 binding from the labeled Δ5'259 probe. However, Δ3'56 and Δ3'158 did not compete, demonstrating that the binding of p40 is specific to the conserved 54-nucleotide element. No binding activity was observed in experiments performed with probe alone, or with cell extracts (serum stimulated and arrested) treated with proteinase K (Fig. 4C), confirming that the p40 band is indeed a protein.

The above findings, together with the results in Fig. 3, suggest that p40 may be involved in the mRNA downregulation mediated by the 54-nucleotide element in arrested cells. To provide further support to this theory, we performed a UV crosslinking with extracts prepared at various time points after serum stimulation of arrested cells. A representative UV crosslinking is shown in Fig. 3E, and the average results of 3 quantified experiments are illustrated in Fig. 3F. We can clearly see that p40 binding is decreased between 0 and 6 hours after cell growth is stimulated, and other proteins interact with the 54-nucleotide element as cells progress through the cell cycle. When we overlay the time course of p40

binding with *dnmt1* mRNA levels following serum stimulation (Fig. 4G), we observe an inverse correlation between p40 binding and *dnmt1* mRNA levels; *dnmt1* mRNA increases shortly after p40 levels fall, and remains elevated while p40 binding is low. Although these results do not prove that p40 is responsible for the change in *dnmt1* mRNA levels, the facts that p40 binding is highly induced in growth-arrested cells and that it is specific for the same region that is required for mRNA downregulation are consistent with this hypothesis.

The rate of mRNA decay in vitro is accelerated in the presence of the 54 nucleotide region and arrested cell extracts- Since mRNA posttranscriptional regulation can occur at multiple levels, we addressed the question of how does the *DNMT1* 3'UTR downregulate mRNA levels in growth-arrested cells? We decided to investigate the role of the *DNMT1* 3'UTR in mRNA decay for two reasons. First, the 3'UTR contains several AREs, which have been shown to modulate the stability of a wide variety of mRNAs, and second, alterations in mRNA half -life involving AREs have been found to involve changes in RNA-protein interactions.

We used an *in vitro* or cell-free RNA degradation assay (Buzby et al., 1999) to assess the decay rates of the various *DNMT1* 3'UTR deletion constructs. This method measures the decay of *in vitro* synthesized radiolabeled RNA transcripts that are incubated with extracts prepared from cells grown under various conditions. This technique has several advantages over other methods of measuring mRNA decay (Wilson and Brewer, 1999), the most important being that it avoids the *in vivo* use of transcriptional inhibitors such as

actinomycin D and DRB, both of which have been found to alter the stability of certain mRNAs (Baker et al., 2000; Kessler and Chasin, 1996; Noe et al., 1999; Searles et al., 1999), and would therefore confound any half-life measurements. In our hands, actinomycin D stabilized the *DNMT1* mRNA and therefore could not be used. We also tried using stable lines expressing tetracycline-repressible *DNMT1* constructs, however all the cell lines we obtained expressed background levels of *DNMT1* in the presence of the repressor.

Fig. 5, A and B show the results of the decay experiment performed with the various 3'UTR deletions in the presence of extracts prepared from arrested Balb/c cells (arrested) or in decay buffer alone (control). We observe that the entire 3'UTR (3'UTR), and the 54-nucleotide element (Δ5'259), decay with half-lives of 35 min and 60 min respectively. However, the half-lives of Δ3'158 and Δ3'56, both of which lack the conserved sequence, were significantly longer (100 min and 132 min respectively), suggesting that in the presence of arrested cell extracts, this element can accelerate the degradation of the *DNMT1* 3'UTR *in vitro*. No significant decay was observed with any of the RNA transcripts in buffer alone. The fact that the half-life of the full 3'UTR is shorter than that of the 54-nucleotide element implies that other regions upstream this sequence may enhance its destabilizing properties.

To test whether this decay is affected by cell growth, we compared the stability of Δ5'259 RNA in the presence of growth-arrested and serum-stimulated cell extracts (Fig. 5, C and D). The half-life of the RNA increases from 58 min when it is incubated with arrested

extracts to 105 min when the RNA is incubated with growth-induced extracts. These data are consistent with the hypothesis that the likely mechanism by which Δ5'259 downregulates mRNA in arrested cells *in vivo* (Fig. 3) is by decreasing mRNA stability.

The 3'UTR inhibits DNMT1 induced transformation of NIH-3T3 cells - It has been well documented that *DNMT1* is overexpressed in a variety of tumors and cancer cell lines (Belinsky et al., 1996; Issa et al., 1993; Kautiainen and Jones, 1986), and that ectopic expression of *DNMT1* can transform both NIH-3T3 cells (Wu and Santi, 1985) and human fibroblasts (Vertino et al., 1996). However, only the coding region of *DNMT1* was used in these latter two studies.

DNMT1 interacts with various cell cycle regulators, such as PCNA (Chuang et al., 1997), and Rb-E2F (Robertson et al., 2000a), and recent data suggest that the cell cycle regulation of *DNMT1* is disrupted in colorectal cancer cells (De Marzo et al., 1999) and in ER-negative breast cancer cells (Nass et al., 1999). We therefore tested the hypothesis that the loss of this cell cycle regulation is the main reason for *DNMT1*'s transforming properties, and expression of a *DNMT1* construct with its full 3'UTR, which should be properly regulated, would not result in changes in cellular identity.

We generated stable NIH-3T3 cell lines expressing human *DNMT1* with the full 3' UTR (hDNMT1), or without the 3'UTR (hDNMT1ΔUTR) under the direction of a tetracycline repressible promoter. Our first interesting observation was that the hDNMTΔUTR clones (n=10) grew at a much faster rate than the hDNMT1 clones. Then, after several passages,

many of the transfected lines exhibited signs of cell death, indicating that expression of hDNMT1ΔUTR had toxic effects. This is not surprising, since others have also observed harmful consequences of *DNMT1* overexpression (Vertino et al., 1996; Wu et al., 1996). To inhibit expression of the transfected constructs and prevent this effect, we cultured the transfectants in the presence of doxycycline until 48 hours prior to each experiment.

We first examined whether the constructs were differentially regulated with cell growth as would be expected. Fig. 6A depicts the results of a competitive RT-PCR performed to quantify the levels of *DNMT1* mRNA in transfectants that were either serum-starved or serum-stimulated for 20 hours. Since the PCR primers used amplify both the mouse and human *DNMT1*, we then hybridized our PCR with a human specific *DNMT1* ^{32}P- labeled oligonucleotide probe to specifically detect the hDNMT1 constructs. Similar to the results obtained with the adenovirally expressed *DNMT1* and the β-*globin* chimeras (Fig. 3), no *DNMT1* mRNA is detected in arrested cells expressing the full hDNMT1 construct (Fig. 6A, *left panel*). However, in cells expressing hDNMT1ΔUTR, mRNA is present at competitor concentrations of 10^{-14}-10^{-16}nM. As expected, in growth-stimulated cells, both constructs show a similar expression profile (Fig. 6A, *right panel*).

We then studied in detail the growth characteristics of the different clones with respect to cell cycle distribution, growth rates, and morphology. Fig. 6B displays a growth curve of representative hDNMT1 and hDNMT1ΔUTR expressing clones, as well as the parental NIH-3T3 cells. While both the control cells and the hDNMT1 clone exhibit similar growth rates, which begin to level off after 10 days, the hDNMT1ΔUTR clone grows at a

faster rate and does not reach saturation with high density. Accordingly, a FACS analysis carried out in the presence or absence of serum (Fig. 6C), reveals that a higher percentage of hDNMT1ΔUTR cells are found in S phase both in the presence and absence of serum. The observation that the parent cell line grew slightly faster that the hDNMT1 clone could be because the hDNMT1 clone was maintained in the presence of puromycin (the selection drug), which we found slowed down cell growth. But perhaps the most striking are the morphological changes observed in the hDNMT1ΔUTR clone. As can be seen in Fig. 6D, while both the control and hDNMT1 cells form an organized monolayer and are contact inhibited upon reaching confluence, the hDNMT1ΔUTR clone grows in a disorganized pattern and continues to do so after confluence is reached, forming large foci. Similar results were observed with 6 other clones. These results suggest that the cell cycle regulatory activity of the 3'UTR inhibits the cellular changes observed upon ectopic expression of *DNMT1*, thus supporting the hypothesis that deregulation of the cell cycle dependent expression of *DNMT1* is important for cellular transformation.

DISCUSSION

Since hyperactivation of DNMT1 has been implicated in cellular transformation (Szyf, 1994), understanding how this enzyme is regulated with cell growth is of obvious interest. In this study, we first confirm previous findings that the *dnmt1* mRNA is regulated with the cell cycle (Robertson et al., 2000b; Szyf et al., 1991). *dnmt1* mRNA is essentially absent in arrested cells, then increases as cells are induced by serum and reaches its maximum level just before the peak of S phase; this coordination would

ensure that sufficient DNMT1 protein is synthesized so as to carry out its maintenance function during replication. Second, we demonstrate that the 3' UTR of *DNMT1* confers a growth-dependent regulation on both its own mRNA, and a heterologous stable β-*globin* mRNA. Third, we identified a highly conserved 54-nucleotide element within the 3'UTR that is required for mediating the downregulation of *DNMT1* mRNA in arrested cells. Thus, *cis*-acting sequences in the 3' UTR are likely responsible for the changes in *dnmt1* mRNA which occur during the cell cycle. Fourth, the 3' UTR is destabilized *in vitro* in the presence of extracts prepared from arrested cells, suggesting that a protein that is present primarily in arrested cells targets the 3'UTR for degradation. The decrease in half-life of Δ5'259 cannot be attributed to the fact that it is shorter than Δ3'158 and Δ3'56, because the full 3'UTR decays at a faster rate. One conceivable explanation for the reduced destabilizing activity of Δ5'259 relative to the full 3'UTR is that AU-rich instability sequences exist upstream of Δ5'259. A possible model is that the 54-nucleotide element is responsible for the growth-dependent stability changes and region(s) upstream enhance its destabilizing activity.

Whereas we were able to show that the 3'UTR downregulates *DNMT1* mRNA in growth-arrested cells, we were not able to demonstrate that this occurs by destabilization of the mRNA in living cells. Actinomycin D is routinely used to block *de novo* synthesis of RNA in order to measure mRNA decay. However, we found that actinomycin D stabilized the *DNMT1* mRNA. This is not uncommon, and has been reported for several other mRNAs (Baker et al., 2000; Kessler and Chasin, 1996; Noe et al., 1999; Searles et al., 1999). We also could not obtain repressible *DNMT1* transfectants that show zero

DNMT1 expression in the repressed state, which could be used for measuring stability of mRNA in cells. However, the fact that the same element that reduces the presence of *DNMT1* mRNA in arrested cells also destabilizes *DNMT1* mRNA *in vitro* supports the conclusion that the 3'UTR acts as a cell cycle regulated destabilizing element.

Many studies have shown that mRNA posttranscriptional regulation plays an important role during development (Surdej et al., 1994; Zhou et al., 1998) and cell cycle progression (Joseph et al., 1998; Maity et al., 1997) and exerts an important control over the expression of protooncogenes (Jones and Cole, 1987; Treisman, 1985) and cytokines (Ming et al., 1998; Shaw and Kamen, 1986). Several pathological states are associated with aberrant regulation at this level, including various immune and inflammatory diseases and several types of cancer (Conne et al., 2000). One potential mechanism by which the 3'UTR may function is by the differential binding of *trans* acting factors during the cell cycle; these RNA-protein complexes could then either increase or decrease mRNA levels. Studies involving several mRNAs such as *PTH* (Sela-Brown et al., 2000), *GM-CSF* (Buzby et al., 1996), and *CYP7A1* (Baker et al., 2000) have found that changes in mRNA stability are correlated with the binding of proteins within the 3'UTR. In accordance with this postulated mechanism, we show, using an RNA mobility gel shift assay, that a specific RNA-protein complex is formed within the 3'UTR sequence only in growth-arrested cells. In addition, our UV crosslinking experiments reveal a protein of approximately 40kDa (p40), which exhibits specific binding to the conserved 54-nucleotide element. This binding is highly induced in arrested cells and decreases at the same time as *dnmt1* mRNA increases during cell cycle progression.

Although our data do not demonstrate a causal link between the p40-3'UTR interaction and *dnmt1* mRNA downregulation, our results are in accordance with this hypothesis. Further purification and characterization of p40, which is currently underway in our lab, may provide us with a better understanding of its mechanism of action.

It has been recently suggested that the deregulation of DNMT1 with the cell cycle rather than its overall levels are important for cellular transformation by DNMT1 (Robertson et al., 2000b; Szyf, 2001c; Szyf et al., 2000). The data presented here are consistent with this hypothesis. In agreement with previous studies (Vertino et al., 1996; Wu et al., 1993), we found that expression of an exogenous *DNMT1* construct comprising only the coding region, which is expressed under conditions of serum deprivation, is able to alter the growth properties of NIH 3T3 cells; the cells grow at a faster rate, and a higher percentage of cells are in S phase. They also exhibit a loss of contact inhibition and do not reach saturation even at high density. In contrast, cells expressing a *DNMT1* construct that includes the 3'UTR and is not expressed in serum-starved cells maintain the growth properties of the control cells.

The mechanisms responsible for cellular transformation by DNMT1 are still unresolved. However, one proposed hypothesis is that elevated levels of DNMT1 lead to the hypermethylation and inactivation of tumor suppressor genes (Merlo et al., 1995). It is possible that ectopic expression of *DNMT1* in arrested cells results in *de novo* methylation and silencing of a critical tumor suppressor gene. However if this is the mechanism, it is not clear why such a gene is methylated only when *DNMT1* is expressed

in arrested cells. An alternative hypothesis is that ectopic expression of *DNMT1* causes cell transformation by interfering with cell cycle regulatory circuits through DNMT1 protein-protein interactions. Since DNMT1 forms a complex with Rb and E2F (Robertson et al., 2000a) as well as histone deacetylase 1 and 2 (Fuks et al., 2000; Rountree et al., 2000) it can inhibit the expression of tumor suppressors by a mechanism that does not involve DNA methylation, as has been previously shown (Milutinovic et al., 2000). DNMT1 can also displace p21 from PCNA and allow replication to occur (Chuang et al., 1997), and inhibition of DNMT1 has been found to inhibit DNA replication (Jackson-Grusby et al., 2001; Knox et al., 2000). Thus, aberrant expression of *DNMT1* during the Go/G1 phase may override the silencing of tumor suppressors and eliminate normal arrest signals, leading to the inappropriate entry into the cell cycle that is observed in cancer cells, as has been previously suggested (Szyf, 1998).

Although further studies are required to confirm this hypothesis, our findings clearly illustrate the importance of the *DNMT1* 3'UTR in regulating its expression with the state of cell growth.

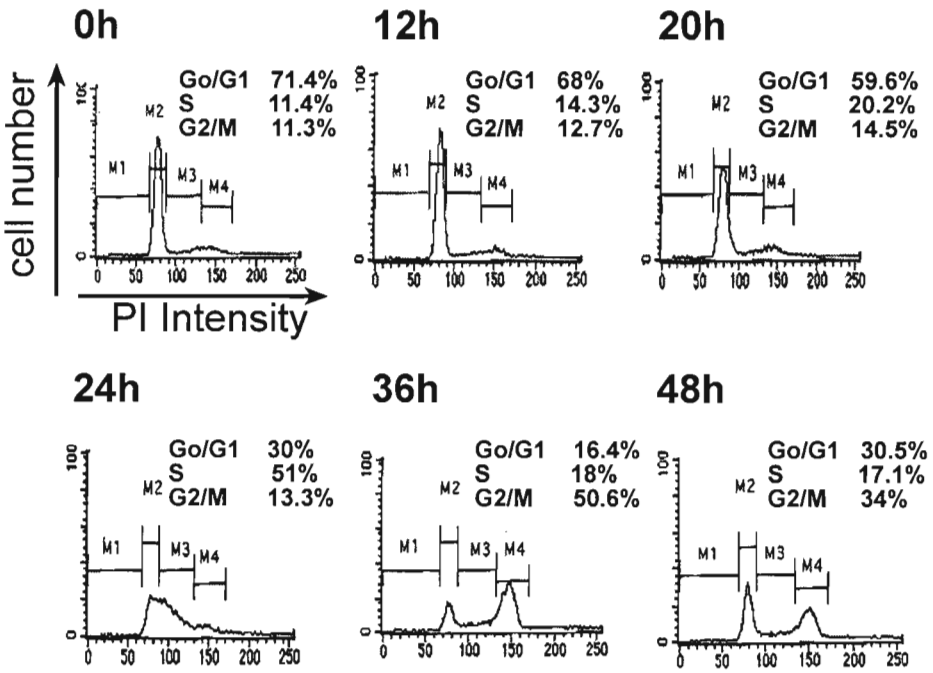
Acknowledgements :

This work was supported by a grant from the National Institute of Cancer Canada to M. S. N. D. is supported by a fellowship from the CIHR. We thank Dr. G. Shaw for his kind gift of the pRBG-AT and pRBG-GC plasmids, and Dr. L . Chasin for the pUD-1 plasmid. We also thank Andrew Slack for assistance with preparation of the DNMT1 adenoviral constructs.

FIGURE LEGENDS

Fig. 1. Induction of *dnmt1* mRNA by serum stimulation of growth-arrested Balb/c-3T3 cells. A. FACS analysis of serum-stimulated cells. Balb/c-3T3 cells were arrested by serum depletion as described in "materials and methods" and then incubated with serum for the indicated time points and subjected to a FACS analysis to determine the percentage of cells at different stages of the cell cycle. *M1*, apoptosis; *M2*, Go/G1; *M3*, S; *M4*, G2/M. **B.** Induction of *dnmt1* mRNA by serum stimulation of growth-arrested cells. Balb/c-3T3 cells were treated as in (A); RNA was then extracted and subjected to a Northern blot analysis using a mouse *dnmt1* cDNA probe (bp 844-2544). To normalize to the amount of total RNA in each lane, the blot was then hybridized with an 18s rRNA oligonucleotide probe. **C.** Correlation between *dnmt1* mRNA levels and stages of the cell cycle. Northern blots were quantified by densitometric scanning and the *dnmt1* signal was normalized to that of 18s. Shown are the *dnmt1* mRNA levels (arbitrary units) and the percentage of cells at each stage of the cell cycle.

A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20h
24h
36h
48h
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S 14.3%
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Go/G1 59.6%
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S 51%
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M1
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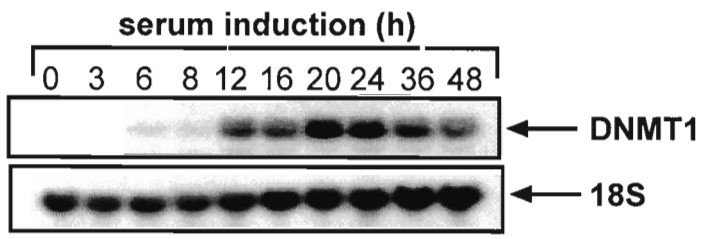
B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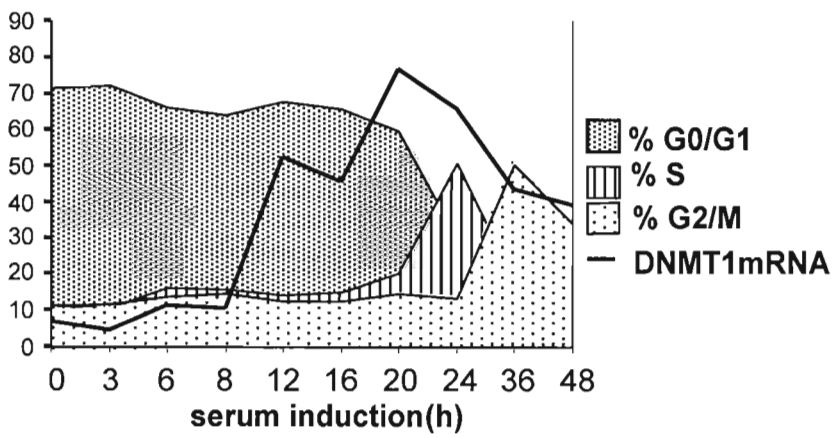
DNMT1
18S

C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80
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50
40
30
20
10
0
0 3 6 8 12 16 20 24 36 48
serum induction(h)
% G0/G1
% S
% G2/M
DNMT1mRNA

Fig. 2. Homology between the *DNMT1* 3' UTR of different species.

Alignments were performed with the following cDNA sequences (GenBank™ accession numbers indicated): mouse (*m*), 5077-5238 (X14805.1); human (*h*), 5249-5405 (NM001379.1); rat (*r*), 5072-5215 (AB012214.1); xenopus (*x*), 4987-5020 (D78638.1); chicken (*c*), 4936-4990 (D43920.1); daniorerio (*dr*), 2333-2363 (AF097875). *Shaded area* denotes a region of 100% conservation between human and chicken *dnmt1*. *Underlined* and *boxed* sequences indicate potential RNA destabilizing motifs. Region in *boldface* marks the 54-nucleotide element of *DNMT1* studied in detail in this report.

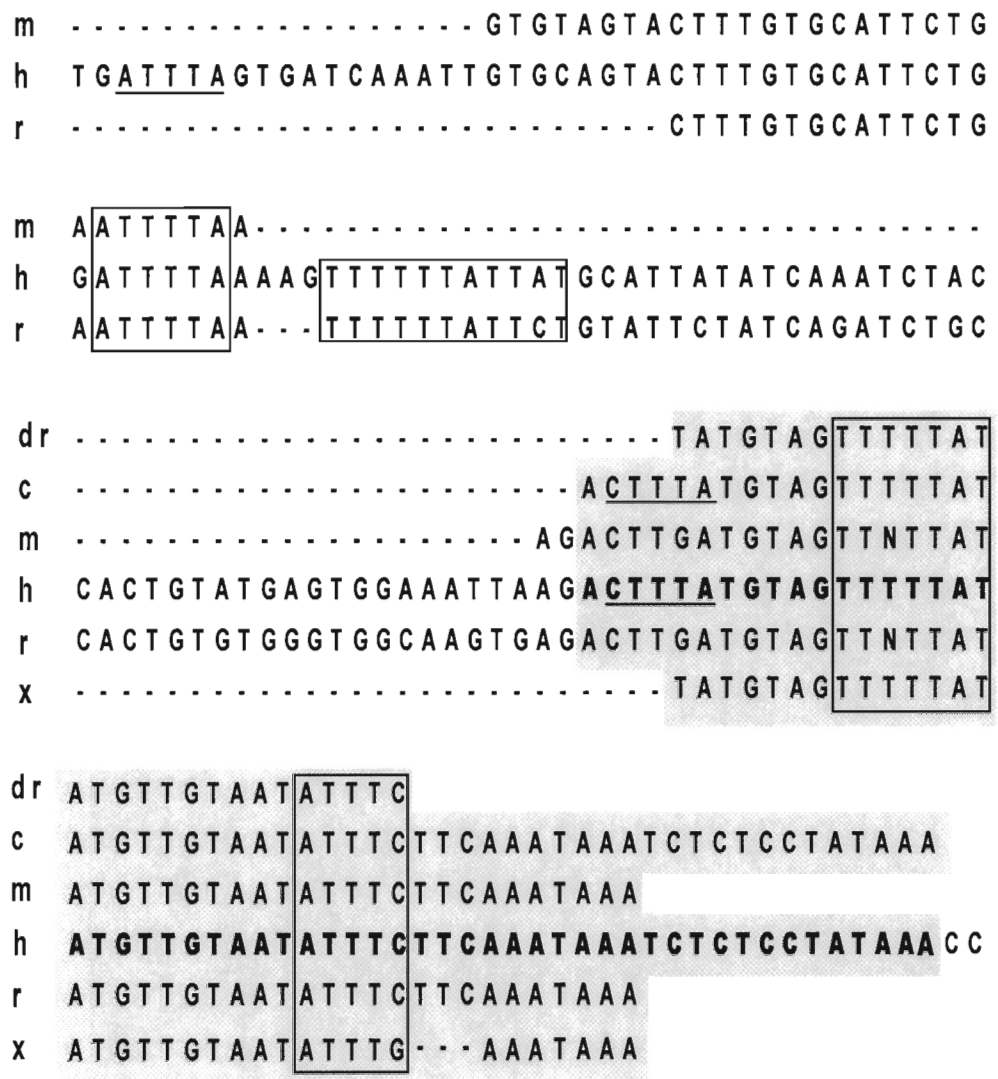
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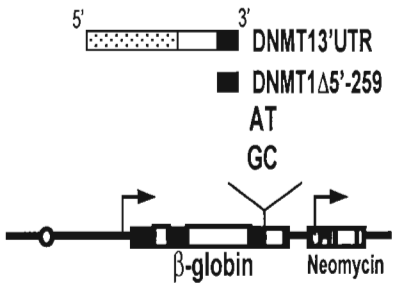
Fig. 3. The *DNMT1* 3'UTR downregulates mRNA levels in a growth-dependent manner. **A.** Map of the pRBG constructs. Constructs were used to create stable transfectants in Balb/c-3T3 cells. The site of insertion of the DNMT1 3' UTR, DNMT1Δ5'259, GM-CSF destabilizer (AT), and GC control sequences are indicated. **B.** Representative Northern analysis of chimeric DNMT13'UTR–β-globin expression. Balb/c-3T3 cells were transfected with either one of the following plasmids: pRBG-DNMT13'UTR, pRBG-DNMT1Δ 5'259, pRBG-AT or pRBG-GC, and stable G418 resistant transfectants were isolated as described in "materials and methods". RNA was extracted from growth-arrested cells (-), or from arrested cells that had been incubated in serum for 20 h (+). RNA was subjected to a Northern blot analysis using standard protocols and hybridization to a labeled β–globin probe (500 bp *Xho*I-*Bam*HI fragment from pRBG-GC). Blots were then stripped and rehybridized with an18s oligonucleotide probe. **C.** Quantification of β-*globin* mRNA expressed in the stable transfectants. The amount of β-*globin* mRNA expressed in each of the transfectants was quantified by scanning densitometry and normalized to the signal obtained following hybridization with an 18s probe. All β-*globin* levels were then calculated as a percentage of the GC control. The experiment was repeated with similar results. **D.** Map of the adenoviral constructs. *DNMT1* deletion constructs shown were cloned into the *Xba*1 site of pAdTrack-CMV. **E.** Representative Northern blot of infected Balb/c-3T3 cells. Arrested cells were infected with the indicated adenoviral constructs and were then either maintained in serum- deprived conditions (*arrested*), or stimulated with 10% serum for 20 h (*+serum*). RNA was extracted and subjected to a Northern blot analysis by

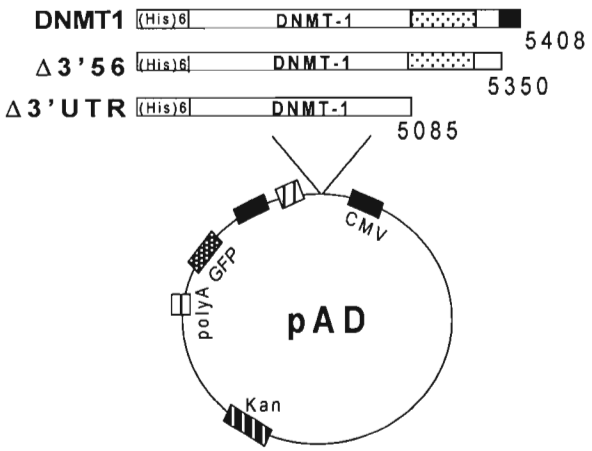
hybridization with a labeled *DNMT1* cDNA probe (bp 601-2191). Blots were then stripped and rehybridized with a probe for GFP (765 bp *Nhe*I-*Hind*III from pEGFP-C1).

F. Quantification of *DNMT1* mRNA levels in the infected cells. The amount of *DNMT1* mRNA expressed in each infection was quantified and normalized to the signal obtained following hybridization with the GFP probe. The graph shows an average of 3 independent experiments.

A


5'
3'
DNMT13'UTR
DNMT1Δ5'-259
AT
GC
β-globin
Neomycin

D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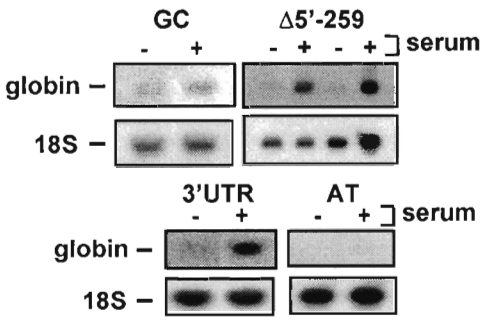
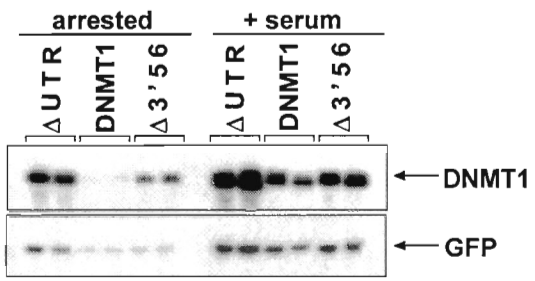
(His)6
DNMT-1
5408
Δ3'56
5350
Δ3'UTR
5085
CMV
GFP
polyA
pAD
Kan

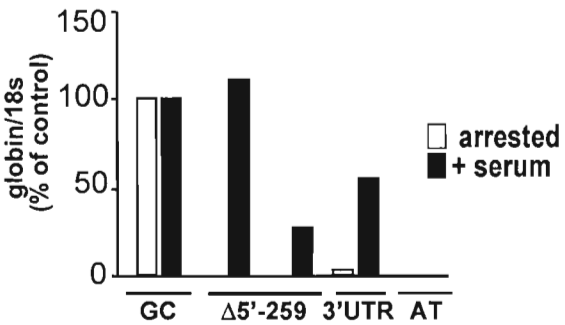
B


GC
Δ5'-259
serum
globin
18S
3'UTR
AT

E


arrested
+ serum
ΔUTR
DNMT1
Δ3'56
DNMT1
GFP

C


globin/18s
(% of control)
150
100
50
0
arrested
+ serum
GC
Δ5'-259
3'UTR
AT

F

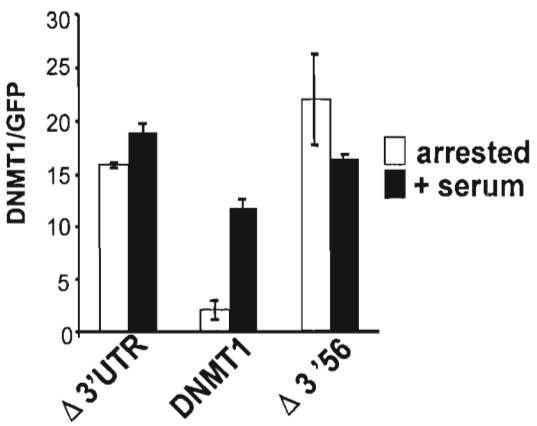

DNMT1/GFP
30
25
20
15
10
5
0
arrested
+ serum
Δ3'UTR
DNMT1
Δ3'56

Fig. 4. Specific RNA-protein interactions within the *DNMT1* 3'UTR. A. The different deletion fragments used to synthesize the RNA probes. The *solid box* indicates the 54-nucleotide highly conserved region. **B.** RNA mobility gel shift assay of arrested Balb/c-3T3 cells upon serum stimulation. An RNA gel shift assay was performed using ^{32}P-labeled *in vitro* transcribed *DNMT1* 3' UTR and extracts prepared from cells treated as in Fig.1A. Time indicates hours after serum stimulation of arrested cells. The complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. The complex specific to arrested cells is indicated by an *arrow*. **C.** UV crosslinking assay. Cells were arrested for 48 h (-) and serum-stimulated for 20 h (+). Extracts were then prepared and subjected to a UV crosslinking assay using a ^{32}P-labeled Δ5'-259 probe, and RNA-protein complexes were resolved on an SDS-polyacrylamide gel. Proteinase K treatments (*+PK*) were performed for 1 h at 37 °C. *Control* indicates reaction performed in absence of cell extract. **D.** Competition UV crosslinking. The assay was performed as above, using extracts prepared from arrested cells. Cold competitors were synthesized from templates shown in (A) and used in 1000x excess. **E and F.** Time course of p40 binding. The assay was performed as stated above, using extracts prepared from cells at various times after serum stimulation. p40 levels were quantified by densitometric scanning, and normalized to the total protein in each lane, which was measured by scanning the gels after staining with Coomassie Blue. A representative experiment is shown in (E), and an average of 3 experiments is shown in (F). **G.** Correlation between p40 binding, *dnmt1* mRNA levels, and cell growth. Results from Fig.1 and the results in (F) were plotted on the same graph to compare the pattern of p40 binding to *dnmt1* mRNA levels. In (C) -(E), the molecular weights in kilodaltons are indicated.

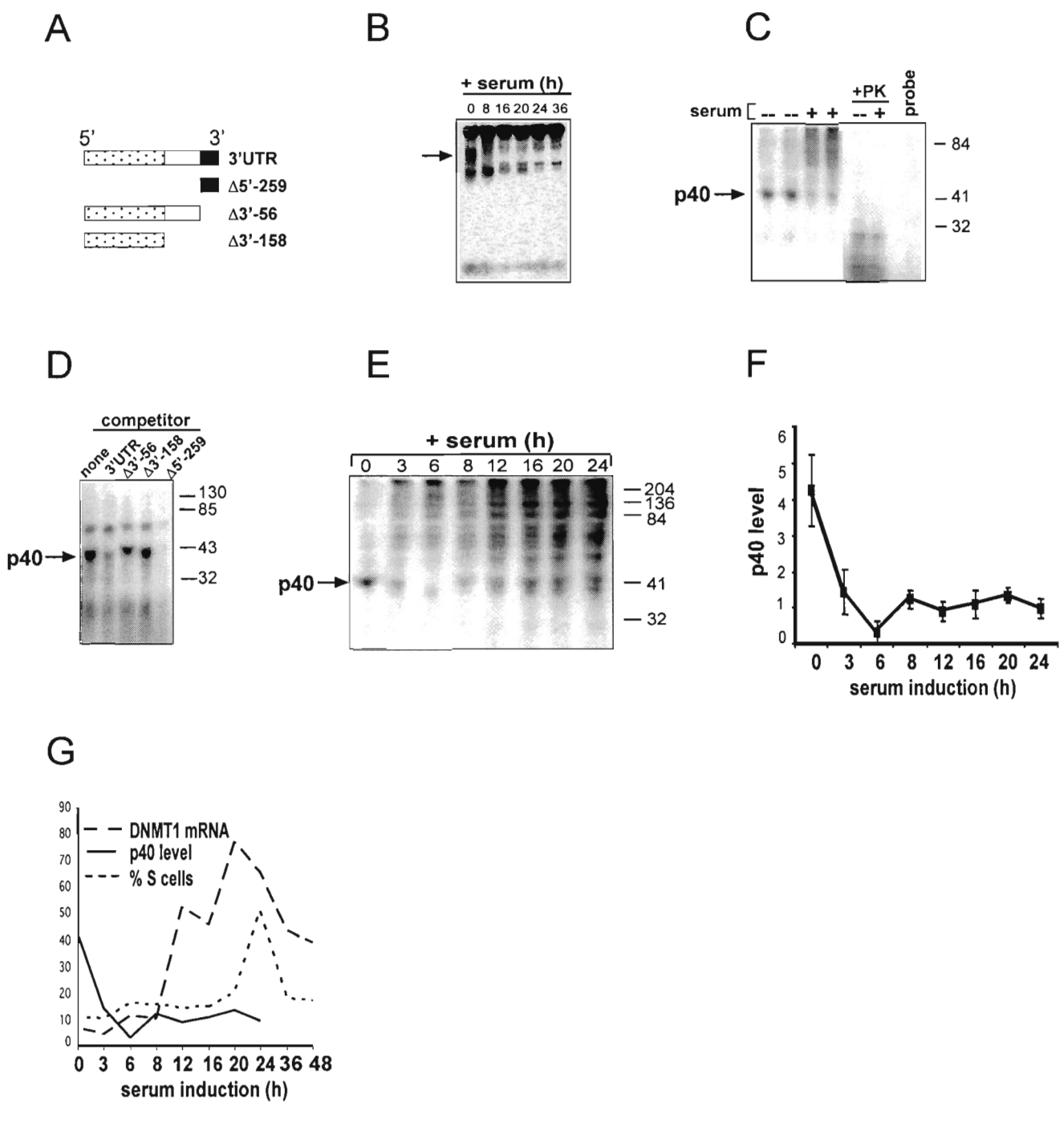

A
5'
3'
3'UTR
Δ5'-259
Δ3'-56
Δ3'-158
B
+ serum (h)
0 8 16 20 24 36
C
serum
+PK
probe
p40
84
41
32
D
competitor
none
3'UTR
Δ3'-56
Δ3'-158
Δ5'-259
p40
130
85
43
32
E
+ serum (h)
0 3 6 8 12 16 20 24
p40
204
136
84
41
32
F
p40 level
serum induction (h)
G
DNMT1 mRNA
p40 level
% S cells
serum induction (h)

Fig. 5. Differential *DNMT1* 3' UTR decay rates *in vitro*. A. *In vitro* decay assay using 3' UTR deletions and arrested cell extract. Radiolabeled 3'UTR RNA transcripts were incubated with 10 μg of whole cell extract from arrested Balb/c cells (*arrested*), or with incubation buffer alone (*control*) for various lengths of time and electrophoresed on an acrylamide gel. The signals were detected by autoradiography and quantified by densitometry. The constructs used and their sizes are indicated beside each experiment. **B.** Half-life measurements. Experiments in (A) were quantified and the signal at each time point was calculated as the percentage of RNA remaining relative to the RNA at time 0. The half-life was calculated as the time point when 50 % of the RNA had been degraded. *Squares*, Δ3'56; *diamonds*, 3'UTR; *circles*, Δ 3'158; *triangles*, Δ 5'259. **C.** *In vitro* decay assay using Δ5'259 and arrested or growth-stimulated extracts. Radiolabeled Δ5'259 transcript was incubated with 10 μg of whole cell extract from arrested or serum stimulated (+ *serum*) Balb/c cells for various lengths of time and processed as in (A). **D.** Δ 5'259 half-life measurements. The experiment in shown in (C) was quantified and half-lives were calculated as in (B). *Squares*, +serum; *diamonds*, -serum.

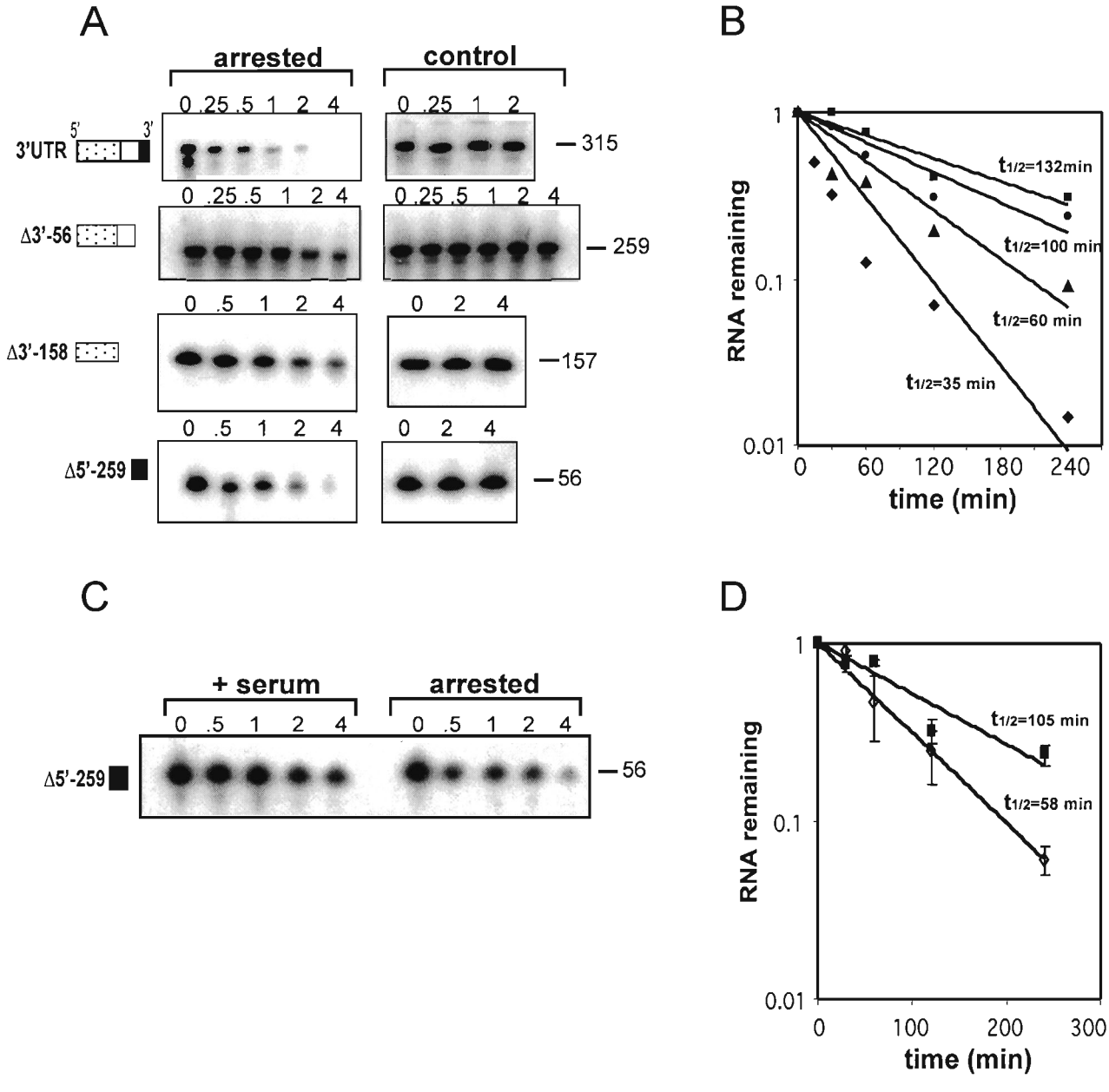
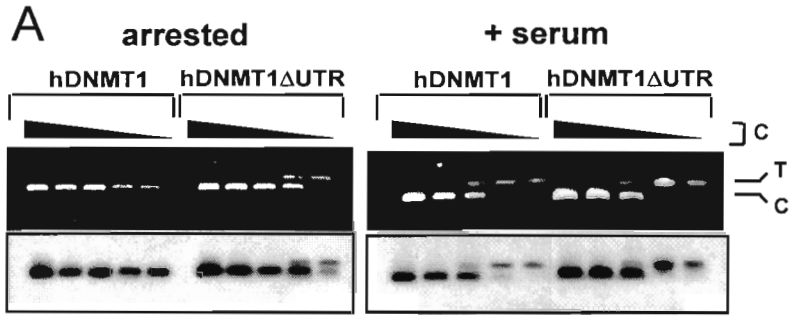
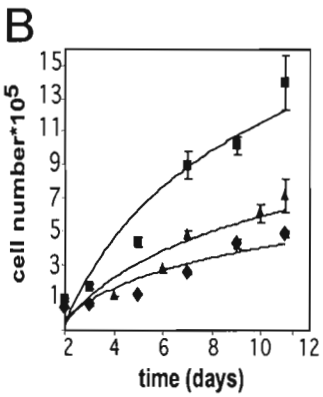
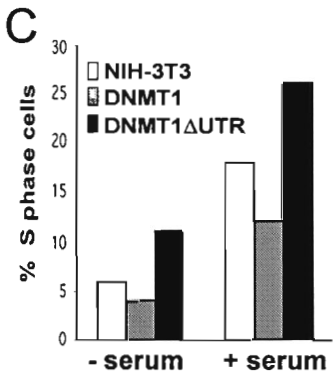
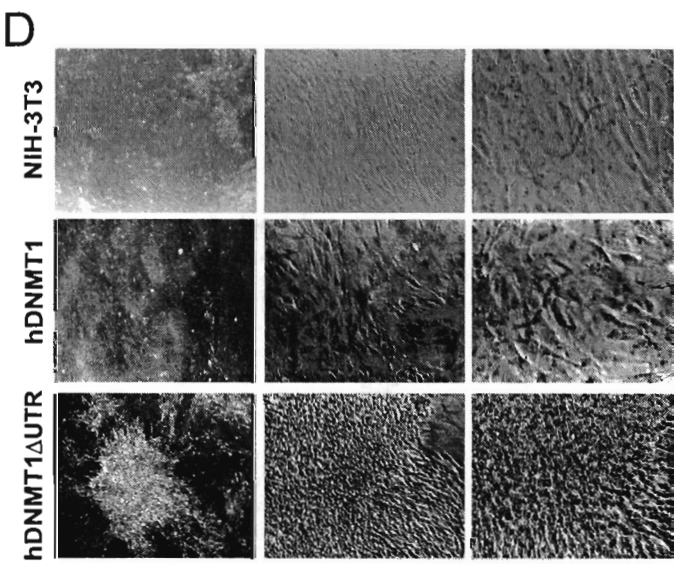

A
arrested
control
0 .25 .5 1 2 4
0 .25 1 2
3'UTR
5'
3'
315
Δ3'-56
259
0 .5 1 2 4
0 2 4
Δ3'-158
157
Δ5'-259
56
B
RNA remaining
1
0.1
0.01
0 60 120 180 240
time (min)
t1/2=132min
t1/2=100 min
t1/2=60 min
t1/2=35 min
C
+ serum
arrested
0 .5 1 2 4
Δ5'-259
56
D
RNA remaining
1
0.1
0.01
0 100 200 300
time (min)
t1/2=105 min
t1/2=58 min

Fig. 6. Effects of the 3' UTR on cell growth following ectopic expression of *DNMT1* in NIH-3T3 cells. A. Measurement of *DNMT1* mRNA levels by competitive PCR. 1μg of RNA from TetOff NIH-3T3 hDNMT1 or hDNMT1ΔUTR stable lines, either growth-arrested or serum-stimulated, was reverse transcribed and equal amounts of cDNA (as determined by [^{35}S]-dATP incorporation) were subjected to DNMT1 PCR amplification in the presence of decreasing concentrations (10^{-12} –10^{-16} nM) of a competitor DNA fragment (*upper panels*). To differentiate between the endogenous *dnmt1* and transfected *DNMT1*, the PCR was transferred to Hybond N+ membrane and hybridized with an oligonucleotide corresponding to bases 3595-3617 of the full length human *DNMT1* (*lower panels*). *T*, target; *C*, competitor. **B.** Growth curves of the stable and parent lines. Cells were plated in 6 -well culture dishes at a density of 25 000 cells per well (day 0). Cells were then counted on 6 subsequent occasions throughout the span of 11 days. An average of 4 measurements was obtained for each time point. *Squares*, hDNMT1ΔUTR; *triangles*, NIH-3T3; *diamonds*, hDNMT1. **C.** Percentage of cells in S phase of the cell cycle. Serum-starved (*-serum*) or growth-stimulated (+ *serum*) cells were harvested and stained with propidium iodide, and the percentage of cells in S phase was determined by flow cytometry. **D.** Morphology of hDNMT1 transfectants. Phase contrast light microscopy at increasing magnfications on day 11 of the growth curve described in (B). *Upper panels*, NIH-3T3; *middle panels*, hDNMT1; *bottom panels*, hDNMT1ΔUTR.


A
arrested
+ serum
hDNMT1
hDNMT1ΔUTR
hDNMT1
hDNMT1ΔUTR
C
T
C


B
cell number*10^5
15
13
11
9
7
5
3
1
2
4
6
8
10
12
time (days)


C
% S phase cells
30
25
20
15
10
5
0
NIH-3T3
DNMT1
DNMT1ΔUTR
- serum
+ serum


D
NIH-3T3
hDNMT1
hDNMT1ΔUTR

The preceding chapter identifies a novel regulatory element within the *DNMT1* mRNA that regulates its expression with cell growth, and provides evidence to suggest that deregulation of *DNMT1* expression with the cell cycle may contribute to cancer development. This provides an alternative explanation for DNMT1's role in cellular transformation, in addition to the previous models of tumor suppressor silencing, and suggests another level at which therapeutic intervention may be possible. With regards to tumorigenesis, the early findings of regional hypermethylation have resulted in the majority of studies focusing on the role of DNMT1. However, sine both regional hypermethylation and global hypomethylation coexist in cancer cells, understanding the mechanisms responsible for both processes is required for the proper design of therapeutic strategies targeting the DNA methylation machinery. The significance of this phenomenon has not received adequate attention, and relatively few investigations have determined the function MBD2/dMTase. Considering that this protein has been shown to demethylate DNA and is overexpressed in many cancers, it is highly possible that MBD2/dMTase is involved in the global hypomethylation characteristic of cancer progression. On the other hand, since other studies have found this protein to be a transcriptional repressor, it may also be involved in mediating regional hypermethylation and gene silencing. Since we must properly determine the function of MBD2/dMTase before we can understand its role in cancer, chapter 2 investigates this protein's apparently contradictory function. The data indicate that the ability of MBD2/dMTase to act as a demethylase and transcriptional activator is dependent on several parameters, which suggests that both the processes of global hypomethylation and local repression might involve the same multifunctional protein.

Chapter 2

Promoter Specific Activation and Demethylation by MBD2/ Demethylase

Nancy Detich, Johanne Theberge and Moshe Szyf

This chapter has been published in the Journal of Biological Chemistry (2002) 277, 35791-35794.

MBD2 is the only member of a family of methyl-CpG-binding proteins that has been reported to be both a transcriptional repressor and a DNA demethylase (dMTase). To understand the apparently contradictory function of MBD2/dMTase, we studied the effects of MBD2/dMTase overexpression on the activity of various *in vitro* methylated promoters transiently transfected into HEK 293 cells. We found that forced expression of a MBD2/dMTase expression vector (His-dMTase) differentially activated two methylated reporters, pSV40-CAT (the SV40 enhancerless promoter adjacent to the CAT reporter gene), and pGL2T+I4xTBRE (a region of the p21 promoter next to the luciferase reporter gene) in a time- and dose-dependent manner. His-dMTase increased pSV40-CAT expression by 3-10 fold after 96 hours, while pGL2T+I4xTBRE expression was increased by 2-3 fold after only 48 hours and did not further increase at 96 hours. Gene activation was not universal since no effect was seen with the p19-ARF promoter. We then assessed whether activation might be due to demethylation within the promoter region. Using bisulfite mapping we found that exogenous expression of His-dMTase induced demethylation at 8 of the 10 CpG sites within the SV40 promoter. The observation that His-dMTase increases the demethylase activity in the cells was also confirmed using an *in vitro* CpG demethylase assay with a mC32pG oligonucleotide substrate and purified Q-Sepharose fractions from HEK 293 cells transfected with His-dMTase or empty pcDNA3.1His vector. We propose that a single protein possessing both repressor and demethylase functions has evolved to coordinate a program that requires suppression of some methylated genes and activation of others.

INTRODUCTION

The epigenome consists of an additional component that is part of the covalent structure of the genome, a coating of methyl groups. In vertebrates, 80% of cytosine residues within the dinucleotide sequence CpG are modified by methylation in a pattern that is tissue specific and that is formed during development and maintained in somatic cells (Razin and Szyf, 1984). It has been well established that the DNA methylation pattern is maintained exclusively by DNA methyltransferase activities, but we have recently proposed that DNA demethylase activities might also participate in the process (Bhattacharya et al., 1999; Cervoni et al., 2002; Cervoni and Szyf, 2001) and that the methylation pattern is a steady state balance of reversible methylation-demethylation reactions (Szyf, 2001b; Szyf, 2001c). We have shown that histone acetylation promotes active demethylation of ectopically methylated genes (Cervoni and Szyf, 2001) and that inhibitors of histone acetylation inhibit demethylation (Cervoni et al., 2002).

It is well documented that the state of activity of a gene, the chromatin structure, and DNA methylation are correlated (Razin, 1998), such that areas of the genome that are methylated are usually less expressed. One molecular mechanism that explains this relationship has been recently elucidated. Repressor complexes are recruited to methylated DNA via the binding of methyl-CpG-binding domain proteins (MBDs). These complexes contain proteins that have histone deacetylase and chromatin remodeling activities, leading to the formation of a more compact and transcriptionally inactive chromatin (Nan et al., 1998). The earliest discovered MBD protein, MeCP2, has

been found to associate with the Sin3a corepressor complex and can also repress transcription in a histone deacetylase-independent manner (Yu et al., 2000). MBD1, MBD2, and MBD3 were later discovered and were also shown to be involved in transcriptional repression (for review see ref.(Ballestar and Wolffe, 2001)).

In contrast, we reported that MBD2 is an enzyme (dMTase) capable of actively demethylating DNA (Bhattacharya et al., 1999). This activity was shown both *in vitro* (Bhattacharya et al., 1999) and *in vivo* (Cervoni and Szyf, 2001). A demethylase is expected to activate genes by removing the repressive methyl residues. The assignment of a demethylase function to a protein that was independently discovered as a recruiter of repressor complexes has triggered obvious controversy in the field (Ng et al., 1999), and several groups reported that they failed to confirm the demethylase activity of MBD2 (Boeke et al., 2000; Ng et al., 1999; Wade et al., 1999).

In this study we tested the hypothesis that MBD2 is a multifunctional protein and that its activity might depend on the context of the promoter with which it interacts. By examining the effects of MBD2/dMTase expression on the activity of various reporter constructs methylated *in vitro* and transfected into HEK 293 cells, we found that MBD2/dMTase differentially activated some but not all promoters in a time- and concentration-dependent manner. Using bisulfite mapping, we found that exogenous expression of MBD2/dMTase induced demethylation within the SV40 promoter, and we also confirmed its demethylase activity *in vitro*. These data support our hypothesis that the complex functional role of this protein depends on the promoter context.

MATERIALS AND METHODS

In vitro methylation of substrates- Enhancerless pSV40-CAT (Genbank™ accession no. X65320), pGL2T+I4xTBRE (Datto et al., 1995), or p19-ARF-LUC (kindly provided by Dr. V. Lobanenkov) were methylated in vitro by incubating 10 μg of plasmid DNA with 12 units of Sss1 CpG methyltransferase (New England Biolabs) in the recommended buffer containing 800 μM S-adenosylmethionine (AdoMet) for 3 h at 37 °C. Another 12 units of Sss1 and 0.16 μmol of AdoMet were then added and the reaction was further incubated another 3 h. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection from HpaII digestion.

Transient transfections and reporter assays- HEK 293 cells were plated at a density of $7x10^4$/well in a six-well dish and transiently transfected with 100 ng of reporter plasmid (methylated or mock methylated) and 1.2 μg of either of the following plasmids: pcDNA 3.1HisB vector (Invitrogen), pcDNA-His-dMTase (Bhattacharya et al., 1999), which contains a His tagged human MBD2b/demethylase cDNA as described in the reference, AdTrack-MeCP2 (constructed from a GST-MeCP2 kindly provided by Dr. X. Nan (Nan et al., 1993)), or pcDNA 3.1-Sp1 (Vallian et al., 1998) using the calcium phosphate precipitation method as described previously (Rouleau et al., 1992). 0.3 μM Trichostatin A (TSA) was added 24 h post-transfection, and cells were harvested after 48 or 96 h. Chloramphenicol acetyltransferase (CAT) assays were performed as described (Rouleau et al., 1992), and luciferase activity was assessed using the Promega luciferase assay

system. The activity of each extract was measured in triplicate and then normalized to the protein concentration. Fold induction was calculated relative to the activity observed with the HisB vector alone. Experiments were performed several times using different cultures of HEK 293 cells and different preparations of plasmids. For dose curve experiments, transfections were performed in triplicate using increasing amounts of HisB or His-dMTase vector (0.05, 0.1, 0.6,1.2, 3 μg). Cells were then harvested after 96 h for CAT or luciferase assays. Fold induction was calculated relative to the activity observed with the HisB vector alone at each concentration.

Bisulfite mapping- Bisulfite mapping was performed as described (Clark et al., 1994) with minor modifications. The SV40 promoter sequence was amplified from 5 μg of sodium bisulfite-treated DNA using the following primers: 5'AAGGGGGATGTGTTGTAAG3' (sense), 5'CTAAAATACCTCAAAATATTCTT3' (antisense). PCR products were then used as templates for subsequent nested PCR reactions using the primers: 5'GGTTAGTGAATTTTAGATTTGT3' (sense), 5'TATATCCAATAATTTTTTTCTCC3' (antisense). PCR products were subcloned using the TA Cloning Kit (Invitrogen), and clones were then sequenced using the T7 sequencing kit (Amersham Biosciences).

Western blot analysis- Whole cell extracts were prepared using radioimmune precipitation assay buffer according to the protocol from Santa Cruz Biotechnology. 50 μg of extract was resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Transfected His-dMTase protein was detected either by anti-MBD2 sheep

polyclonal IgG (Upstate Biotechnologies no. 07198) according to the manufacturer's protocol, or by anti-Xpress mouse monoclonal IgG antibody (Invitrogen R910-25, which recognizes the Xpress epitope within the pcDNA3.1His vector) at 1:5000, followed by peroxidase-conjugated antimouse IgG (Jackson Immunoresearch) at 1:20 000. Enhanced chemiluminescence detection kit was used for both (Amersham Biosciences).

Extraction of demethylase activity- HEK 293 cells were transfected with 10 μg of His-dMTase/10 cm plate (x10) or left untransfected, and nuclear extracts were prepared 48 h later as described (Szyf and Bhattacharya, 2002a). Approximately 8.5 mg of extract (1.4 ml) was diluted to 50 mM NaCl with 10 ml of buffer L (10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.8) containing 1 μg/ml of each of the following protease inhibitors: Pefabloc, aprotinin, and leupeptin. 2 ml of Q-Sepharose beads (Amersham Biosciences) were washed 3x with 8 ml buffer L + 50 mM NaCl and then pre-equilibrated 30 min in the same buffer. Each extract (HEK or HEK+dMTase) was then subjected to 4 x 40 min bindings, each with 0.25 ml of Q-Sepharose, rotating at 4 °C. The beads were then pooled (1 ml/sample) and washed 5 x 10 min, each with 4 ml of buffer L + 50 mM NaCl. Batch elution was then performed with 5 x 1 ml buffer L, each containing the following concentrations of NaCl (M): 0.2, 0.4, 0.6, 0.8,1.0, for 10 min each. The different fractions, flow through, and washes were then assayed for demethylase activity.

Demethylation assay- Demethylase activity was measured using a methylated (m) C32pG oligonucleotide substrate as described (Szyf and Bhattacharya, 2002b) with minor modifications. 1 μl of a mC32pG oligonucleotide substrate ([α^{32}P]-dGTP-labeled) was

incubated with 30 µl of buffer L and 20 µl of each of the purification samples for 48 h at 37 °C. Samples were then subjected to a phenol/chloroform extraction followed by ethanol precipitation and resuspension in 8 µl of ddH_20. 1 µl of 10x micrococal nuclease buffer (250 mM Tris-HCl, 10 mM $CaCl_2$) and 1 µl of micrococcal nuclease were added, followed by an overnight incubation at 37 °C. 2 µl of each sample was then resolved by thin layer chromatography and visualized by autoradiography.

RESULTS

Recombinant MBD2/dMTase can activate certain promoters in a time-dependent manner- Since MBD2b was found to act as a DNA demethylase (Bhattacharya et al., 1999; Cervoni and Szyf, 2001), and since promoter demethylation is associated with gene activation (Plumb et al., 2000), we first wanted to determine whether ectopic expression of MBD2/dMTase would lead to promoter activation. We performed various co-transfection experiments using several *in vitro* methylated reporter constructs. A long line of data has established that *in vitro* methylation can suppress genes when ectopically introduced into vertebrate cells (Cedar et al., 1983). As expected, both the SV40 and 4xTBRE (a 4x repeat of the TGF-β response element of the p21 promoter) reporter constructs (Fig. 1A) were inhibited considerably by *in vitro* methylation (Fig. 1B). Co-transfection of His-dMTase led to activation of the SV40 promoter, but only after 96 hours, while the 4xTBRE promoter was activated almost equally at both 48 and 96 hours post-transfection. Similar results were obtained with TSA, which activates transcription by inhibiting histone deacetylases, and has also been found to induce DNA demethylation

(Cervoni and Szyf, 2001). Sp1 was used as a positive control for activation since both of these promoters are highly enriched in Sp1 sites (Fig. 1A). The transcriptional activation seen with MBD2/dMTase is not universal since exogenous MBD2/dMTase was not able to activate the p19-ARF promoter even after 96 hours (Fig. 1C). Thus MBD2/dMTase acts differently on different CG-rich ubiquitous promoters. If the corepressors required for suppressing methylated promoters by MBDs are saturated in HEK 293 cells, then this might explain why MBD2 does not suppress methylated promoters in these cells. To exclude this possibility, we tested whether another member of the MBD family, MeCP2, could repress these methylated promoters. The results shown in Fig. 1D illustrate that MeCP2 represses the SV40 promoter, consistent with previous findings (Yu et al., 2000), and thus the machinery necessary for repressing methylated genes by MBDs is active in HEK 293 cells. In summary, our results show that MBD2/dMTase can differentially activate certain methylated promoters.

Dose-dependent activation by MBD2/dMTase- To provide further evidence that MBD2/dMTase can act as a transcriptional activator, we transfected the methylated SV40 or 4xTBRE reporters along with different amounts of pcDNA3.1-His-dMTase. As seen in Fig.2, A and B, while we observed a general dose-dependent increase in activation of both promoters, these promoters also varied slightly in their sensitivity to the amount of transfected His-dMTase as well as in the magnitude of their response, similar to the observations presented in Fig. 1. A Western blot using an antibody against MBD2 confirms that the His-dMTase construct is expressed in HEK 293 cells (Fig. 2C) and demonstrates that the protein levels correlate with the transcriptional activation observed.

These results, together with those in Fig. 1, indicate that MBD2/dMTase can function as a dose-dependent activator of gene transcription, which is both time- and promoter-dependent.

Purified His-dMTase from HEK 293 cells exhibits in vitro demethylase activity- One explanation for the transcriptional activation by His-dMTase is that it causes promoter demethylation. To demonstrate that the His-dMTase construct produces an active demethylase enzyme in our system, we subjected nuclear extracts from HEK 293 cells transfected with His-dMTase to chromatography on Q-Sepharose to partially purify the demethylase, and compared the demethylase activity to untransfected HEK 293 cells. Fractions were eluted with a step gradient of NaCl, tested for the presence of His-dMTase by a Western blot using anti-Xpress antibody, and also assayed for demethylase activity. The latter was carried out by measuring the conversion of mdCMP to dCMP within a 24 bp mC32pG double stranded oligonucleotide substrate using thin layer chromatography. As shown in Fig. 3A, His-dMTase elutes at 0.2 and 0.4 M NaCl. These fractions exhibit a significant increase in demethylase activity: 42% of the mdCMP is converted to dCMP in the 0.2 M fraction in comparison to the untransfected control, which displays minimal demethylase activity (<5% in the 0.2 M fraction) (Fig. 3, B and C).

Expression of His-dMTase increases demethylation of the SV40 promoter region- Since demethylation within a promoter is associated with transcriptional activation (Plumb et al., 2000), we next wanted to determine if this was a possible mechanism by which MBD2/dMTase overexpression led to the activation of the SV40 promoter.

Following transfection of methylated SV40-CAT along with His-dMTase or empty vector as a control, we used bisulfite mapping to examine the 10 different CpG sites within the SV40 promoter (Fig. 4A). As shown in Fig. 4B, expression of His-dMTase increased the level of demethylation at 8 out of the 10 CpG sites. Remarkably, most of the CpG sites remained fully or almost (<5%) fully methylated in the control clones, with the exception of site 6. Since between 20-30 clones were sequenced from 3 independent experiments, it is highly unlikely that this is a random event. These results are also consistent with previous studies demonstrating that exogenous expression of MBD2/dMTase leads to increased demethylation within a promoter (Cervoni and Szyf, 2001) and that expression of MBD2/dMTase is correlated with demethylation within the promoters of *c-erbB-2* and *survivin* genes (Hattori et al., 2001). We also assessed the CAT activity from the same transfections used for bisulfite mapping and found that, as in our previous experiments, dMTase overexpression led to transcriptional activation of the SV40 promoter (Fig. 4*C*). This supports the hypothesis that the likely mechanism by which MBD2/dMTase causes transcriptional activation is by demethylating the promoter and allowing an open chromatin configuration free of transcriptional repressor complexes.

DISCUSSION

Our paper provides evidence that MBD2/dMTase can act as a transcriptional activator, consistent with its role as a DNA demethylase. However, the activation observed is critically dependent on several parameters, including promoter identity and length of

transfection time (Fig.1). Our results are consistent with previous data demonstrating that MBD2/dMTase is an active demethylase *in vitro* (Bhattacharya et al., 1999), and that transfection of exogenous dMTase can lead to demethylation in living cells with a concomitant increase in gene expression (Cervoni and Szyf, 2001).

The activation of methylated promoters by MBD2/dMTase is partial as is the demethylation. This suggests the presence of other factors that protect the DNA from complete demethylation and activation of transcription.

Although it is accepted in recent publications that MBD2 acts as a transcriptional repressor (Boeke et al., 2000; Feng and Zhang, 2001; Ng et al., 1999; Sekimata et al., 2001), the data presented here do not necessarily contradict previously published data once the experimental conditions stated in these reports are carefully considered. First, in all the studies demonstrating repression by MBD2/dMTase, transcriptional assays were performed anywhere from 24 to 48 hours post-transfection. Since we do not see activation of the SV40 promoter until 96 hours post-transfection, it is possible that MBD2/dMTase could activate some of the promoters in these reports provided that the transfection time is extended. The fact that a longer time is necessary for activation by MBD2/dMTase may be explained by previous data showing that demethylation of ectopically methylated DNA in living cells is a slow process (Cervoni and Szyf, 2001). Second, previous studies (Ng et al., 1999; Sekimata et al., 2001) used the Gal4 DNA binding domain to bind MBD2-GAL4 chimeras to their promoters rather that looking at the effect of cognate MBD2 on methylated DNA as done in this study; this may also

account for some of the discrepancies. Third, not all promoters are repressed by MBD2 and not all concentrations of transfected MBD2 bring about repression. For example, Boeke *et al.* (2000) demonstrated that although the TK promoter was repressed by methylation, transfection of MBD2 had no effect; and in a study by Sekimata *et al.* (2001), certain concentrations of transfected Gal4-MBD2 alone did not repress their reporter construct. The data presented above are consistent with our findings that MBD2/dMTase had no effect on the p19-ARF promoter (Fig. 1C) and that the effect on transcription we observed is dose dependent, where the dose causing activation varies with the promoter type (Fig. 2, A and B). Last, although MBD2 has been found to associate with the NuRD repressor complex (Feng and Zhang, 2001), it was not originally purified as part of this complex (Zhang et al., 1998). Also, there is evidence that the NuRD complex may associate with different DNA binding proteins, not only MBD2, depending on the physiological state of the cell (Feng and Zhang, 2001). Thus, it is possible that under certain cellular conditions and within certain promoters MBD2/dMTase may act as a transcriptional repressor by recruiting the NuRD complex. However, it is equally possible that in a different cell environment and within different promoters MBD2 may act independently of NuRD as a demethylase and activator. In support of the latter, expression of MBD2/dMTase is correlated with demethylation within the promoter of *c-erbB-2* and *survivin* genes (Hattori et al., 2001). In addition, a recent report demonstrated that the Drosophila homolog of MBD2, dMBD2/3, formed foci that associated with DNA at the cellular blastoderm stage, concurrent with the activation of the embryonic genome, and also associated with the active Y chromosome (Marhold et al., 2002).

Several proteins have been found to possess dual repressor and activator functions, such as E2F (Muller and Helin, 2000), ATF4 (Hai and Hartman, 2001), and the Sp family of proteins (Black et al., 2001), so it is similarly feasible that MBD2 is a protein with dual functions. It is possible that both repressor and demethylase functions reside in one protein to coordinate a program of gene expression that requires suppression of some methylated genes and activation of others. Further studies will be necessary to determine what are the key factors involved in determining the role of MBD2/dMTase in transcription.

Acknowledgements:

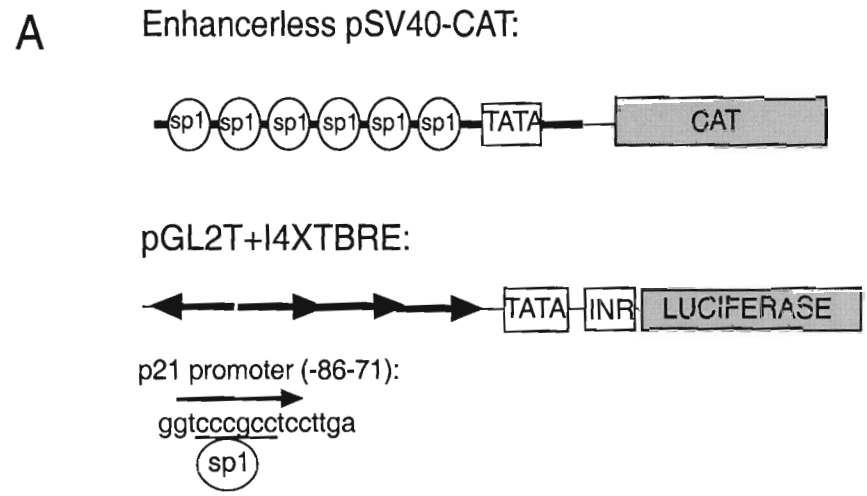
This research was supported by the Canadian Institute of Health Research and the National Cancer Institute of Canada.

N.D. is a recipient of the Canadian Institute of Health Research Doctoral Fellowship.

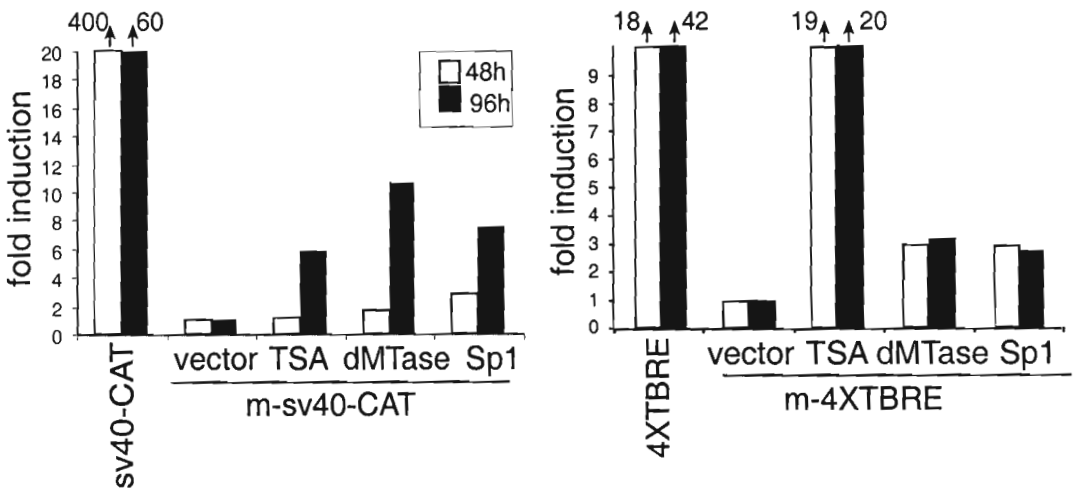
FIGURE LEGENDS

Fig. 1. Recombinant MBD2/dMTase can activate certain promoters in a time-dependent manner. A. Structure of the pSV40-CAT and pGL2T+I4xTBRE constructs. **B, C, and D.** HEK 293 cells were transiently transfected with SV40-CAT, pGL2T+I4xTBRE, or p19-ARF-LUC reporter plasmids (m, methylated in vitro with Sss1), and either empty HisB vector or plasmids expressing His-dMTase, MeCP2 or Sp1 using the calcium phosphate precipitation method. 0.3 μM TSA was added 24 h post-transfection. Cells were harvested after 48 or 96 h and each extract was assayed for CAT or luciferase activity and then normalized to the protein concentration. Fold induction was calculated relative to the activity observed with the HisB vector alone. Experiments were performed several times using different cultures of HEK 293 cells and different preparations of plasmids with similar results.

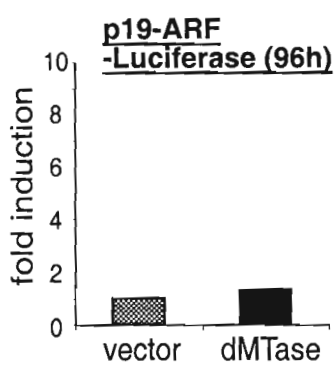
A


Enhancerless pSV40-CAT:
sp1
sp1
sp1
sp1
sp1
sp1
TATA
CAT
pGL2T+I4XTBRE:
TATA
INR
LUCIFERASE
p21 promoter (-86-71):
ggtcccgcctccttga
sp1

B


400
60
48h
96h
fold induction
sv40-CAT
vector TSA dMTase Sp1
m-sv40-CAT
18
42
19
20
4XTBRE
vector TSA dMTase Sp1
m-4XTBRE

C


p19-ARF
-Luciferase (96h)
fold induction
vector
dMTase

D

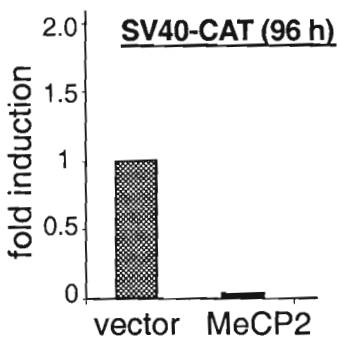

SV40-CAT (96 h)
fold induction
vector
MeCP2

Fig. 2. Dose-dependent activation by MBD2/dMTase. A and B. Transfections were performed as in Fig. 1 using increasing amounts of HisB or His-dMTase vector. Cells were harvested after 96 h for CAT or luciferase assays. Fold induction was calculated relative to the activity observed with the HisB vector alone at each concentration. **C.** Cells were transfected as in (A) and cell extracts were subjected to Western blotting using anti-MBD2 antibody (Upstate Biotechnologies).

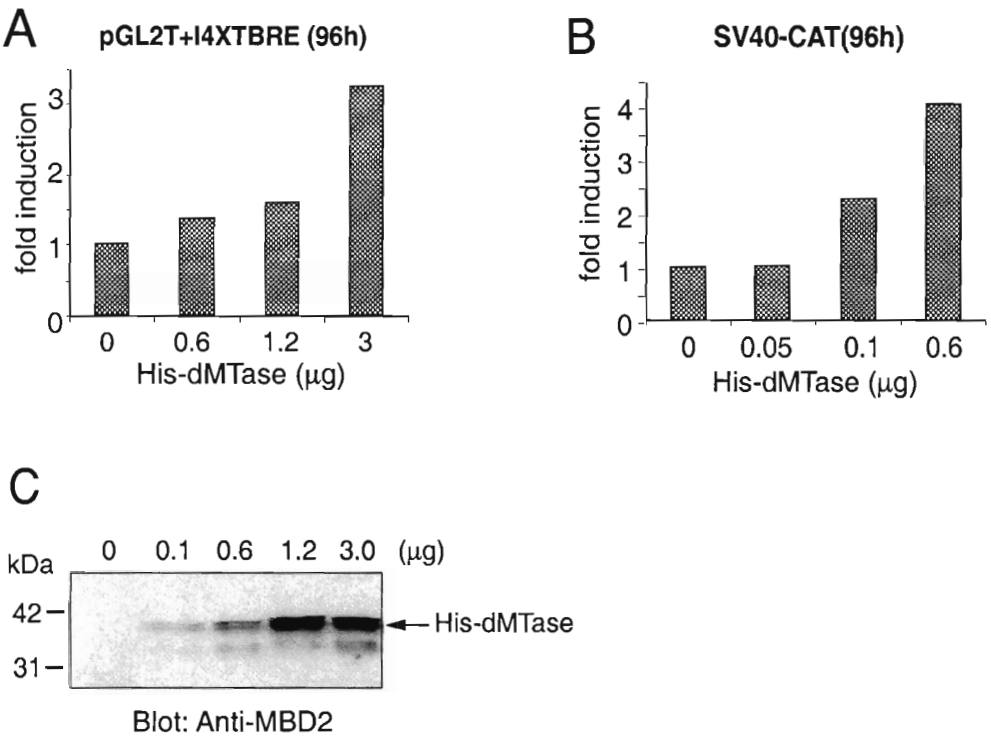
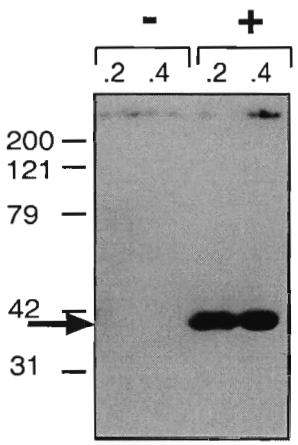
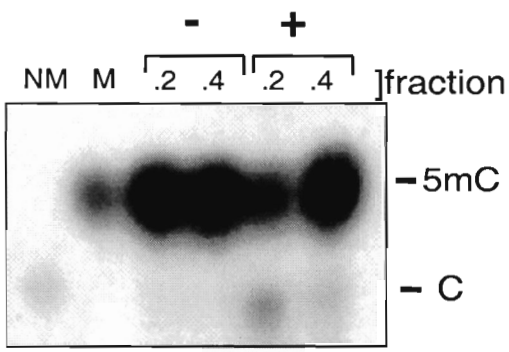

A
pGL2T+I4XTBRE (96h)
fold induction
0
1
2
3
0
0.6
1.2
3
His-dMTase (μg)
B
SV40-CAT(96h)
fold induction
0
1
2
3
4
0
0.05
0.1
0.6
His-dMTase (μg)
C
kDa
0
0.1
0.6
1.2
3.0
(μg)
42
31
His-dMTase
Blot: Anti-MBD2

Fig. 3. Purified His-dMTase from HEK 293 cells exhibits *in vitro* demethylase activity. A. HEK 293 cells were transfected with His-dMTase (+) or harvested untransfected (-). Nuclear extracts were subjected to chromatography on Q-Sepharose and the active demethylase fractions were eluted with a step gradient of NaCl. His-dMTase elutes at 0.2 and 0.4 M NaCl steps. The fractions were tested by a Western blot using anti-Xpress antibody (Invitrogen) to demonstrate the presence of His-dMTase in the active fractions (indicated by the *arrow*). **B.** The fractions were assayed for demethylase activity using a mC32pG oligonucleotide substrate. Following demethylation, the DNA was digested to 3'-mononucleotides and separated on thin layer chromatography. *M*, methylated control; *NM*, non-methylated control; *5mC*, 5-methyldeoxycytidine 3'-monophoshpate; *C*, deoxycytidine 3'-monophoshpate. **C.** The 0.2 M NaCl fraction was assayed in triplicate and the degree of demethylation was quantified by densitometry.

A


-
+
.2
.4
.2
.4
200
121
79
42
31

B


-
+
NM
M
.2
.4
.2
.4
]fraction
5mC
C

C

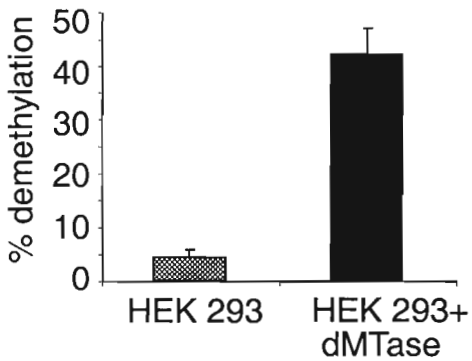
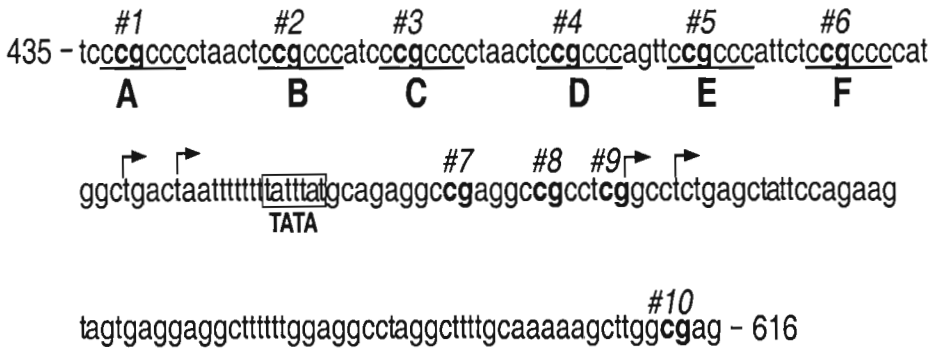

% demethylation
50
40
30
20
10
0
HEK 293
HEK 293+
dMTase

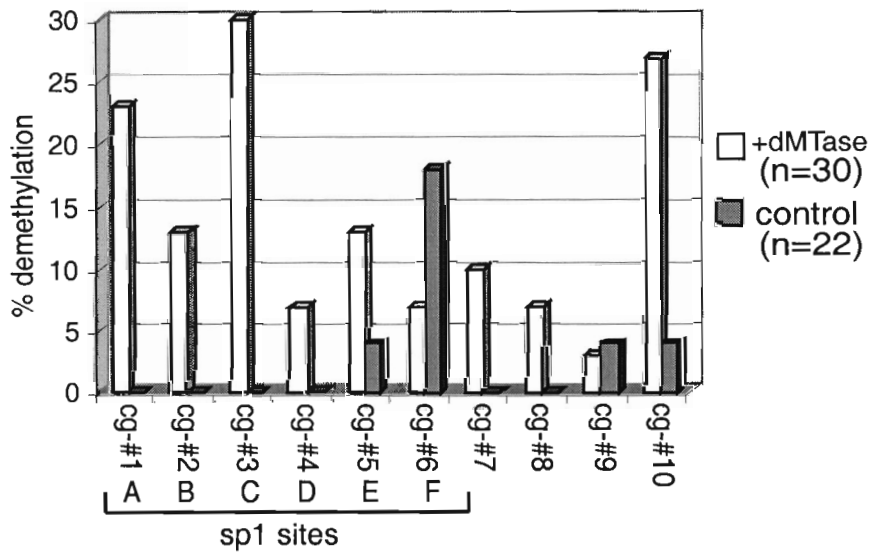
Fig. 4. Expression of His-dMTase increases demethylation of the SV40 promoter.

A. Nucleotide sequence of SV40 promoter region analyzed by bisulfite mapping. CpG dinucleotides whose methylation status was determined are numbered (*1-10*) and Sp1 sites are marked *A-F*. **B.** The SV40 construct in Fig. 1A was methylated *in vitro* and transfected into HEK 293 cells with either His-dMTase (+*dMTase*) or HisB vector (*control*). Cells were harvested 96 h post-transfection, and DNA was isolated and treated with sodium bisulfite. The region in (A) was amplified by PCR, subcloned, and sequenced. The graph indicates the average percentage of demethylation at each CpG site. Results are an average of 3 independent experiments, where *n* is the total number of clones sequenced. **C.** Representative CAT assay from one of the three experiments analyzed by bisulfite mapping in (B), *a.u.*, arbitrary units.

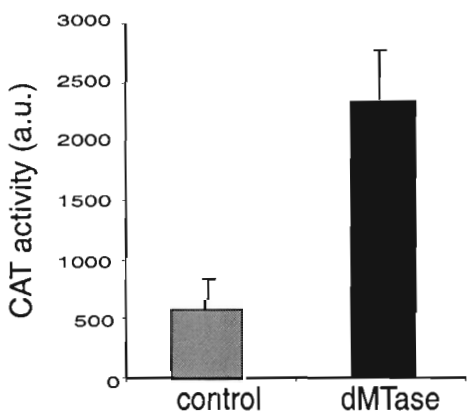
A


#1 #2 #3 #4 #5 #6
435 - tccgccctaactccgcccatccgccctaactccgcccagttccgcccattctccgccccat
A B C D E F
#7 #8 #9
ggctgactaatttttttatttatgcagaggccgaggccgcctcggcctctgagctattccagaag
TATA
#10
tagtgaggaggctttttggaggcctaggcttttgcaaaaagcttggcgag - 616

B


% demethylation
30
25
20
15
10
5
0
cg-#1
cg-#2
cg-#3
cg-#4
cg-#5
cg-#6
cg-#7
cg-#8
cg-#9
cg-#10
A
B
C
D
E
F
sp1 sites
+dMTase (n=30)
control (n=22)

C


CAT activity (a.u.)
3000
2500
2000
1500
1000
500
0
control
dMTase

Data presented in the former chapter confirm that MBD2/dMTase can demethylate DNA and activate gene expression, and further indicate that the promoter context may be critical. The existence of such an enzyme points to the possibility that DNA methylation is a reversible process and that the methylation pattern is a balance of DNA methylation and demethylation reactions. An increase in DNA methylation could therefore be accomplished not only by stimulating DNMTs but also by inhibiting demethylases. Although this concept of reversibility has long been accepted for other biological modifications, it has not been considered as a potential mechanism for the modulation of DNA methylation by physiological and pharmacological agents. However, if it is possible to correct aberrant methylation patterns by regulating the action of demethylases, then we are provided with another opportunity for therapy in diseases such as cancer. Chapter 3 identifies the first inhibitor of demethylases *in vitro* and in living cells, S-adenosylmethionine (AdoMet). While the prevailing model has been that increased AdoMet levels augment DNA methylation by stimulating DNMT activity, the data presented in the following manuscript leads to the conclusion that AdoMet might increase DNA methylation by inhibiting active demethylation. In addition, AdoMet is also able to revert cellular transformation, suggesting that hypomethylation triggered by demethylases may indeed be critical for tumorigenesis.

Chapter 3

The methyl donor S-adenosylmethionine inhibits active demethylation of DNA; a candidate novel mechanism for the pharmacological effects of S-adenosylmethionine.

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This chapter has been published in The Journal of Biological Chemistry, Apr 3, 2003, JBC Papers in Press, M211813200.

*The first two authors contributed equally to the manuscript. Authors are listed by alphabetical order.

S-adenosylmethionine (AdoMet) is the methyl donor of numerous methylation reactions. Exogenous administration of AdoMet was shown to cause hypermethylation of DNA and to protect against liver disease and liver cancer in rodent models. The current model is that an increased concentration of AdoMet stimulates DNA methyltransferase (DNMT) reactions, triggering hypermethylation and protecting the genome against global hypomethylation, a hallmark of cancer. Using an assay of active demethylation in HEK 293 cells, we show that AdoMet inhibits active demethylation and expression of an ectopically methylated CMV-GFP plasmid in a dose dependent manner. The inhibition of GFP expression is specific to methylated GFP; AdoMet does not inhibit an identical but unmethylated CMV-GFP plasmid. S-adenosylhomocysteine (AdoHcy), the product of methyltransferase reactions utilizing AdoMet, does not inhibit demethylation or expression of CMV-GFP. *In vitro*, AdoMet but not AdoHcy inhibits methyl-CpG-binding protein 2/DNA demethylase (MBD2/dMTase) as well as endogenous demethylase activity extracted from HEK 293, suggesting that AdoMet directly inhibits demethylase activity and that the methyl residue on AdoMet is required for its interaction with demethylase. We also demonstrate that AdoMet inhibits anchorage independent growth, an indicator of tumorigenesis, of HEK 293 and A549 cells. Taken together, our data support an alternative mechanism of action for AdoMet as an inhibitor of intracellular demethylase activity, which results in hypermethylation of DNA. The antitumorigenic effects of AdoMet are consistent with the hypothesis that demethylase activity is required for tumorigenesis.

INTRODUCTION

S-Adenosylmethionine (AdoMet) is the main methyl donor in numerous methyltransferase reactions in all organisms (Lu, 2000). The reduced derivative of 5,10-methylenetetrahydrofolate, 5-methyltetrahydrofolate, provides the methyl group for methionine and AdoMet synthesis (Finkelstein, 2000). A series of rodent experiments as well as epidemiological data have suggested a correlation between diets deficient in folate or in sources of methyl groups (i.e. foods containing methionine, one-carbon compounds, and choline) and the risk for colorectal adenomas and cancer (Potter, 2002). Such diets, referred to collectively as methyl-deficient, have been shown to promote liver cancer in rodents (Mato et al., 1997; Poirier, 2002), while AdoMet treatment was shown to prevent the development of liver cancer in rat (Pascale et al., 2002).

In light of the clinical and epidemiological data suggesting a link between AdoMet levels and cancer, it is important to understand the tumor protective mechanism of action of AdoMet, as well as the tumor promoting action of methyl-deficient diets. This is of importance not only for realizing the therapeutic potential AdoMet, but also for unraveling basic mechanisms of tumorigenesis, especially the role of methyl group metabolism. AdoMet is the cofactor for transmethylation reactions including DNA methylation (Cheng and Roberts, 2001; Chiang et al., 1996), while S-adenosylhomocysteine (AdoHcy) is the product of transmethylation reactions and an inhibitor of DNA methyltransferases (DNMT)(Chiang, 1998). A current model is that exogenous administration of AdoMet increases the intracellular ratio of AdoMet to

AdoHcy, thus stimulating DNMT activity resulting in increased DNA methylation (Garcea et al., 1989; Pascale et al., 2002). An increase in AdoHcy concentrations, even without a concomitant reduction in AdoMet, results in inhibition of DNMT and DNA hypomethylation (Caudill et al., 2001). Methyl-deficient diets decrease intracellular AdoMet concentration, increase AdoHcy concentrations, and trigger DNA hypomethylation (Pogribny et al., 1995; Poirier, 2002; Steinmetz et al., 1998). A genetic link was established between polymorphisms in the *Methylenetetrahydrofolate Reductase* gene encoding the enzyme catalyzing the synthesis of 5-methyltetrahydrofolate, and DNA hypomethylation (Friso et al., 2002; Stern et al., 2000). Global hypomethylation of DNA is a hallmark of cancer (Ehrlich, 2002a; Feinberg et al., 1988). If the mechanism of action of methyl-rich diets in cancer chemoprevention and methyl-deficient diet in cancer promotion is through changing genomic methylation status, then it implies that global hypomethylation plays a causal role in cancer. This hypothesis is supported by the observation that 5-azaC, a DNMT inhibitor (Jones and Taylor, 1980) can reverse AdoMet mediated chemoprevention of liver carcinogenesis (Pascale et al., 1991).

Although it has been controversial, there is now little doubt (Lu, 2000) that exogenous AdoMet increases the intracellular AdoMet levels. AdoMet uptake into cells has also been verified through a high performance liquid chromatography analysis (Watson et al., 1999). A number of data support the notion that exogenous AdoMet causes hypermethylation of DNA (Fuso et al., 2001; Garcea et al., 1989).

Whereas this model provides an attractively simple explanation as to the possible relationship between exogenous AdoMet administration and DNA methylation, there are

a number of unresolved issues. First, increased AdoMet should increase DNMT activity only if the normal intracellular concentration of AdoMet is below the *Km* for the enzyme, which has not been demonstrated as of yet. However, it is possible that the main mechanism by which elevating AdoMet levels increases DNMT activity is by competing with AdoHcy, an inhibitor of DNMT. Such an indirect mechanism of activation might be relevant even if the basal level of AdoMet is above the *Km*. Second, even if exogenous administration of AdoMet increases the activity of DNMT, it is not clear whether the normal level of enzyme is limiting or whether the specificity of DNA methylation patterns is determined by the molecular activity of DNMT. There is no evidence to suggest that specific sites remain unmethylated in vertebrate genomes simply for the reason that the molecular activity of DNMT is limiting. Third, methyl-deficient diets cause hypomethylation in the liver whose cells are mostly postmitotic and do not replicate (Poirier, 2002). If the mechanism of this hypomethylation involves only inhibition of DNMT, it could take effect only in cells that actively synthesize DNA. Since measurable demethylation is seen with these diets, this could only occur if a significant fraction of liver cells proliferate during the treatment. Whereas an increase in proliferation in the liver is seen as a consequence of methyl-deficient diets, it is not clear whether proliferation precedes or follows global hypomethylation.

The current hypothesis on the mechanism of action of AdoMet is based on the assumption that DNA methylation is a unidirectional and irreversible reaction, which is catalyzed by DNMT exclusively. However, an increasing list of data supports the hypothesis that DNA methylation *in vivo* is also fashioned by demethylase activity

(Oswald et al., 2000). A DNA demethylase activity was partially purified from human lung carcinoma A549 cells (Ramchandani et al., 1999) and the protein MBD2 was shown to bear demethylase activity (Bhattacharya et al., 1999). The demethylase activity of MBD2 was disputed by several groups (Ng et al., 1999; Zhang et al., 1999), but our recent data have demonstrated that ectopic MBD2/dMTase causes DNA demethylation in a promoter specific manner (Detich et al., 2002). We therefore propose that DNA methylation is a reversible reaction, and that the steady state DNA methylation status of a gene reflects a balance of methylation and demethylation (Szyf, 2001a; Szyf, 2001b). Thus, it is possible that AdoMet increases methylation by inhibiting demethylation.

It is impossible to determine whether hypermethylation of a gene *in vivo* following a chronic drug treatment is caused by either an increase in DNMT activity, as proposed by the current model, or inhibition of DNA demethylase. A model system is required to study either methylation or demethylation in isolation from the reverse activity. We have recently described such a model (Fig. 1). When an unmethylated CMV-GFP plasmid is transiently transfected into the human embryonal kidney cell line HEK 293, it remains unmethylated throughout the transient transfection period up to 96 hours, demonstrating that DNMTs do not target extrachromosomal DNA under the conditions of our experiment (Cervoni and Szyf, 2001). When an *in vitro* methylated CMV-GFP plasmid is transiently transfected into these cells, it generally remains methylated. However, when histone acetylation is induced by Trichostatin A (TSA), the plasmid is fully and actively demethylated by an endogenous demethylase activity. Since the plasmid does not replicate during the time frame of the experiment, this assay measures only active

demethylation (Cervoni and Szyf, 2001). This system thus allows us to determine the impact that different factors might have on demethylase activity. We have recently used this assay to illustrate that a protein that inhibits histone acetylation inhibits active demethylation in living cells (Cervoni et al., 2002).

In this study we took advantage of this assay to test the hypothesis that exogenous administration of AdoMet inhibits demethylase activity in living cells. Using an *in vitro* demethylase assay, we then tested whether AdoMet inhibits recombinant MBD2/dMTase activity extracted from infected SF9 insect cells, as well as endogenous demethylase activity from HEK 293 cells. We also show that concentrations of exogenous AdoMet that inhibit demethylase activity also inhibit the transformation potential of HEK 293 and A549 cells *in vitro*, as determined by a soft agar assay. Taken together, our results support an alternative hypothesis for the mechanism of action of AdoMet as a DNA hypermethylating and an antitumorigenic agent.

MATERIALS AND METHODS

In vitro methylation of substrates- CMV-GFP (pEGFP-C1 from Clontech; Genbank™ accession no. U55763) was methylated *in vitro* by incubating 10 μg of plasmid DNA with 12 units of SssI CpG methyltransferase (New England Biolabs) in the recommended buffer containing 800 μM AdoMet for 3 h at 37 °C. Another 12 units of SssI and 0.16 μmol of AdoMet were then added and the reaction was further incubated for an additional 3 h. The methylated plasmid was recovered by phenol/chloroform extraction

and ethanol precipitation, and complete methylation was confirmed by observing full protection from *Hpa*II digestion.

Cell culture and transient transfections – Human embryonal kidney HEK 293 cells (ATCC CRL 1573) were plated at a density of $7.5x10^4$/well in a six-well dish and transiently transfected with 80 ng of CMV-GFP (methylated or mock-methylated) using the calcium phosphate precipitation method as described previously (Rouleau et al., 1992). 0.3 μM TSA (Sigma) was added 24 h post-transfection. After an additional 24 h, cells were treated with or without various concentrations of AdoMet or AdoHcy (2 – 8 mM) (Sigma). Cells were harvested 72 h post transfection. Each experiment was performed in triplicate and experiments were performed several times using different cultures of HEK 293 cells.

Western blot analysis- Whole cell extracts were prepared using Radio Immuno Precipitation Assay buffer according to the Santa Cruz Biotechnology protocol, and protein concentrations were determined using the Bradford Reagent (BIO RAD). 2.5 μg of protein were resolved on a 12.5% SDS-polyacrylamide gel and then transferred to PVDF membrane (Amersham Biosciences). After blocking the nonspecific binding with 5% skim milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti rabbit IgG (Sigma) at 1:5000, and enhanced chemiluminescence detection kit (Amersham Biosciences). Membranes were stained with 0.2% Ponceau S (Sigma) to determine loading of total protein in each lane. Both the Western blots and Ponceau stained membranes were

quantified using NIH Image 1.62 software, and the GFP signal was normalized to the total protein (which varied only slightly) in each lane.

Southern blot analysis- DNA was extracted from HEK 293 cells using the DNeasy Tissue Kit (Quiagen). DNA was first digested with 50 units of *Eco*R1, followed by digestion with 20 units of either *Hpa*II or *Msp*I restriction enzymes. Samples were subjected to electrophoresis on a 1.5 % agarose gel and then transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were probed with a ^{32}P-labeled CMV-GFP cDNA probe (*Ava*II-*Cfr*101 fragment) synthesized using a random priming labeling kit (Roche Molecular Biochemicals). Membranes were hybridized at 68 °C for 4-6 h in a buffer containing 0.5 M sodium phosphate pH 6.8, 1 mM EDTA, 7% SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min in a solution of 5% SDS, 0.04 M sodium phosphate pH 6.8, 1mM EDTA, and then four times for 10 min in the same solution containing 1% SDS. The demethylation assay measures the fraction of GFP molecules that were demethylated using *Hpa*II restriction enzyme, which cleaves unmethylated CCGG but does not cleave methylated CCGG sequences. The methylated GFP DNA remains intact following *Hpa*II digestion and is identical to the fragment obtained following *Eco*RI digestion (indicated by M in Fig. 2B), whereas the unmethylated GFP DNA is cleaved by *Hpa*II resulting in a 0.5 kb fragment (indicated by U in Fig. 2B). We scanned each *Hpa*II digested lane and measured the intensity of the total signal hybridizing with the GFP probe in the same *Hpa*II lane (including the unmethylated U and methylated M fragments), this value is equal to 100% of GFP molecules in the lane. We then determined the intensity of the unmethylated

signal per *Hpa*II lane, and divided this value (U) by the total signal for GFP (U+M) in the same lane. The results of 3 independent experiments were quantified by densitometry (NIH Image 1.62). The percent methylation for each was determined as [M / (U+M)] *100.

AdoMet preparations for in vitro studies- AdoMet was prepared as a 50 mM solution by dissolving lyophilized powder (Sigma) in distilled water. AdoHcy was purchased from Sigma and dissolved in distilled water to a concentration of 50mM.

Purification of recombinant MBD2/dMTase from SF9 cells- A fragment containing human MBD2/dMTase was excised from pCR2.1-dMTase (Bhattacharya et al., 1999) with *Bam*HI and *Xho*I and transferred to the Baculovirus expression transfer vector pBlueBacHis2 C (Invitrogen). PBlueBacHis2 C-MBD2/dMTase and Bac-N-Blue viral DNA were cotransfected into the SF9 insect cell line and recombinant viruses were isolated, identified and amplified according to the manufacturer's protocol (Invitrogen) with no modifications. High titer P3 viral stocks were used for infections. Insect SF9 cells were cultured in spinner flasks to a density of $2.5x10^6$ cells/ml in Grace's Insect Cell Culture Medium Supplemented (1X) from Life Technologies. For infection, $5x10^6$ cells were plated in 10 cm tissue culture plates (Sarstedt) and allowed to settle and attach for 30 minutes. The culture medium was removed and was replaced with 10 ml of the same medium containing MBD2/dMTase virus at a multiplicity of infection of 10. The cells were cultured with the virus for 5 days at 27 °C and were then harvested by scraping in cold phosphate buffered saline. Cell pellets from 10 plates were frozen and kept at –70

°C until they were used for enzyme purification. Frozen pellets were thawed in 5 ml of lysis buffer (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 500 mM NaCl, 0.05% Tween 20, 10% glycerol and 10 mM imidazole) containing 1μg/ml of the following protease inhibitors: aprotinin, leupeptin and pefablock. Protease inhibitors were added to all the solutions used in the purification. The homogenates were subjected to two cycles of freezing and thawing (5 minutes per step). DNA in the homogenate was sheered by passing through an 18.5 gauge needle 10 times. The extracts were then subjected to 15 cycles of sonication (10 seconds burst, 10 seconds gap per cycle at 20% of maximal output). The extracts were centrifuged at 10, 000 x g for 35 min. The supernatant was transferred into a fresh tube and was recentrifuged for additional 25 min at 15, 000 x g. The extract was filtered through a 5 micron filter to remove any particulate matter and the buffer was exchanged on a PD-10 buffer exchange column (Amersham Biosciences) with buffer L (10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$) containing 50 mM NaCl. Recombinant MBD2/dMTase was partially purified by Q-Sepharose (Amersham Biosciences) ion exchange chromatography. Q-Sepharose beads (1 ml of swollen beads) were washed extensively and pre-equilibrated with buffer L containing 50 mM NaCl and divided into 3 equal aliquots. The cell extracts were sequentially bound three times to the 3 aliquots of Q-Sepharose beads in batch in 15 ml tubes by shaking gently on a Nutator for 45 min at 4 ^{0}C. Following each binding step, the bound beads and unbound supernatant were separated by centrifugation for 2 min at 1000 x g and the supernatant was transferred into a new tube and bound with new pre-equilibrated beads. The bound beads from the three binding steps were joined and resuspended in lysis buffer. The beads were washed in batch 4 times with 5 ml buffer L + 50 mM NaCl. For each

washing step, the beads were incubated with the wash solution for 15 min and were then separated from the wash supernatant by centrifugation for 2 min at 1000 x g. Following washing, the proteins were eluted in batch (30 min per step) with a stepwise NaCl gradient in buffer L. Each elution step was analyzed for *in vitro* demethylase activity and for the presence of the recombinant His-tagged MBD2/dMTase by a Western blot analysis using the anti-Xpress antibody from Invitrogen as previously described (Detich et al., 2002 Sep 27). MBD2/dMTase peak elution is at the 0.4 M NaCl step. No demethylase activity was observed in the same fractions prepared in a similar manner from uninfected SF9 cells. For concentration of the 0.4 M NaCl fraction, a Microcon YM-10 concentrator (Millipore) was used at 3300 x g and 4 °C. Spinning time varied according to the volume, and was 25 min for 500 µl.

Extraction of endogenous demethylase activity from HEK 293 cells - 10 x 10 cm tissue culture plates of HEK 293 cells were used to prepare cell extracts as outlined in the previous section. Q-Sepharose fractionation was performed also as described above and as previously described (Detich et al., 2002 Sep 27).

Preparation of substrate DNA for in vitro demethylation assay- (a) Methylation of substrate: Typically, 25 µg of DNA from *micrococcus lysodeikticus* Type XI (Sigma) were methylated with M.SssI (60 units, New England Biolabs) and AdoMet (3.2 mM, New England Biolabs) in methylation buffer (NEbuffer2) in the presence of 50 mM EDTA for 3-4 h at 37 °C. Fresh AdoMet (3.2 mM) and enzyme (40 units) were then added before incubating at 37 °C for additional 3-4 h. To achieve complete methylation,

the methylation reaction is repeated after AdoHcy, a product of the methylation reaction and inhibitor of DNA methylation, is removed using a Microcon 10 concentrator (Millipore) according to manufacturer's protocol. The degree of methylation was verified by methylation sensitive restriction enzyme analysis (*MspI*/*Hpa*II digestion) on aliquots of the reaction mixture. The DNA was purified by phenol/chloroform extraction, and unincorporated nucleotides were removed by Nap5 gel filtration chromatography (Amersham Biosciences). The Nap5 column was equilibrated with demethylation buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.0). DNA containing fractions were combined, concentrated on a Microcon 10, and subjected to a second Nap5 desalting column. DNA containing fractions were again concentrated as described above.

(b) [α-^{32}P]-dGTP labeling of DNA: We then prepared either methylated or unmethylated DNA that is ^{32}P labeled at G, the 3' neighbor of the methylated C, as previously described (Szyf and Bhattacharya, 2002b) with the following modifications: 5 µg of either methylated or unmethylated DNA in 35 µl double distilled water were denatured and annealed to a hexanucleotide primer by boiling for 10 min in the presence of 3 µl random hexanucleotide mix (0.2 A_{260}) (Roche Molecular Biochemicals). The primed DNA was then subjected to template directed extension with the Klenow fragment of DNA polymerase I in the presence of labeled [α-^{32}P]-dGTP (Perkin Elmer Life Sciences), dTTP, dATP, and either m-dCTP for methylated DNA or dCTP for unmethylated DNA. The 3' phosphate of all the 5' neighbors of G including either C or mC is labeled by this procedure. In detail, the labeling was performed for 3 h at 37 °C in polymerase buffer (50 mM NaCl, 6.6 mM Tris-HCl, 6.6 mM $MgCl_2$, 1 mM DTT, pH 7.4) with Klenow

fragment I (10 units, Roche Molecular Biochemicals), [α-^{32}P]-dGTP (50 μCi), and 1 mM of each of the following: dATP, dTTP, m-dCTP/dCTP. The reaction mixture was then extracted with phenol and chloroform. TCA precipitation (2 ml 10% trichloroacetic acid, 20 μg herring sperm DNA) of an aliquot typically showed 80-95 % labeling efficiency (260,000-320,000 cpm/μl, total of 150 μl). The DNA was purified by eluting it twice from a Nap5 column and concentrating on a Microcon 10 as described above with the following variation: distilled water was used for prewashing and elution in the second column. The final concentration was 5 ng/μl and the specific activity was typically 8.0-8.8 x10^6 cpm/μg.

In vitro Demethylation Assay – A typical reaction mixture (50 μl) consisted of 25 ng of ^{32}P-prelabeled DNA (prepared as described above) incubated in demethylation buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.0) with either the purified MBD2/dMTase (5 μl, ~5 ng), or the purified demethylase activity from HEK 293 cells (5 μl) for 24 h at 37 °C in either the absence or presence of AdoMet or AdoHcy. The DNA was extracted from the enzyme by incubation in 2 volumes of DNA extraction buffer (10 mM Tris-HCl, 0.5 M NaCl, 1% SDS) containing 0.1units proteinase K (Roche Molecular Biochemicals) at 50 °C for 2 h. Subsequent phenol/chloroform extraction in the presence of tRNA (50 μg) as a carrier and ethanol precipitation with salt and 95% ethanol resulted in almost quantitative recovery of the input DNA. The DNA pellets were resuspended in distilled water (8 μl) and digested with micrococcal nuclease to ^{32}P-labeled 3' mononucleotides as described elsewhere (Detich et al., 2002 Sep 27; Szyf and Bhattacharya, 2002b). The labeled mononucleotides were separated by thin layer chromatography and visualized by

autoradiography on a phosphorimager plate, and the levels of cytosine (C) and 5-methylcytosine (mC) were quantified by the MCID-M4 software (Imaging Research Inc.). The percent demethylation [C/(C+mC)] was calculated per each sample and then normalized to the value obtained for the demethylase reaction in the absence of inhibitor (0 mM AdoMet/AdoHcy).

Soft agar assay- HEK 293 and A549 cells were plated at a density of $7.5x10^4$ in a six-well dish and were treated the following day with either 4 mM AdoMet or AdoHcy for an additional 24 h. For analysis of growth in soft agar, $3 x10^3$ cells were seeded in triplicate onto a six-well dish in 4 ml of complete medium containing 0.33% agar solution at 37 °C (Freedman and Shin, 1974). Cells were fed with 2 ml of medium twice weekly. The assay was performed in triplicate and colonies were counted 21 days after plating for A549 cells, and 7 days after plating for HEK 293 cells.

RESULTS

AdoMet inhibits TSA induced active demethylation of ectopically methylated and transiently transfected CMV-GFP in a dose-dependent manner- There have been several reports demonstrating that exogenous administration of AdoMet leads to DNA hypermethylation (Cai et al., 1998; Fuso et al., 2001; Pascale et al., 1991). Similarly, other studies have shown that a decrease in dietary folate or a depletion of intracellular AdoMet results in DNA hypomethylation (Pascale et al., 2002; Pogribny et al., 1995; Sibani et al., 2002; Zhao et al., 1997). However, it is not known whether AdoMet's effects on methylation are due to changes in DNMT or DNA demethylase activities.

We utilized a previously described transient transfection based assay system (Fig.1 and (Cervoni and Szyf, 2001)) to study the effects of AdoMet on the active demethylation of ectopically methylated DNA. In prior studies we have shown that *in vitro* methylated CMV-GFP reporter plasmid is actively demethylated 72 hours following transfection into HEK 293 cells when histone hyperacetylation is induced with TSA (Cervoni and Szyf, 2001). Since CMV-GFP does not replicate nor is it *de novo* methylated in HEK 293 cells (Cervoni and Szyf, 2001), this assay specifically measures active demethylation in living cells.

We first determined the effects of increasing doses of AdoMet, or the product of AdoMet dependent methyltransferase reactions, AdoHcy, on the demethylation of methylated CMV-GFP (Fig. 2). DNA was isolated from HEK 293 cells transfected with methylated CMV-GFP DNA and treated with either TSA and AdoMet, or TSA and AdoHcy. DNA was first linearized with the *Eco*RI restriction enzyme, followed by digestion with *Msp*I (which cleaves the sequence CCGG) or *Hpa*II (which cleaves the sequence CCGG only when it is not methylated). The demethylated *Hpa*II digested 529bp fragment (U) and the methylated undigested DNA (M) were quantified within each sample, and the percent methylation for total CMV-GFP DNA in each lane was determined as [M / (U+M)] *100. As can be seen in Fig. 2, A and C, the addition of TSA results in nearly complete demethylation of CMV-GFP by endogenous demethylase activity, as indicated by the complete *Hpa*II digestion of CMV-GFP to the 529 bp fragment (U). Upon the addition of increasing concentrations of AdoMet (Fig. 2, B and D), the percentage of methylated

GFP remaining increases in a dose-dependent manner, illustrated by the decrease in the ratio of the 529 bp *Hpa*II fragment (U) to the undigested DNA (M). AdoHcy has an insignificant effect on the demethylation of CMV-GFP (Fig. 2, B and D), indicating that the methyl moiety of AdoMet is required for inhibition of demethylation.

We then determined whether AdoMet stimulates *de novo* methylation of an identical unmethylated CMV-GFP substrate. Fig. 2E illustrates that unmethylated CMV-GFP, transfected under identical conditions, does not get *de novo* methylated even in the presence of 8 mM AdoMet. This indicates that AdoMet does not cause an increase in DNMT activity on ectopic CMV-GFP. Thus, the likely mechanism by which AdoMet causes hypermethylation of CMV-GFP in comparison with the TSA treated control is by inhibiting its active demethylation by resident demethylases.

AdoMet reduces TSA induced expression of methylated CMV-GFP in a dose-dependent manner, but has no effect on unmethylated CMV-GFP- A number of studies have shown that an increase in AdoMet inhibits gene expression (Fuso et al., 2001 Nov 23; Watson et al., 1999), however it is not clear whether AdoMet specifically affects genes whose methylation state it alters exclusively, or whether it has a non specific effect on gene expression. We took advantage of the CMV-GFP system described above to address this question. We determined whether AdoMet influences the expression of either methylated CMV-GFP, whose methylation state is affected by AdoMet, or unmethylated CMV-GFP, whose methylation state is not affected by AdoMet. HEK 293 cells were transiently transfected and treated with TSA and either AdoMet or AdoHcy, as described in the

previous section. Extracts were then prepared and subjected to a Western blot analysis using an antibody directed against the GFP protein.

Fig. 3, A and C illustrate that methylated CMV-GFP is completely repressed in untreated HEK 293 cells. This is as expected, since it is well documented that DNA methylation leads to gene silencing. The addition of TSA leads to a dramatic induction of GFP expression as expected from the complete demethylation following TSA treatment. Upon the addition of increasing amounts of AdoMet, GFP expression is decreased in a dose-dependent fashion. AdoHcy has no significant effect on the expression of methylated GFP, consistent with its lack of effect on DNA demethylation.

Since our system measures expression and demethylation that is dependent on histone hyperacetylation, there are two possible mechanisms by which AdoMet could exert its effects on demethylation (Fig. 1). AdoMet could directly inhibit a demethylase activity, or it could inhibit histone acetylation, which we have previously shown leads to an inhibition of demethylation (Cervoni et al., 2002). If the latter were true, then AdoMet should also inhibit the TSA induced expression of unmethylated GFP, whose expression is induced by histone acetylation as well. Fig. 3, B and D indicate that this is not the case, since AdoMet has an insignificant effect on the induction of unmethylated GFP by TSA. The fact that AdoMet specifically affects the expression of a methylated copy of CMV-GFP, and not an unmethylated copy, supports the model that AdoMet inhibits gene expression by directly inhibiting the active demethylation of methylated CMV-GFP.

AdoMet inhibits the tumorigenic properties of two different transformed cells lines: A549 human lung carcinoma cells and HEK 293 human embryonal kidney cells- It is well documented that a correlation exists between reduced intracellular AdoMet (either as a consequence of decreased folate intake or pharmacological intervention) and an increase in cell proliferation and tumorigenesis (Cai et al., 1998; Martinez-Chantar et al., 2002; Pascale et al., 2002 Jul; Sibani et al., 2002). However, there is no direct demonstration that AdoMet treatment of a cancer cell line can revert its state of transformation. We therefore decided to test whether treatment with AdoMet could inhibit the transformation potential of HEK 293 cells, which were shown here to be responsive to AdoMet inhibition of demethylase. We also tested the effects of AdoMet on the transformed state of the A549 cell line to exclude the possibility that AdoMet's effects are an idiosyncrasy of HEK 293 cells. Cells were treated for 24 hours with either AdoMet or AdoHcy at a concentration of 4 mM. An equal number of living cells were seeded onto soft agar to test their anchorage independent growth, an indicator of tumorigenesis *in vitro* (Freedman and Shin, 1974). As shown in Fig. 4, both untreated HEK 293 and A549 cells formed colonies in soft agar, which is characteristic of cancer cell lines. Remarkably, after only a single 24-hour treatment with AdoMet, no colonies were formed with A549 cells and only a minimal number of colonies were observed with HEK 293 cells. In contrast, AdoHcy did not inhibit anchorage independent growth with either cell line and even had a stimulatory effect. Since we have shown here that 4 mM AdoMet inhibits cellular demethylase activity in HEK 293 cells whereas AdoHcy has no effect at the same concentration, the inhibition of cell transformation is consistent with the hypothesis that hypomethylation is critical for tumorigenesis. AdoMet's chemoprotective effects against

colorectal cancer might therefore be due to its inhibition of active demethylation.

AdoMet but not AdoHcy inhibits demethylation activity in vitro- In order to further confirm that the observed effect of AdoMet and AdoHcy is due to inhibition of active demethylation and not an indirect effect, in vitro studies with a recombinant MBD2/dMTase (the only demethylase characterized thus far) were performed (Bhattacharya et al., 1999). Since it is not certain whether MBD2/dMTase is responsible for the demethylation seen in HEK 293 cells, we also performed these studies with purified endogenous demethylase activity from HEK 293 cells. Together, these in vitro experiments should test whether AdoMet can act as an inhibitor of one or more demethylase activities.

First, His tagged MBD2/dMTase was partially purified by chromatography on Q-Sepharose from cell extracts of SF9 cells infected with the recombinant MBD2/dMTase construct as described in "materials and methods". Fractions were eluted with a stepwise gradient of NaCl and assayed for demethylation activity with a ^{32}P-prelabeled methylated DNA from micrococcus lysodeikticus (Fig. 5A, left panel). Conversion of mdCMP to dCMP, while not extensive, was almost exclusively detected with the 0.4 M NaCl fraction (16% demethylation). This correlates with the peak presence of the His-tagged recombinant MBD2/dMTase protein in this fraction as demonstrated by a Western blot analysis (Fig. 5B) using an anti-Xpress antibody. To confirm that the 0.4 M NaCl fraction contained demethylase activity, the fraction was concentrated 10 fold on a Microcon concentrator. As expected, the demethylase activity in the 0.4 M NaCl fraction

increased accordingly (Fig.5A, right panel); 5 µl of the concentrated fraction completely converted mdCMP to dCMP for the same amount of DNA as used before (Fig.5A, left panel).

Next, we determined whether AdoMet inhibits the demethylation activity of MBD2/dMTase. The aforementioned DNA was incubated with MBD2/dMTase from SF9 cells in the presence of increasing AdoMet concentrations, and conversion of mdCMP (mC) to dCMP (C) was assessed as above. Fig. 6A presents the autoradiography and quantification of one representative experiment. Due to the numerous steps involved in this assay it is impossible to avoid small loading differences between samples. To determine percent activity, the percent demethylation [C/(C+mC)] was calculated within each sample in order to control for these differences and then normalized to the value obtained for the demethylase reaction in the absence of inhibitor (0 mM AdoMet/AdoHcy). Conversion of mdCMP to CMP was greatly reduced at 0.5 mM AdoMet and abolished completely at concentrations higher than 0.7 mM.

In contrast to AdoMet, no inhibition of demethylation occurred in the presence of increasing concentrations of AdoHcy (Fig. 6B). These results indicate that the small differences in the chemical structure (methyl group and positive charge on the sulfur) between AdoMet and AdoHcy are responsible for their different interactions with the MBD2/dMTase.

To test whether AdoMet inhibits endogenous HEK 293 demethylase activity, we extracted demethylase from HEK 293 cells using Q-Sepharose fractionation as previously described (Detich et al., 2002), and incubated it with increasing concentrations of AdoMet. The results shown in Fig. 6C indicate that, similar to recombinant MBD2/dMTase, the demethylase activity extracted from HEK 293 cells is inhibited by 50% at 0.5 mM AdoMet. Taken together, the above experiments demonstrate that AdoMet can inhibit the in vitro demethylation activity of recombinant MBD2/dMTase as well as endogenous demethylase extracted from HEK 293 cells.

AdoMet and AdoHcy compete for binding to the catalytic site on DNMTs. It was therefore proposed that the ratio of AdoHcy to AdoMet determines DNMT activity as discussed in the introduction. AdoHcy inhibits DNMTs whereas increased AdoMet offsets this inhibition. We therefore determined whether a similar relationship applies to MBD2/dMTase. A competition experiment between AdoMet and AdoHcy is presented in Fig. 6D. Increasing concentrations of AdoHcy were added in the presence of an inhibitory concentration of AdoMet (10 mM) in a series of demethylation reactions. The results of this experiment illustrate that even a tenfold concentration excess of AdoHcy to AdoMet does not diminish inhibition of the demethylase reaction by AdoMet. This is consistent with the hypothesis that AdoMet has a higher affinity for MBD2/dMTase as compared with AdoHcy. Additional studies are necessary to elucidate the mode of inhibition: whether AdoMet is a competitive inhibitor with the substrate DNA or an allosteric inhibitor as was demonstrated for Methylene Tetrahydrofolate Reductase.

Furthermore, we do not know how MBD2/dMTase recognizes AdoMet on a structural basis.

DISCUSSION

The currently accepted mechanism for the effects of the methyl donor AdoMet on DNA methylation and tumorigenesis is founded on the assumption that the DNA methylation reaction is irreversible and defined exclusively by the DNMT. Taking advantage of our previously developed assay of demethylase activity in living cells (Fig. 1), we tested an alternative hypothesis that AdoMet inhibits demethylase activity. If the steady state methylation status of DNA is maintained by an equilibrium of DNMT and demethylase activities (Szyf, 2001a), then inhibition of the demethylase side of the equilibrium should result in hypermethylation. Therefore, the reported DNA hypermethylation effects of exogenous AdoMet might be caused in part by inhibiting the level of demethylase activity in tumor cells. The main advantage of the system used in this study is that it measures active demethylation exclusively, without interference from either replication dependent passive demethylation or *de novo* methylation (Cervoni and Szyf, 2001).

We show here that exogenous AdoMet inhibits TSA stimulated demethylation of ectopically methylated and transiently transfected CMV-GFP DNA (Fig. 2, B and E). Since methylation inhibits the expression of CMV-GFP, inhibition of demethylation of CMV-GFP results in reduction of GFP protein expression (Fig. 3, A and C), illustrating that AdoMet affects both demethylation of DNA and gene expression. This association of inhibition of demethylation and silencing of gene expression prompted us to rule out

the possibility that AdoMet has a general methylation-independent inhibitory effect on gene expression, or a general toxic effect, which might also result in inhibition of expression.

It is possible that AdoMet increases histone methyltransferase activity, resulting in hypermethylation of K9 on H3 histones, which has been shown to correlate with inhibition of acetylation (Dillon and Festenstein, 2002; Nakayama et al., 2001). Inhibition of acetylation was shown to inhibit expression and demethylation of CMV-GFP (Cervoni et al., 2002). To address this alternative possibility, we measured in parallel the effects that AdoMet might have on methylated as well as unmethylated CMV-GFP plasmid, both transfected and treated with exogenous AdoMet under equivalent conditions. We first show that AdoMet treatment does not result in *de novo* methylation of unmethylated CMV-GFP (Fig. 2E). Thus, exogenous AdoMet does not stimulate DNA methylation as might be predicted by the current hypothesis of AdoMet's mechanism of action. Second, we show that exogenous AdoMet does not inhibit expression of unmethylated CMV-GFP (Fig. 3, B and D) under conditions where a clear inhibition of expression of methylated CMV-GFP is observed (Fig. 3, A and C). Thus AdoMet specifically affects the expression of methylated genes. To our knowledge, this is the first demonstration that AdoMet specifically targets methylated DNA. This result also rules out the possibility that AdoMet exerts a general toxic effect on the cell. Our data therefore demonstrate that exogenous AdoMet specifically affects methylated DNA and prevents its expression. This most probably occurs by inhibiting an endogenous demethylase activity, resulting in hypermethylation of CMV-GFP and methylation-

dependent repression.

We used the product of AdoMet dependent methyltransferase reactions, AdoHcy, as a control. AdoHcy differs from AdoMet by a single methyl group. We show that AdoHcy has no effect on either gene expression (Fig. 3, A and C) or demethylation (Fig. 2, B and D). Taken together, these results indicate that both activities of AdoMet, inhibition of demethylation and inhibition of gene expression, are tightly associated and that they are both dependent on the methyl moiety in AdoMet.

We also demonstrate that AdoMet inhibits the tumorigenic potential of both HEK 293 and A549 cells at the same concentration that it inhibits demethylation and expression of methylated DNA (Fig. 4). This is consistent with epidemiological and clinical data, as well as with animal experiments using liver cancer models (Cai et al., 1998; Martinez-Chantar et al., 2002; Pascale et al., 2002 Jul; Sibani et al., 2002). Since we show that AdoMet specifically inhibits the expression of methylated genes, we suggest that part of the antitumorigenic effects of AdoMet could be explained by the inhibition of demethylation and expression of genes that are required for anchorage independent growth. Further experiments will be needed to identify the genes whose expression are inhibited by AdoMet and are required for anchorage independent growth and tumorigenesis.

In addition, we show that AdoMet directly inhibits recombinant MBD2/dMTase as well as demethylase activity extracted from HEK 293 cells in a dose dependent manner using

an *in vitro* assay (Fig. 6). AdoHcy does not inhibit MBD2/dMTase at the same concentrations (Fig. 6). Since an increase in intracellular AdoHcy was previously shown to be associated with hypomethylation (Caudill et al., 2001), we tested the possibilities that AdoHcy a) stimulates MBD2/dMTase activity, and b) competes with AdoMet binding to MBD2/dMTase and relieves AdoMet inhibition. Our results suggest that AdoHcy does not interact with MBD2/dMTase and that it has no effect on AdoMet inhibition of this enzyme *in vitro*. Our results support the conclusion that the methyl group in AdoMet is required for its interaction with MBD2/dMTase. Although our results demonstrate that exogenous AdoMet inhibits demethylase activity *in vitro* and in living cells, there is no evidence that the intact AdoMet is the inhibitor. Since AdoMet is not intrinsically stable, particularly at physiological pH, it is difficult to assess whether AdoMet or a breakdown product is the inhibitory compound. At the AdoMet concentrations (mM) used in our studies, micromolar or even nanomolar concentrations of breakdown products of AdoMet may be present. Additional experiments are required to test this possibility. Nevertheless, our experiments demonstrate that pharmacological administration of AdoMet inhibits active demethylation and alters gene expression.

Although further studies are necessary to determine whether MBD2/dMTase is responsible for demethylation of our methylated plasmid in HEK 293 cells, the fact that AdoMet inhibits both recombinant MBD2/dMTase and endogenous demethylase activities (Fig.6), provides support for the hypothesis that demethylase(s) is inhibited by AdoMet. Thus, in addition to its role as a cofactor for transmethylation reactions, AdoMet can also act as a regulator of DNA methylation by inhibiting demethylase

activity.

Our data further emphasize that the demethylase side of the methylation equilibrium has to be taken into account when dissecting the mechanism of action of drugs that modify the DNA methylation pattern. Based on our data, we suggest that AdoMet can alter DNA methylation patterns by inhibiting demethylase, which is expressed in some or most cells (Fig.7). In this case, a reduction in the intracellular levels of AdoMet by methyl-deficient diets removes this inhibition and increases the demethylase tone, resulting in active demethylation of DNA that could take place even in postmitotic tissue. Interestingly, AdoMet has recently been shown to inhibit the overall demethylation of a CG site in the 5' region of the *myogenin* gene during C2C12 differentiation (Fuso et al., 2001). However, this report did not determine whether AdoMet stimulated DNMT or inhibited DNA demethylase.

The finding that AdoMet inhibits anchorage independent growth (Fig. 4) suggests that active demethylation might be especially important for tumorigenesis. This is consistent with the well-documented observations of global hypomethylation in cancer cells (Ehrlich, 2002 Aug 12). There is evidence that AdoMet's tumor protective mechanism involves DNA methylation since this protection is removed when the animals are co-treated with 5-azaC (Pascale et al., 1991). In accordance with this hypothesis, we have recently shown that antisense inhibition of MBD2/dMTase inhibits tumorigenesis (Slack et al., 2002). It is tempting to speculate that certain genes that are required for anchorage independent growth might be inhibited by methylation and activated by a demethylase

activity. Inhibition of the demethylase tone by AdoMet is proposed to result in silencing of these genes. Future experiments should focus on the identification of cancer promoting genes that are suppressed by AdoMet. If AdoMet's mechanism of action in inhibiting tumorigenesis involves inhibition of demethylation, it would support the hypothesis that demethylation plays a causal role in tumorigenesis, and serve as a warning against using inhibitors of DNA methylation as anticancer agents.

Acknowledgements:

Supported by the National Cancer Institute of Canada and NSERC Canada.

We wish to thank Johanne Theberge for her excellent technical help and support. N.D. is a recipient of the Canadian Institute of Health Research Doctoral Fellowship and the McGill Faculty of Medicine Internal Fellowship. S.H. wishes to thank the Deutsche Forschungsgemeinschaft for a postdoctoral research stipend and G.J. for financial support from his grants.

FIGURE LEGENDS

Fig. 1. Outline of the model system used to assess the effects of AdoMet on active demethylation in living cells. (a) CMV-GFP plasmid is methylated *in vitro* and transiently transfected into HEK 293 cells. (b) Histone acetylation is induced with TSA, which results in DNA demethylation by endogenous demethylase activity (Cervoni and Szyf, 2001). (c) The degree of demethylation is then measured by methylation sensitive restriction digestion using *Hpa*II enzyme, which is followed by Southern blot analysis using a GFP specific probe (*Ava*II-*Cfr*101 fragment). The possible mechanisms of action of AdoMet are indicated: Either a direct inhibition of demethylase activity (1), or an indirect mechanism by first stimulating histone methylation and inhibiting histone acetylation, which would then inhibit active demethylation (2).

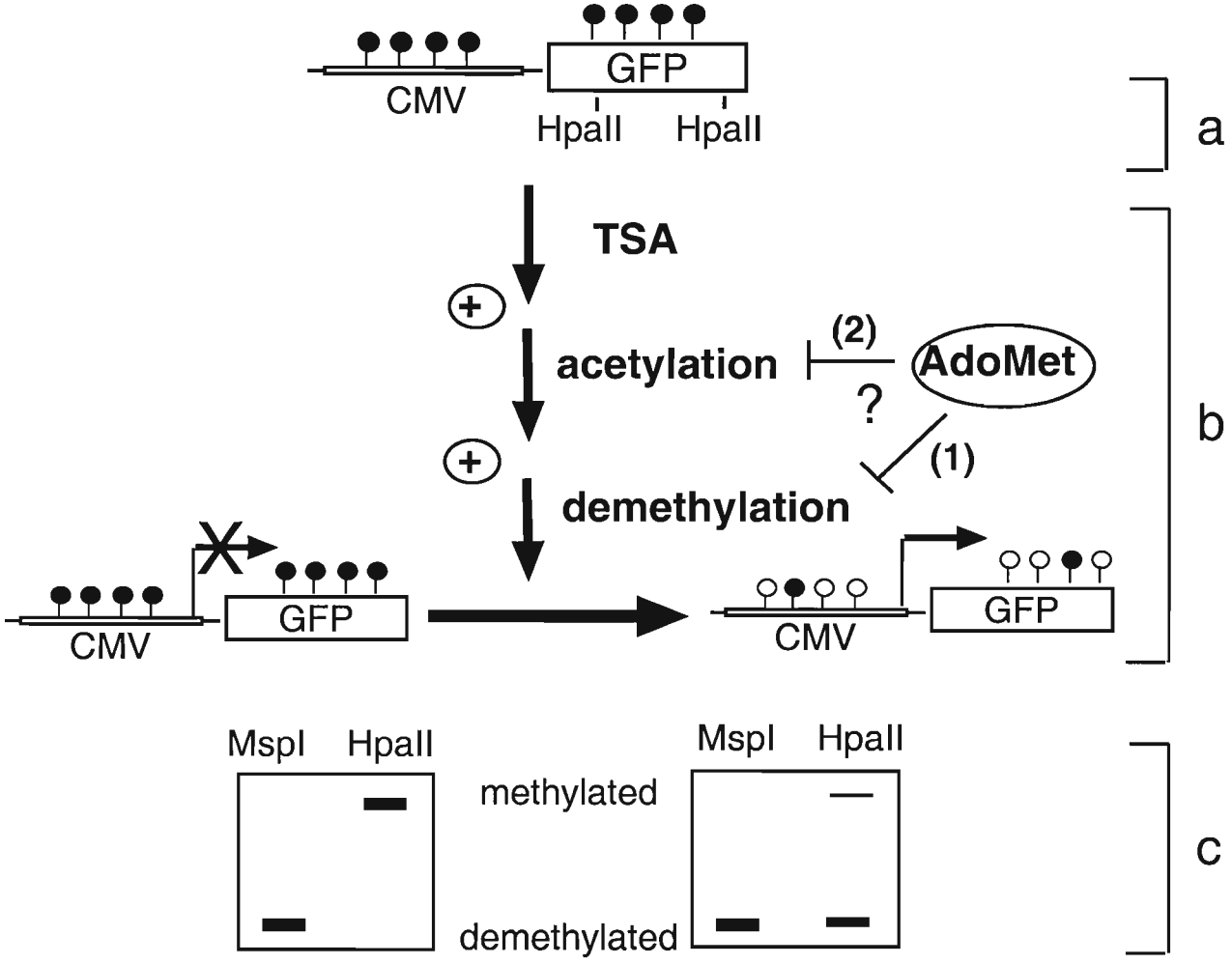
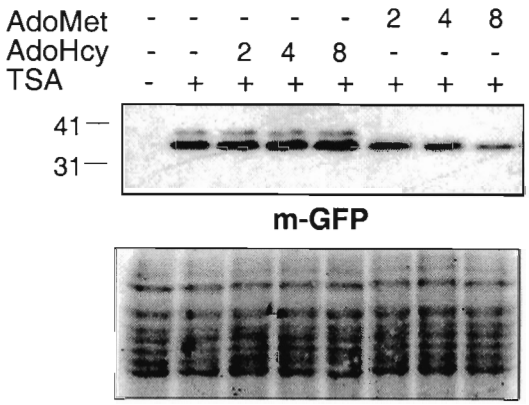
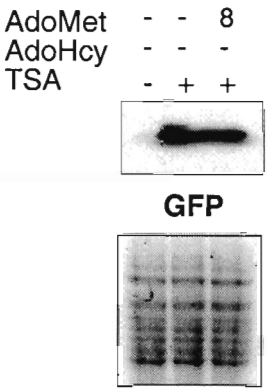

GFP
CMV
HpaII
HpaII
a
TSA
+
acetylation
(2)
AdoMet
?
+
(1)
demethylation
b
GFP
CMV
GFP
CMV
MspI
HpaII
methylated
demethylated
MspI
HpaII
c

Fig. 2. AdoMet inhibits active demethylation of CMV-GFP. A, B, E. Either *in vitro* methylated CMV-GFP plasmid (*m-GFP*) (A, B), or unmethylated CMV-GFP (*GFP*) (*E*) were transiently transfected into HEK 293 cells. Cells were treated with a final concentration of 0.3 μM TSA (+ *TSA*) or left untreated (- *TSA*), and increasing concentrations of either AdoMet or AdoHcy (2, 4, 8 mM) were then added. Cells were harvested 72 h post-transfection and the methylation status of CMV-GFP was determined by *Msp*I/*Hpa*II restriction digestion and Southern blot analysis as outlined in "materials and methods" and Fig.1. *M*, methylated and *Hpa*II undigested GFP; *U*, unmethylated and *Hpa*II fully digested GFP (529bp). **C.** The results of 3 independent experiments as shown in (A) were quantified by densitometry and the average percent methylation remaining for each sample was calculated as outlined in "materials and methods" and charted +/- S.D. **D.** The results of 3 independent experiments as shown in (B) were quantified as in (C) and the averages +/- S.D. are presented. **O**, AdoMet; □, AdoHcy.

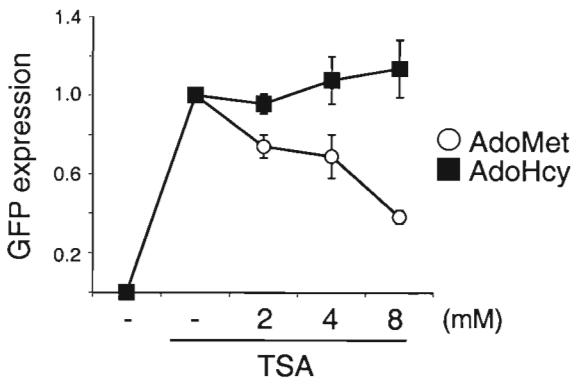
A


AdoMet - - - - - 2 4 8
AdoHcy - - 2 4 8 - - -
TSA - + + + + + + +
41
31
m-GFP

B


AdoMet - - 8
AdoHcy - - -
TSA - + +
GFP

C


GFP expression
1.4
1.0
0.6
0.2
AdoMet
AdoHcy
- - 2 4 8 (mM)
TSA

D

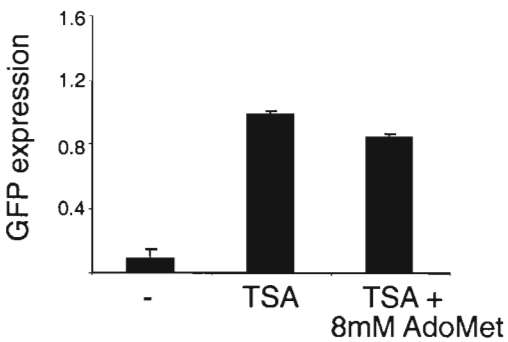
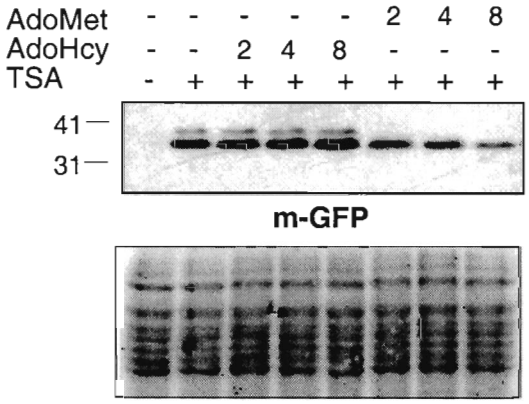
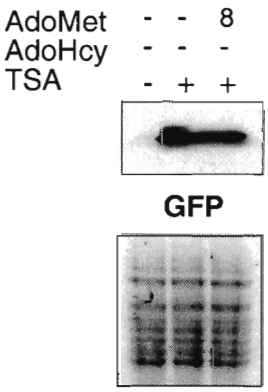

GFP expression
1.6
1.2
0.8
0.4
-
TSA
TSA +
8mM AdoMet

Fig. 3. AdoMet reduces TSA induced expression of methylated CMV-GFP. A and B. HEK 293 cells were transiently transfected with either methylated CMV-GFP plasmid (*m-GFP*) (A), or unmethylated CMV-GFP (*GFP*) (B) and treated with 0.3 μM TSA plus the indicated concentrations (mM) of AdoMet or AdoHcy. Total cell extracts were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to PDVF membrane and GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334). Membranes were stained with Ponceau S to show equal loading. **C.** Experiments such as those shown in (A) were performed in triplicate and quantified by densitometry. Results are presented as arbitrary units relative to the level of GFP expression in the presence of TSA alone, which was normalized to one. O, AdoMet; □, AdoHcy. **D.** Quantification of triplicate experiments as shown in (B); the averages +/- S. D. are presented.

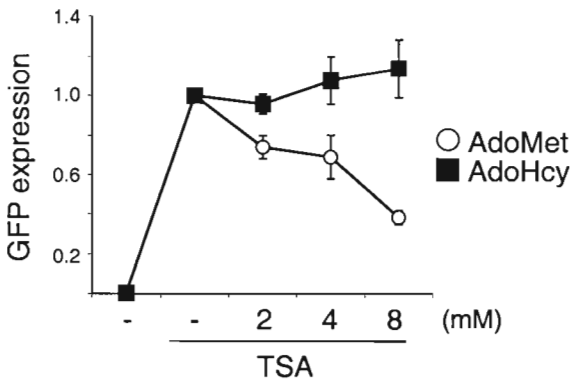
A


AdoMet - - - - - 2 4 8
AdoHcy - - 2 4 8 - - -
TSA - + + + + + + +
41
31
m-GFP

B


AdoMet - - 8
AdoHcy - - -
TSA - + +
GFP

C


GFP expression
1.4
1.0
0.6
0.2
AdoMet
AdoHcy
- - 2 4 8 (mM)
TSA

D

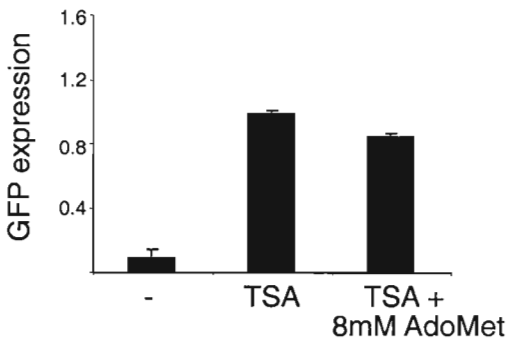
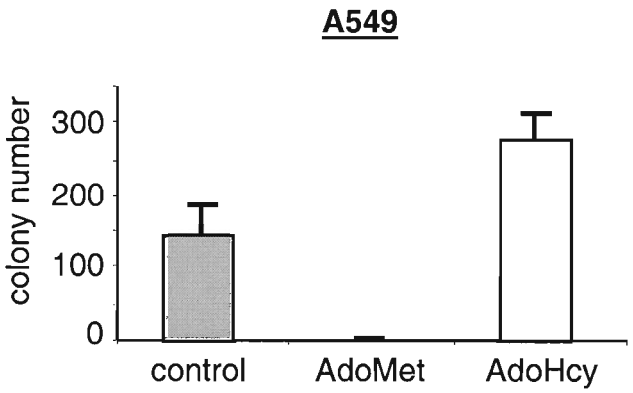

GFP expression
1.6
1.2
0.8
0.4
-
TSA
TSA +
8mM AdoMet

Fig. 4. AdoMet inhibits the growth of A549 and HEK 293 cells in soft agar. A549 cells (A) and HEK 293 cells (B) were treated with either 4 mM AdoMet or AdoHcy for 24 h, or left untreated (*control*). 3 x 10^3 live cells were then seeded in triplicate onto a six-well dish in 4 ml of complete medium containing 0.33% agar solution. Colonies were counted 7 days after plating for HEK 293 cells, and 21 days after plating for A549 cells. Results shown are the average colony number/well from triplicate assays.

A


A549
colony number
300
200
100
0
control
AdoMet
AdoHcy

B

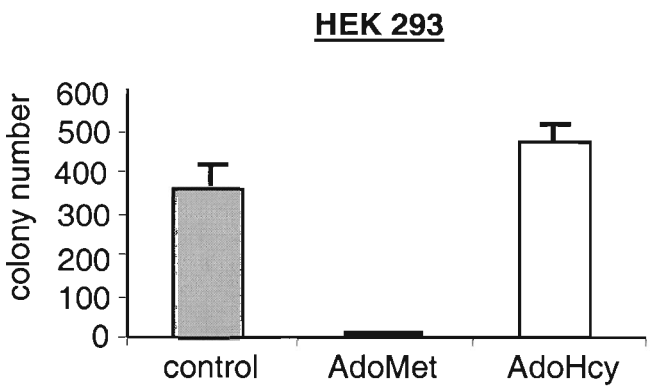
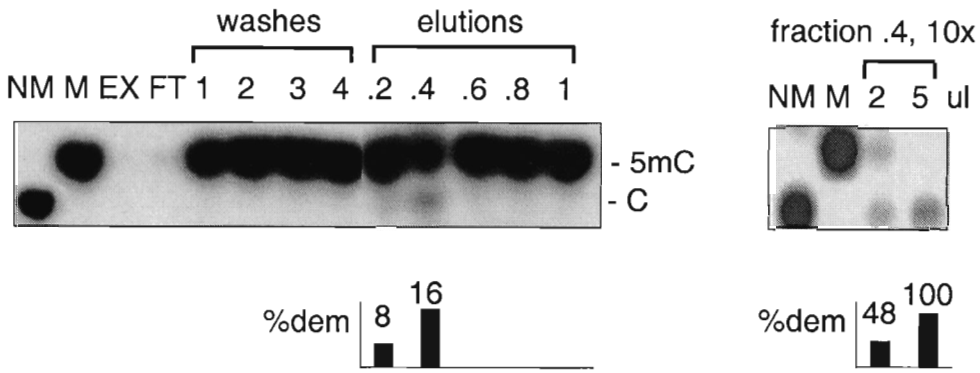

HEK 293
colony number
600
500
400
300
200
100
0
control
AdoMet
AdoHcy

Fig. 5. Partial purification of His-MBD2/dMTase from SF 9 cells. A. Cell extracts prepared from SF9 cells infected with MBD2/dMTase baculovirus were subjected to chromatography on Q-Sepharose and eluted with a step-wise gradient of NaCl. Fractions were assayed for demethylase activity using a ^{32}P-prelabeled methylated DNA from *micrococcus leisodeikticus*. Demethylation activity elutes almost exclusively at 0.4 M NaCl with some activity present in the 0.2 M NaCl fraction (*left panel*). The 0.4 M NaCl fraction was concentrated 10 fold and re-assayed (*right panel*). Following demethylation, the DNA was digested to 3'-mononucleotides and separated by thin layer chromatography. The DNA that was incubated with the whole cell extract (*EX*) and with the flow through (*FT*) could not be recovered for activity analysis, most likely due to nuclease activities in the fractions. The MBD2/dMTase tightly binds to Q-Sepharose as shown by the fact that the washes are free of demethylation activity. **B.** The fractions were analyzed by Western blot using anti-Xpress antibody (Invitrogen) to demonstrate the presence of His-MBD2/dMTase (indicated by the *arrow*). The presence of the protein correlates with its activity in the elution profile, with almost all the MBD2/dMTase detected at 0.4 M NaCl. Lower molecular weight bands in the extract and the bindings might be due to partial degradation of the protein during purification. *NM*, unmethylated control; *M*, methylated control; *5mC*, 5-methyldeoxycytidine 3'-monophosphate; *C*, deoxycytidine 3'-monophosphate, *EX*, extract; *FT*, flow through.

A


washes
elutions
NM M EX FT 1 2 3 4 .2 .4 .6 .8 1
- 5mC
- C
%dem
8
16
fraction .4, 10x
NM M 2 5 ul
%dem
48
100

B

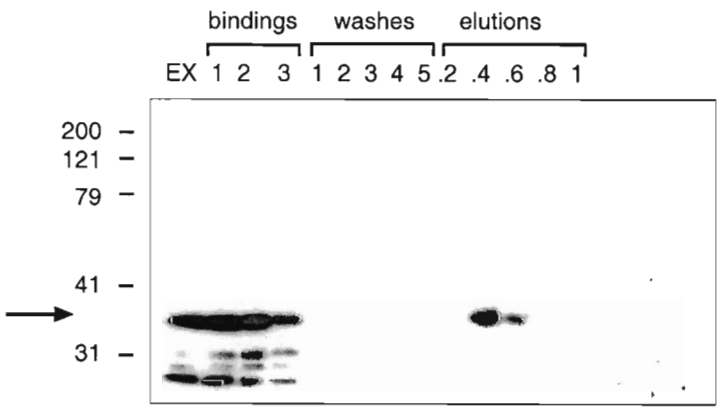

bindings
washes
elutions
EX 1 2 3 1 2 3 4 5 .2 .4 .6 .8 1
200
121
79
41
31

Fig. 6. AdoMet, but not AdoHcy, inhibits demethylation activity *in vitro*. A and C. ^{32}P-prelabeled methylated DNA from *micrococcus leisodeikticus* was incubated with either MBD2/dMTase (A) or HEK 293 cells' extracted demethylase (C), and increasing concentrations of AdoMet. The autoradiography and quantification of one representative experiment is shown. Percent activity was calculated as described in "materials and methods". **B.** Increasing concentrations of AdoHcy were added to the demethylase reaction; a representative experiment is shown. **D.** Increasing concentrations of AdoHcy were added to reaction mixtures containing 10 mM AdoMet and demethylase activity was determined. *NM*, unmethylated control; *M*, methylated control; *5mC*, 5-methyldeoxycytidine 3'-monophosphate; *C*, deoxycytidine 3'-monophosphate.

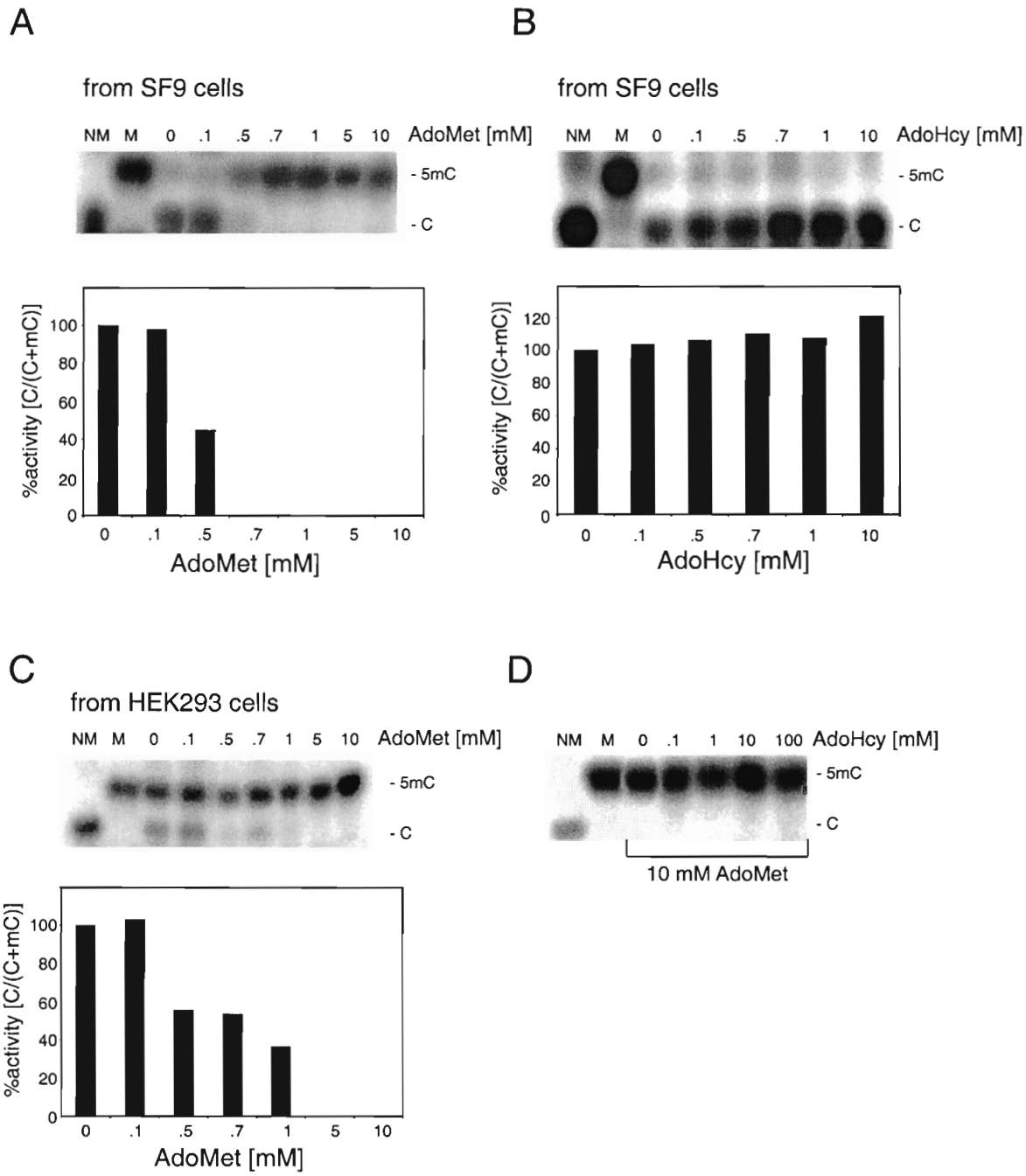
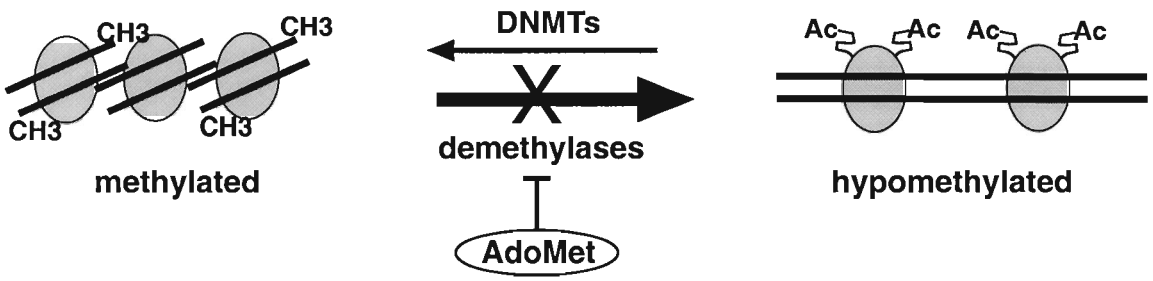

A
from SF9 cells
NM M 0 .1 .5 .7 1 5 10 AdoMet [mM]
- 5mC
- C
%activity [C/(C+mC)]
100
80
60
40
20
0
0 .1 .5 .7 1 5 10
AdoMet [mM]
B
from SF9 cells
NM M 0 .1 .5 .7 1 10 AdoHcy [mM]
- 5mC
- C
%activity [C/(C+mC)]
120
100
80
60
40
20
0
0 .1 .5 .7 1 10
AdoHcy [mM]
C
from HEK293 cells
NM M 0 .1 .5 .7 1 5 10 AdoMet [mM]
- 5mC
- C
%activity [C/(C+mC)]
100
80
60
40
20
0
0 .1 .5 .7 1 5 10
AdoMet [mM]
D
NM M 0 .1 1 10 100 AdoHcy [mM]
- 5mC
- C
10 mM AdoMet

Fig. 7. Possible model depicting how AdoMet may alter DNA methylation patterns and exert a chemoprotective effect. The steady state methylation pattern of a gene is determined by an equilibrium of DNMTs and DNA demethylases acting upon it. In cells where DNA demethylase is overexpressed, certain genes may have a tendency to become hypomethylated, and some of these genes may promote anchorage independent growth and tumorigenesis. In this case, the administration of AdoMet would have a tumor protective effect by inhibiting demethylation and shifting the equilibrium to the normally methylated state.


CH3
CH3
CH3
CH3
methylated
DNMTs
demethylases
AdoMet
Ac
Ac
Ac
Ac
hypomethylated

The former manuscript describes an alternative mechanism of action for AdoMet as an inhibitor of intracellular demethylase activity, which results in hypermethylation of DNA. This finding forces us to take into account the demethylase side of the methylation equilibrium when determining the mechanism of action of drugs that modify the DNA methylation pattern. The possibility that epigenomic states might be manipulated therapeutically by modulating demethylase activity has vital implications not only in cancer, but even more importantly, in postmitotic tissues such as the brain. Postmitotic tissues pose an important barrier to epigenetic therapeutic modulation in the fact that the cells do not divide. This is especially imperative in situations where certain genes are abnormally hypermethylated. Since all the DNA hypomethylating drugs that are available to date act by inhibiting DNMT, they induce passive demethylation, and are therefore extremely effective in replicating cells but are irrelevant for cells that do not divide. Thus, the only chance to correct abnormal hypermethylation in adult non-dividing tissues is to identify agents that can pharmacologically stimulate active demethylation by endogenous demethylases. The following manuscript illustrates that the commonly prescribed drug valproate (VPA) can induce replication independent active demethylation of DNA, and also shows that MBD2/dMTase is involved in this process. This study is the first demonstration that an established drug can erase DNA methylation patterns in the absence of DNA replication, and suggests potential new uses for this therapeutic agent.

Chapter 4

Valproate induces replication independent active DNA demethylation.

Nancy Detich, Veronica Bovenzi, and Moshe Szyf

This chapter has been published in The Journal of Biological Chemistry, May 14, 2003, JBC Papers in Press, M303740200.

In this paper, we demonstrate that valproic acid (VPA), a drug that has been used for decades in the treatment of epilepsy and as a mood stabilizer, triggers replication independent active demethylation of DNA. Thus, this drug can potentially reverse DNA methylation patterns and erase stable methylation imprints on DNA in non-dividing cells. Recent discoveries support a role for VPA in the regulation of methylated genes, however the mechanism has been unclear because it is difficult to dissociate active demethylation from the absence of DNA methylation during DNA synthesis. We therefore took advantage of an assay that measures active DNA demethylation independently from other DNA methylation and DNA replication activities in HEK 293 cells. We show that VPA induces histone acetylation, DNA demethylation, and expression of an ectopically methylated CMV-GFP plasmid in a dose dependent manner. This demethylation appears to be selective, since global methylation levels are unaffected. In contrast, valpromide (VPM), an analogue of VPA that does not induce histone acetylation, does not induce demethylation or expression of CMV-GFP. Furthermore, we illustrate that the methyl-CpG-binding protein 2/DNA demethylase (MBD2/dMTase) participates in this reaction, since antisense knockdown of MBD2/dMTase attenuates VPA induced demethylation. VPA treatment of HEK 293 cells also increases the phosphorylation of MBD2/dMTase, possibly augmenting the activity of this enzyme. Taken together, our data support a new mechanism of action for VPA as enhancing intracellular demethylase activity through its effects on histone acetylation, and raises the possibility that DNA methylation is reversible in postmitotic tissues by commonly prescribed drugs.

INTRODUCTION

DNA methylation is a modification of DNA whereby methyl groups are added as part of the covalent structure of the genome, thus providing an extra layer of epigenetic information. A well-documented relationship exists between DNA methylation, chromatin structure, and gene expression (Razin, 1998), such that methylated genes are generally transcriptionally silent. Two mechanisms have been proposed to explain this repression: The first is that methylation interferes with the binding of transcription factors, and has been shown for several proteins such as AP2 (Comb and Goodman, 1990), and c-Myc (Prendergast and Ziff, 1991). The second mechanism involves the recruitment of various repressor complexes to methylated DNA via the binding of methyl-CpG-binding proteins (MBD). These complexes contain proteins that have histone deacetylase and chromatin remodeling activities, leading to the formation of a more compact and transcriptionally inactive chromatin (Nan et al., 1998).

Valproic acid /Valproate/2-n-propylpentanoic acid (VPA) has been used for decades in the treatment of epilepsy, and is also effective as a mood stabilizer and in migraine therapy. Recent data suggests that this drug, in addition to its other known classical actions, can modulate the epigenome by inhibiting histone deacetylases (HDAC) (Gottlicher et al., 2001; Phiel et al., 2001) similar to agents such as Trichostatin A (TSA) and *n*- butyrate, thus triggering an increase in gene expression. Other studies also support a role for VPA in the regulation of methylated genes: It was demonstrated that the *reelin* gene, which encodes a neuronal protein that is down regulated in schizophrenia, is methylated in neuronal precursor cells, accompanied by minimal expression. Following

treatment of these cells by 5'-aza-2'-deoxycytidine (5-aza-CdR), a known DNA methylation inhibitor, or VPA, the expression of *reelin* is induced (Chen et al., 2002). VPA can also revert the down regulation of both *reelin* and *gad67* caused by L-methionine, an agent shown to increase DNA methylation (Tremolizzo et al., 2002). Similarly, both 5-aza-CdR and VPA induce the expression of *5-lipoxygenase* (Manev and Uz, 2002). The data presented above suggest the possibility that VPA may also be able to trigger DNA demethylation.

The prospect of erasing established DNA methylation patterns in somatic non-dividing tissue is extremely attractive and might have important therapeutic applications. However, the generally accepted model is that the DNA methylation pattern is an irreversible reaction maintained only by DNA methyltransferase activities. If such is the case, then demethylation could only come about when replication occurs in the absence of DNA methyltransferase, and it should therefore be impossible to reverse DNA methylation in non-dividing postmitotic tissues such as the brain. VPA, according to this classical model, could only act by inhibiting DNA methylation during DNA synthesis. However, there are several pieces of evidence indicating that DNA demethylation can occur in the absence of cell division (Bruniquel and Schwartz, 2003; Lucarelli et al., 2000; Oswald et al., 2000; Paroush et al., 1990; Szyf et al., 1985a; Wilks et al., 1984). In addition, a DNA demethylase activity was purified in our laboratory from the human lung cancer cell line A549 (Ramchandani et al., 1999) and the protein MBD2/dMTase was found to actively demethylate DNA both *in vitro* (Bhattacharya et al., 1999) and in living cells (Cervoni and Szyf, 2001; Detich et al., 2002). Other groups (Hendrich et al., 2001;

Ng et al., 1999) have nevertheless disputed the demethylase activity of MBD2. Since we have shown that active demethylation can be induced or inhibited by modifying histone acetylation (Cervoni et al., 2002; Cervoni and Szyf, 2001), and in view of the fact that VPA is also known to be an HDAC inhibitor, we hypothesize that it might act by increasing histone acetylation and increasing the accessibility of demethylase to DNA. If VPA acts by this mechanism, it might be used to stably erase methylation of specific genes in tissues where DNA replication does not take place.

It is impossible to determine whether hypomethylation of a gene *in vivo* or in living cells following a drug treatment is caused by either passive demethylation, resulting from inhibition of DNA methylation during DNA synthesis (as with 5-aza-CdR), or by the active removal of the methyl group by an enzyme. We have recently described a model system (Cervoni and Szyf, 2001) that allows one to distinguish between these two mechanisms. When an *in vitro* methylated CMV-GFP plasmid is transiently transfected into the human embryonal kidney cell line HEK 293, it generally remains methylated. Increasing histone acetylation induces active demethylation of the plasmid (Cervoni and Szyf, 2001). Since we have shown that the plasmid does not replicate during the time frame of the experiment, nor is it methylated by DNA methyltransferases, the demethylation measured by this assay is active demethylation and not passive demethylation. We have recently used this system to demonstrate the inhibition of active demethylation by inhibitors of histone acetylation (Cervoni et al., 2002).

In this study, we utilize this method to test the hypothesis that VPA treatment induces active DNA demethylation in living cells. Our data has important implications on the therapeutic reversal of pathological DNA methylation in non-dividing somatic tissues.

MATERIALS AND METHODS

In vitro methylation of substrates- CMV-GFP (pEGFP-C1 from Clontech; Genbank™ accession no. U55763) was methylated *in vitro* with SssI CpG methyltransferase (New England Biolabs), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5´..CG...3´. 10 μg of plasmid DNA was incubated with 12 units of SssI in the recommended buffer containing 800 μM S-adenosyl-methionine (AdoMet) for 3 h at 37 °C. Another 12 units of SssI and 0.16 μmol of AdoMet were then added and the reaction was further incubated for an additional 3 h. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection from *Hpa*II digestion.

Cell culture and transient transfections- Human embryonal kidney HEK 293 cells (ATCC CRL 1573) were plated at a density of $7.5x10^4$/well in a six-well dish and transiently transfected with 80 ng of CMV-GFP (methylated or mock-methylated) using the calcium phosphate precipitation method as described previously (Rouleau et al., 1992). 24 hours post-transfection, cells were treated with one of the following: 1 to 20 mM VPA (sodium salt, Sigma), 0.3 μM TSA (Sigma), 5 mM valpromide (VPM, kindly

donated by Katwijk chemie bv), or left untreated. Fresh media containing VPA and VPM were replaced after an additional 24 h and cells were harvested 72 h post-transfection. To determine the effects of MBD2/dMTase knockdown on the demethylation of CMV-GFP, 1.6 μg of either a pRetroTet On MBD2 antisense expression plasmid (Slack et al., 2002), or the pRetroTet On empty vector (Clontech) was added to the CMV-GFP transfection mixture, followed by treatment with 20 mM VPA. Each experiment was performed in triplicate and experiments were performed several times using different cultures of HEK 293 cells. 5'-aza-2'-deoxycytidine (5-aza-CdR) was purchased from Sigma.

Southern blot analysis- DNA was extracted from HEK 293 cells using the DNeasy Tissue Kit (Quiagen). DNA was first digested with 50 units of *Eco*R1, followed by digestion with 20 units of either *Hpa*II or *Msp*I restriction enzymes. Samples were subjected to electrophoresis on a 1.5 % agarose gel and then transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were probed with a ^{32}P-labeled CMV-GFP cDNA probe (*Ava*II-*Cfr*10I fragment) synthesized using a random priming labeling kit (Roche Molecular Biochemicals). Membranes were hybridized at 68 °C for 4-6 h in a buffer containing 0.5 M sodium phosphate pH 6.8, 1 mM EDTA, 7% SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min in a solution of 5% SDS, 0.04 M sodium phosphate pH 6.8, and 1mM EDTA, and then four times for 10 min in the same solution containing 1% SDS. The demethylation assay measures the fraction of GFP molecules that were demethylated using *Hpa*II restriction enzyme, which cleaves unmethylated CCGG but does not cleave methylated CCGG sequences. The methylated GFP DNA remains intact following *Hpa*II digestion

and is identical to the fragment obtained following *Eco*RI digestion (indicated by Me in Fig. 2C), whereas the unmethylated GFP DNA is cleaved by *Hpa*II resulting in a 0.5 kb fragment (indicated by U in Fig. 2C). We scanned each *Hpa*II digested lane and measured the intensity of the total signal hybridizing with the GFP probe in the same *Hpa*II lane (including the unmethylated U and methylated Me fragments), this value is equal to 100% of GFP molecules in the lane. We then determined the intensity of the unmethylated signal per *Hpa*II lane, and divided this value (U) by the total signal for GFP (U+M) in the same lane. The percent demethylation is thus calculated as [U / (U+Me)] *100. The results of 3 independent experiments were quantified and averaged.

Bisulfite mapping- Bisulfite mapping was performed exactly as described (Cervoni et al., 2002 Jul 12).

Chromatin immunoprecipitation assays- CMV-GFP transfections and VPA treatments were performed essentially as previously described, except that HEK 293 cells were seeded in 10 cm plates (2 per sample) at a density of 5 x 10^5 /plate, and 0.5 μg of methylated CMV-GFP was used. ChIP assays were performed by following the Upstate Biotechnology Chromatin Immunoprecipitation (ChIP) Assay Kit protocol (cat no. 17-295). Chromatin was immunoprecipitated using anti-acetylated histone H3 antibody (Upstate Biotechnology, cat no. 06-599) as recommended by the manufacturer. PCR reactions were repeated exhaustively using serial dilutions (1 to 1:10000) of template to determine the linear range of the PCR, and a final dilution of 1/100 was used. The following primers were used to amplify a 500 bp region of the GFP gene: 5'-

CAAGGGCGAGGAGCTGTT-3' (sense) and 5'-CGGCCATGATATAGACGTTG-3' (antisense), at an annealing temperature of 58 °C.

Radiolabeled [^{3}H]-dCTP extension assay- The assay was performed essentially as described with minor modifications (Pogribny et al., 1999). Briefly, 1.0 μg of DNA was digested overnight with 10 units of *Msp*I or *Hpa*II in a 20 μl reaction volume. A third reaction was carried out in restriction enzyme buffer alone to serve as the background control. 10 μl of the mixture was then used for the extension reaction, which was carried out in a 25 μl reaction volume containing 0.5 units of recombinant Taq polymerase (MBI Fermentas), 1.0 mM MgCl2, 1x PCR buffer (+NH2SO4), 0.1 μl of [^{3}H] dCTP (65.8Ci/mmol, Perkin Elmer). The reactions were applied onto DE 81 ion-exchange filter papers, which were then washed 5 x 5 min with 3-4 ml of PBS and allowed to dry. The [^{3}H]-dCTP incorporated was measured, and the counts obtained from the background controls were subtracted from the samples.

Western blot analysis- GFP Western blots were performed exactly as in (Cervoni and Szyf, 2001). For MBD2 Western blots, nuclear extracts were prepared from HEK 293 cells as described (Szyf et al., 1991). 25 to 50 μg of nuclear proteins were loaded onto a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Anti-MBD2 monoclonal antibody (IMG147, Imgenex, San Diego, CA) was used at a dilution of 1:200 in a solution of TBS (0.05 M Tris, 0.2 M NaCl, pH 7.6) + 0.5% Tween 20 + 5% milk overnight at 4 °C. The blot was then reacted with peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) at a 1:20,000 dilution. For both GFP and MBD2, enhanced chemiluminescence detection kit (Amersham Biosciences) was used.

Membranes were also stained with 0.1% Amido black (in 25% isopropanol, 10% acetic acid) to confirm equal loading of protein in each lane. Both the Western blots and Amido stained membranes were quantified using NIH Image 1.62 software, and the GFP/MBD2 signal was normalized to the total protein in each lane.

Alkaline Phosphatase (CIP) treatment- 25 μg of nuclear extract was incubated with 25 units of alkaline phosphatase (New England Biolabs) in a 20 μl reaction volume containing 1x NEB buffer 3 for 1 h at 37 °C. Control reactions were also carried out in the presence of buffer alone. MBD 2 Western blots were then performed as described above.

RESULTS

The association of CMV-GFP with acetylated histones is increased in the presence of VPA- Several groups have reported that VPA can act as a direct inhibitor of histone deacetylases (Gottlicher et al., 2001; Phiel et al., 2001), leading to an increase in acetylated histones H3 and H4 within cells. However, it is not known whether this increase occurs with H3 and H4 that are bound throughout the genome or whether it is specific to certain genes. Therefore we first determined whether VPA induces histone acetylation of methylated CMV-GFP in our system, using chromatin immuno-precipitation assays (ChIP) with anti-acetyl-histone H3 (anti-Ac-H3). The results presented in Fig. 1A show that, whereas a minimal fraction of the transfected methylated GFP gene is associated with Ac-H3 in control cells (lane 3), the fraction of transfected methylated CMV-GFP associated with Ac-H3 increased 7 fold (lane 6) following a 48 h

treatment with 20 mM VPA (Fig. 1B). Thus, VPA is effective in inducing histone acetylation around the transiently transfected methylated CMV-GFP gene.

VPA stimulates active demethylation of ectopically methylated CMV-GFP in a dose-dependent manner- We next tested the hypothesis that VPA triggers active DNA demethylation of transiently transfected methylated CMV-GFP, similarly to TSA, an HDAC inhibitor (Cervoni and Szyf, 2001). We first determined the effects of increasing doses of VPA on the demethylation of methylated CMV-GFP (Fig. 2). DNA was isolated from HEK 293 cells transfected with methylated CMV-GFP DNA (whose full methylation status was confirmed prior to transfection, Fig. 2A) for 72 h and treated with 0 - 20 mM VPA. Cells were also treated with 0.3 μM TSA as a positive control for demethylation, since we have shown that this leads to nearly 100% demethylation (Cervoni and Szyf, 2001). DNA was first linearized with the *Eco*RI restriction enzyme, followed by digestion with *Msp*I (which cleaves the sequence CCGG) or *Hpa*II (which cleaves the sequence CCGG only when it is not methylated). The demethylated *Hpa*II digested 529bp fragment (U) and the methylated undigested DNA (Me) were quantified within each sample (to control for possible loading differences), and the percent methylation for total CMV-GFP DNA in each lane was determined as [Me / (U+Me)] *100. As can be seen in Fig. 2, C and E, the addition of VPA results in a dose dependent active demethylation of methylated CMV-GFP by an endogenous demethylase activity. This is illustrated by the increase in the ratio of the 529 bp *Hpa*II fragment (U) to the undigested DNA (Me). The addition of 20 mM VPA results in almost complete demethylation, similar to 0.3 μM TSA. Fig. 2B confirms that unmethylated CMV-GFP,

transfected under identical conditions, does not get *de novo* methylated in our system either in the presence or absence of 20 mM VPA, suggesting that VPA does affect DNA methyltransferase activity.

In order to determine whether this demethylation is dependent on VPA's HDAC inhibitory properties, we used our assay to compare the effects of VPA to that of its analogue VPM, which is not an HDAC inhibitor (Phiel et al., 2001). Fig. 2, D and F illustrate that, while 5 mM VPA results in almost 50 % demethylation, the same concentration of VPM has virtually no effect. Together, the above data support the hypothesis that VPA, through its ability to induce histone hyperacetylation, is an inducer of active DNA demethylation.

VPA triggers active demethylation of CMV-GFP but not global demethylation in HEK 293 cells- We next confirmed the results of our Southern blot analysis by an independent bisulfite analysis, which determines the methylation pattern at single base resolution (Clark et al., 1994). The result of these analyses, presented in Fig. 3A, reveal that methylated CMV-GFP retains its methylation pattern in untreated HEK 293 cells (filled circles represent methylated CpGs). In contrast, a 20 mM VPA treatment results in almost complete demethylation of all the methylated CpG sites tested in GFP (open circles), with the exception of 3 different CpG sites in several clones.

Previously reported data appear to indicate that different HDAC inhibitors vary in their effects on global methylation levels. For example, *n*-butyrate has been shown to cause genome wide hypomethylation (Szyf et al., 1985a), while only selective loss of DNA

methylation was found with TSA (Selker, 1998). To assess the effects of VPA on global methylation levels, we performed a radiolabeled [^{3}H]-dCTP extension assay (Pogribny et al., 1999), in which the amount of [^{3}H]-dCTP incorporated into the DNA following digestion with a methylation sensitive restriction enzyme is directly proportional to the amount of demethylation. We used 5-aza-CdR, a known inhibitor of DNA methyltransferase, as a positive control for demethylation. As can be seen in Fig 3B, the amount of demethylation in VPA treated versus control samples is almost equal (46% compared to 44%). In contrast and as expected, treatment with 5-aza-CdR led to an increase in genome wide demethylation (78%). Thus, while VPA does not induce global demethylation in HEK 293 cells, it leads to demethylation that is selective to certain sequences.

The expression of methylated CMV-GFP is induced by VPA and is dose-dependent- Several studies have demonstrated that VPA treatment can lead to the activation of unmethylated genes by either inducing histone acetylation (Phiel et al., 2001), activating the Wnt signaling pathway (Chen et al., 1999b; Phiel et al., 2001), or by activating AP-1 dependent transcription (Asghari et al., 1998). Certain methylated genes are also induced by VPA treatment but it was not determined whether demethylation is involved. We therefore assessed whether demethylation of CMV-GFP by VPA changes its state of expression using a Western blot analysis with anti-GFP antibody.

As shown in Fig. 4, A and B, transiently transfected methylated CMV-GFP is almost fully repressed in untreated HEK 293 cells (lane 1) as expected. Following treatment

with 1 to 20 mM VPA, a dose dependent induction of GFP expression is observed. Thus, the doses of VPA that lead to the demethylation of CMV-GFP also induce gene expression. VPM, which does not induce histone acetylation, has no effect on GFP protein levels (Fig. 3, C and D), indicating that VPA's ability to promote both the demethylation (Fig. 2) and expression of CMV-GFP is dependent upon its HDAC inhibitory activity.

The induction of expression by VPA might be a consequence of demethylation, or alternatively, demethylation and expression are independent effects of the HDAC inhibitory activity of VPA. To address this issue, we compared the induction of methylated CMV-GFP and unmethylated CMV-GFP following identical transfections and 10 mM VPA treatment. If demethylation were not required for the effects of VPA on expression, then one would predict a similar fold activation of methylated and unmethylated CMV-GFP. The induction of unmethylated GFP by VPA is shown in Fig. 4, E and F, and the comparison with the methylated plasmid is presented in Fig. 4G. This experiment demonstrates that VPA induces the expression of methylated CMV-GFP (12 fold) to a greater extent than unmethylated CMV-GFP (3 fold). Taken together, our data are consistent with the hypothesis that VPA induces the expression of methylated genes through its effects on both histone acetylation and DNA demethylation, the latter of which is unique to methylated genes.

MBD2/dMTase is a demethylase enzyme involved in the active demethylation induced by VPA- The next issue we wanted to address is how does VPA trigger active

demethylation? We have previously hypothesized that the open chromatin structure brought about by histone acetylation favors access of demethylase enzymes to the DNA (Cervoni et al., 2002; Cervoni and Szyf, 2001). We determined whether MBD2/dMTase, the only demethylase characterized to date, is involved in the demethylation induced by VPA. The promoter of MBD2 contains several AP-1 sites, and since VPA can activate AP-1 dependent transcription we hypothesized that VPA might induce MBD2/dMTase expression in HEK 293 cells. An increase in levels of this enzyme, together with acetylation promoted DNA accessibility, could reasonably explain the demethylation observed. An MBD2 Western blot using nuclear extracts from cells treated with increasing doses of VPA was performed, and the results are shown in Fig. 5A. To our surprise, while VPA did not increase the overall levels of MBD 2/dMTase, it caused a shift in the relative levels of two forms of MBD2/dMTase (indicated by the double arrows), such that only a slower migrating form of MBD2/dMTase was present at 20 mM VPA. We then decided to examine whether the upper form might be due to alteration by phosphorylation, given that the size difference between the two forms is consistent with this modification, and since VPA has been shown to activate kinase pathways (Yuan et al., 2001) and lead to the phosphorylation of proteins (De Sarno et al., 2002). Fig. 5B illustrates that upon alkaline phosphatase (CIP) treatment, the upper form of MBD2/dMTase is abolished and only the lower form is present, indicating that phosphorylation of MBD2/dMTase is occurring within HEK 293 cells and that this is increased upon treatment with VPA.

The fact that VPA treatment resulted in the modification of MBD2/dMTase prompted us

to examine whether this enzyme mediates the VPA induced demethylation of CMV-GFP, by measuring the effect of antisense knockdown of MBD2/dMTase. HEK 293 cells were transfected with methylated CMV-GFP, together with an MBD2 antisense expression construct (AS-MBD2) (Slack et al., 2002) or an empty vector as a control. Following treatment with 20 mM VPA, cells were harvested and subjected to Southern blot analysis as in Fig 2. The results shown in Fig. 5C and quantified (average of three experiments) in Fig. 5D indicate that antisense knockdown of MBD2/dMTase inhibits the demethylation induced by VPA by approximately 70%. A Western blot analysis using an anti-MBD2 antibody confirmed that MBD2/dMTase levels were reduced by 50% by the antisense treatment (Fig. 5, E and F). These data indicate that MBD2/dMTase mediates, at least in part, the active demethylation triggered by VPA. The residual (30%) demethylation remaining after antisense knockdown of MBD2/dMTase could be due to either incomplete knockdown (as demonstrated in the Western blot analysis) or due to the presence of other as of yet uncharacterized demethylases.

DISCUSSION

VPA is a well-tolerated drug that has been used for many years in the treatment of epilepsy and bipolar disorder. Although several modes of action have been proposed to explain the therapeutic effects of VPA, this paper describes a novel mechanism that could potentially be involved. Our studies imply that VPA activates methylated genes by stimulating active replication-independent demethylation. It has been shown before that VPA can induce hypomethylation in the rat liver (Alonso-Aperte et al., 1999), similar to

the DNA methylation inhibitor 5-aza-CdR, but it was not clear whether it does so by inhibiting DNA methylation or by other mechanisms. This paper tested the hypothesis that DNA methylation is a reversible process, and therefore drugs that affect DNA methylation patterns might do so by either inhibiting DNA methylation or promoting demethylation. We took advantage of an assay previously developed in our laboratory, which measures active demethylation in living cells, to test whether VPA can stimulate active demethylation.

Using a ChIP assay, we first demonstrate that VPA induces acetylation of H3 histones associated with methylated CMV-GFP (Fig. 1), which is in accordance with previous studies demonstrating the HDAC inhibitory activity of VPA (Gottlicher et al., 2001; Phiel et al., 2001). Second, we show that VPA stimulates active demethylation of ectopically methylated and transiently transfected CMV-GFP DNA (Fig. 2, C and E). Third, we illustrate that demethylation is dependent on histone acetylation since the analogue VPM, which is not an HDAC inhibitor, has no effect (Fig. 2, D and F). Bisulfite mapping analysis confirms these data, and indicates that VPA stimulates the erasure of the entire methylation pattern of CMV-GFP (Fig. 3A). We propose that VPA stimulates demethylation by inducing acetylation of H3 histones associated with CMV-GFP and increasing the accessibility of demethylase to the DNA. Previous studies have demonstrated that 3 mM VPA are required to induce significant histone acetylation (Gottlicher et al., 2001; Phiel et al., 2001), and similarly we observe 50% demethylation at 5 mM, however demethylation further increases up to 20 mM VPA. It is unclear whether at higher concentrations, VPA acts exclusively as a histone deacetylase inhibitor,

or whether it may trigger another as of yet unknown process leading to enhanced demethylation.

Fourth, in contrast to a previous report in which global hypomethylation was observed upon VPA treatment (Alonso-Aperte et al., 1999), we did not detect any change in overall genomic methylation (Fig. 3B), suggesting that VPA treatment has some specificity. How can we explain VPA's selective effects on demethylation and the difference between our results and the abovementioned study? It is possible that the genome in HEK 293 cells is better protected from general demethylation than other cell lines. For example, histone methylation on lysine 9 is a modification that is associated with condensed chromatin and is not affected by HDAC inhibitors (Kondo et al., 2003). Genes associated with K9 methylated histones might be inaccessible to demethylase. Previous observations suggest that histone methylation protects the maternal genome from active demethylation following fertilization (Arney et al., 2002). In addition, we have previously shown that INHAT proteins, which inhibit histone deacetylation, also inhibit active demethylation even in the presence of the HDAC inhibitor TSA (Cervoni et al., 2002 Jul 12).

Fifth, we show that VPA induces the expression of methylated CMV-GFP (Fig. 4, A and B), illustrating that VPA concurrently affects both active demethylation of DNA and gene expression. Furthermore, we demonstrate that demethylation directly contributes to the effects on gene expression (Fig. 4G). These data are consistent with a previous report illustrating that the methylated *5-lipoxygenase* gene can be activated by VPA in non-

proliferating cells, while 5-aza-CdR is only effective in cells that divide (Manev and Uz, 2002). However, this study did not determine whether VPA induced demethylation.

Finally, we show that MBD2/dMTase is required for VPA induced demethylation, supporting the hypothesis that demethylase activity is required for the demethylation triggered by VPA. The results of our antisense experiment (Fig. 5, C and D) indicate that MBD2/dMTase is likely the principal enzyme involved in the demethylation of CMV-GFP. MBD2/dMTase has been shown to act as an active demethylase enzyme *in vitro* (Bhattacharya et al., 1999), it is able to stimulate methylated genes in transfection experiments (Cervoni and Szyf, 2001; Detich et al., 2002; Goel et al., 2003), and its expression is associated with demethylation of endogenous genes (Hattori et al., 2001). However, some groups previously reported that they failed to observe demethylase activity *in vitro* for MBD2, though the reason behind this discrepancy is unclear. *Mbd2-/-* knockouts also did not exhibit global differences in DNA methylation (Hendrich et al., 2001; Ng et al., 1999), however this study did not examine the demethylation of specific genes. Therefore it is possible that MBD2/dMTase is involved in specific demethylation events, which would be consistent with our data.

Furthermore, our data reveal that VPA treatment leads to an increase in phosphorylation of MBD2/dMTase (Fig. 5, A and B). Studies have shown that VPA can lead to phosphorylation of proteins such as AKT, GSK-3B (De Sarno et al., 2002), and ERK (Yuan et al., 2001). As for MBD2/dMTase, the exact mechanism by which this occurs remains to be determined. Although at the present time we do not know what the effects

of this modification are, we speculate that it may increase the enzyme's activity. This has been well established for numerous proteins, such as the MAPK and CDK enzymes (Lew, 2003), as well as for transcription factors such as CREB (Quinn, 2002) and c-jun (Papavassiliou, 1993). Based on our data, we can envision a model where VPA induces active demethylation through two mechanisms: First, by inducing histone acetylation, which would lead to a more open chromatin structure and thus allow the demethylases access to the DNA. And second, by phosphorylating MBD2/dMTase, which could possibly increase its demethylase activity.

Thus, VPA is the first therapeutic agent shown to be able to erase DNA methylation patterns in a replication-independent manner by stimulating a demethylase enzyme. An important point to note is that although our assay measures demethylation that is independent of replication, the HEK 293 cells we use are actively dividing. Thus, we do not know for certain whether postmitotic tissues possess the same machinery involved in chromatin organization and demethylation. However, we do know that MBD2/dMTase is expressed in postmitotic tissues such as the brain (Francis et al., 2002; Jung et al., 2002) and liver (Saito et al., 2001). Furthermore, previous studies have demonstrated that VPA can induce both histone acetylation and the expression of methylated genes (*reelin, gad67, 5-lipoxygenase*) in non-dividing tissues or cells at therapeutically relevant concentrations (Manev and Uz, 2002; Tremolizzo et al., 2002). Although further studies are required to determine whether these genes become demethylated, the abovementioned findings together with our data support the possibility that VPA might be used to modify DNA methylation patterns in postmitotic tissues. This observation has important

therapeutic and biological implications since a number of genes such as *FMR1* (El-Osta, 2002), *NF 2* (Kino et al., 2001), and *reelin* (Tremolizzo et al., 2002) were shown to be silenced in the brain by mechanisms that might involve DNA methylation. The only available demethylating drug is 5-aza-CdR, which needs to be incorporated into DNA and inhibit DNA methyltransferase during cell division. Such a mechanism makes this drug, as well as other DNA methyltransferase inhibitors, ineffective in the brain. Several other HDAC inhibitors such as phenylbutyrate, depsipeptide, and SAHA are known, however they are currently only in use in clinical trials (Johnstone, 2002). Interestingly, SAHA is able to cross the blood brain barrier and improves motor deficits in a mouse model of Huntington's disease (Hockly et al., 2003). However, VPA has the advantage in that it is already an established drug whose long-term effects are known. If it is possible to take advantage of well-tolerated HDAC inhibitors, such as VPA, to stimulate the erasure of DNA methylation by resident demethylases, then it might be feasible to therapeutically activate genes silenced by DNA methylation in the brain and other non-dividing tissues.

Acknowledgements:

Supported by the National Cancer Institute of Canada.

N.D. is a recipient of the Canadian Institute of Health Research Doctoral Fellowship and the McGill Faculty of Medicine Internal Fellowship. V.B. is supported by a Canadian Institute of Health/Fragile X Research Foundation of Canada post-doctoral fellowship.

FIGURE LEGENDS

Fig. 1. Histone acetylation within CMV-GFP is induced by VPA. A. HEK 293 cells were transfected with *in vitro* methylated GFP plasmid and treated with a final concentration of 20 mM VPA (*VPA*) or left untreated (*control*). Cells were formaldehyde crosslinked after 72 h and subjected to a ChIP assay using an antibody against Ac-H3. A 500 bp region of the GFP sequence was amplified from the DNA samples by PCR as described in "materials and methods". A representative PCR is shown: *Input*, 10% of total DNA prior to immunoprecipitation; *no ab*, reactions carried out in the absence of antibody; *IP*, immunoprecipitations with anti-Ac-H3 antibody. **B.** Experiment in (A) was quantified (NIH Image 1.63), and results are presented as the ratio of GFP amplified from the Ac-H3 immunoprecipitate relative to the corresponding input.

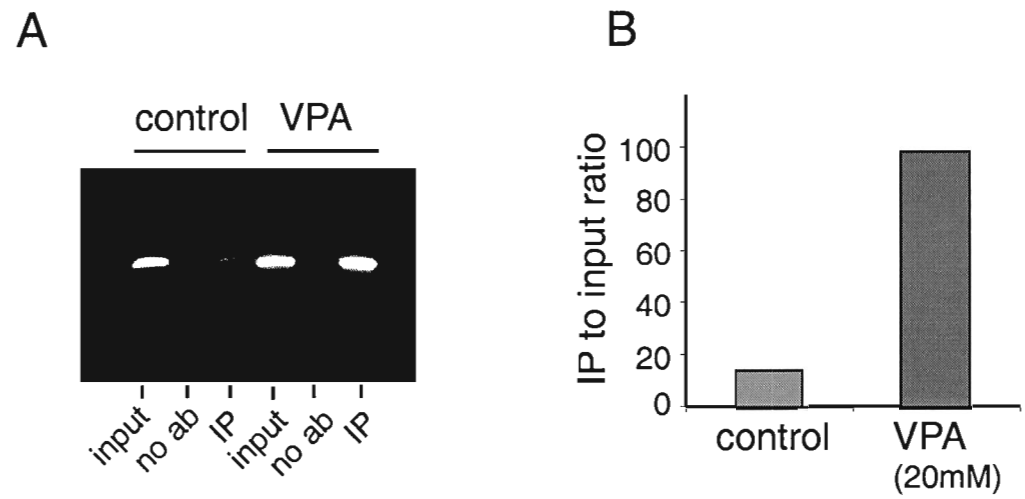

A
control
VPA
input
no ab
IP
input
no ab
IP
B
IP to input ratio
100
80
60
40
20
0
control
VPA
(20mM)

Fig. 2. VPA stimulates active demethylation of CMV-GFP. **A.** *Left panel*, a physical map of the CMV-GFP region with the relevant restriction sites is shown. Probe for Southern blotting is depicted as a *dashed line* flanked by restriction sites *Cfr*10 I and *Ava* II; *arrows* indicate the location of both the outside and nested primers used for bisulfite mapping in Fig. 3. *Right panel*, CMV-GFP plasmid was methylated *in vitro*, and full methylation status was confirmed by *Msp*I/*Hpa*II restriction digestion and Southern blot analysis as outlined in "materials and methods". *Me*, methylated and *Hpa*II undigested GFP fragment; *U*, fully digested GFP fragment (529bp), which appears upon digestion of unmethylated GFP with *Hpa*II or upon digestion with *Msp*I regardless of methylation. **B.** Unmethylated GFP plasmid was transiently transfected into HEK 293 cells. After 24 h, cells were treated with a final concentration of 20 mM VPA or left untreated (*control*). Cells were harvested 72 h post-transfection and GFP methylation status was assessed as in (A). *M*, *Msp*I; *H*, *Hpa*II. **C and D.** HEK 293 cells were transfected with *in vitro* methylated CMV-GFP and treated with either 0.3 μM TSA or increasing concentrations of VPA (C), or with either 5 mM VPA or 5 mM VPM (D). Cells were harvested and methylation of CMV-GFP determined as in (B). **E.** The results of 3 independent experiments as shown in (C) were quantified by densitometry, and the average percent demethylation for each sample was calculated [U/(U+Me)] *100 and charted +/- S.D. **F.** The results of 3 independent experiments as shown in (D) were quantified as in (E) and the averages +/- S.D. are presented.

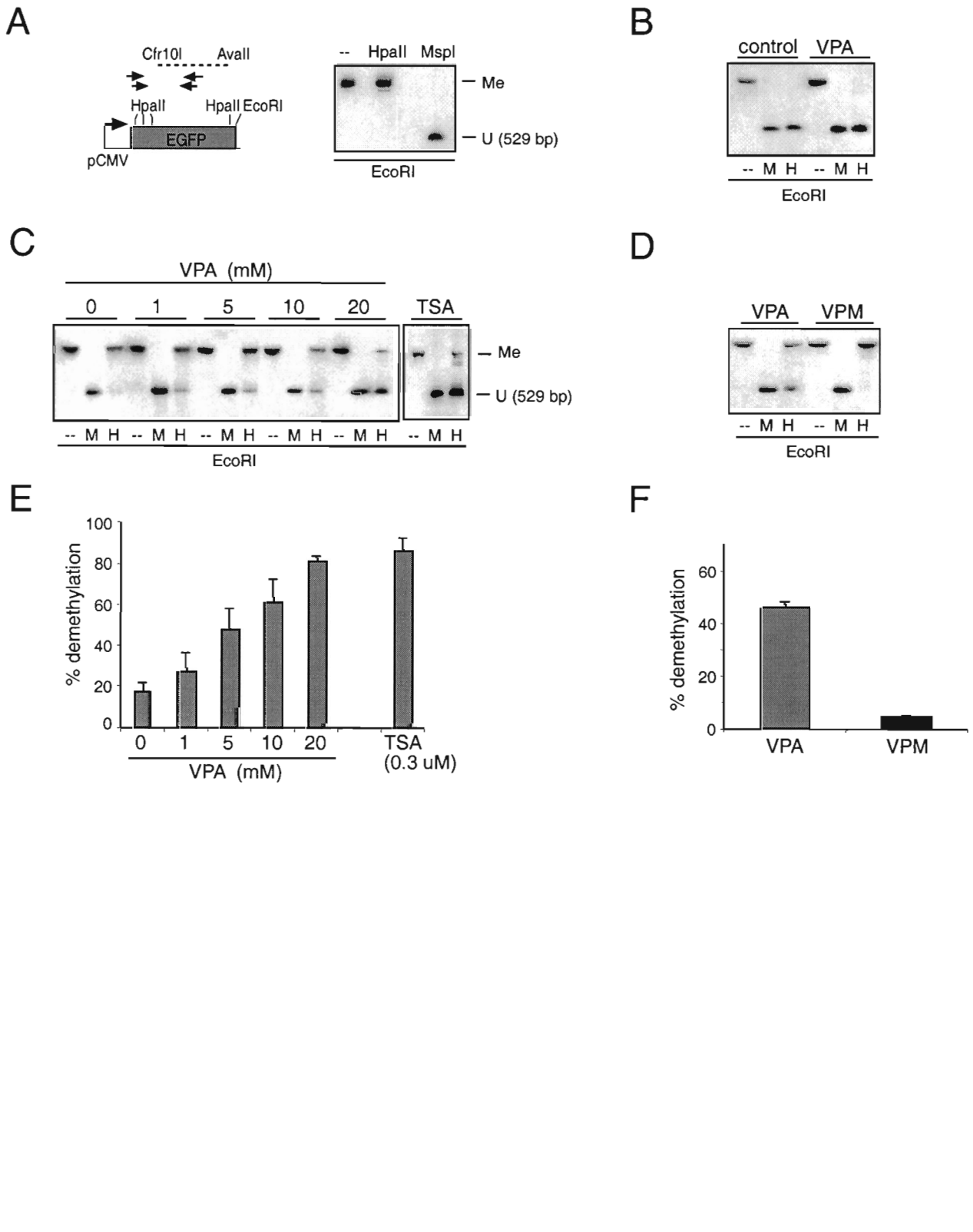
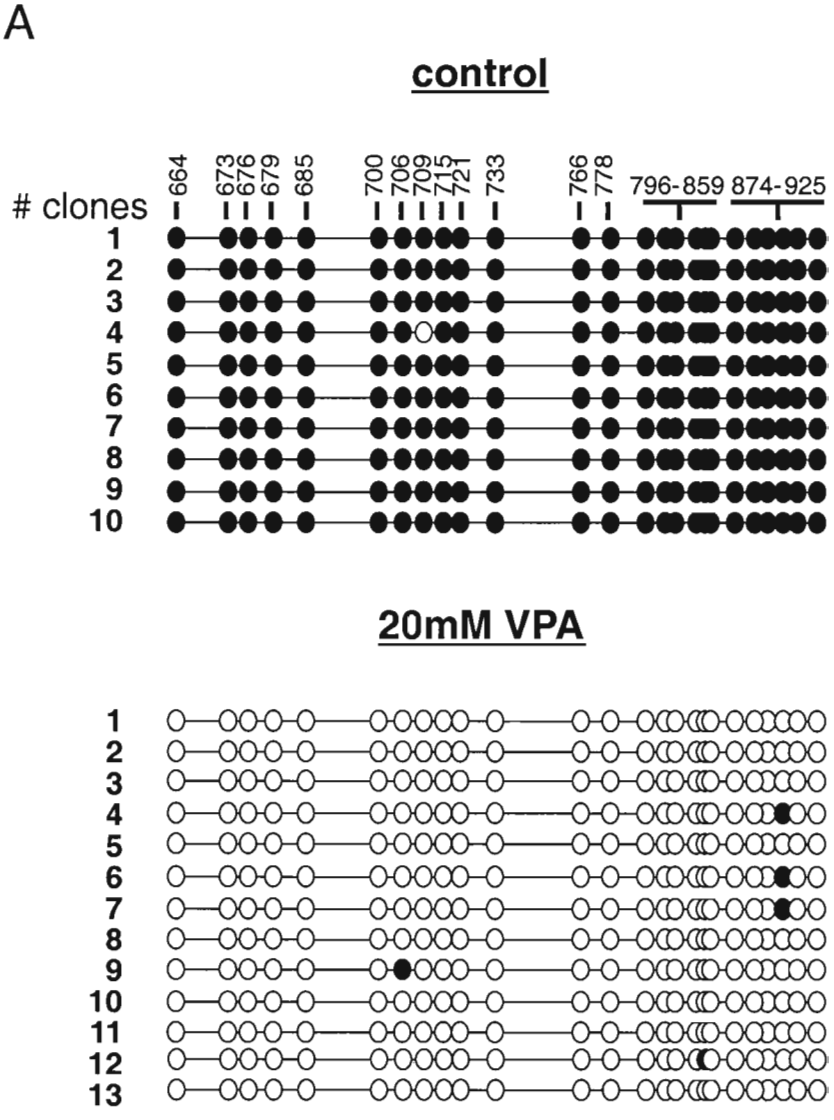

A
Cfr10I
AvaII
HpaII
HpaII EcoRI
EGFP
pCMV
-- HpaII MspI
Me
U (529 bp)
EcoRI
B
control
VPA
-- M H -- M H
EcoRI
C
VPA (mM)
0
1
5
10
20
TSA
Me
U (529 bp)
-- M H -- M H -- M H -- M H -- M H -- M H
EcoRI
D
VPA
VPM
-- M H -- M H
EcoRI
E
% demethylation
100
80
60
40
20
0
0 1 5 10 20
VPA (mM)
TSA
(0.3 uM)
F
% demethylation
60
40
20
0
VPA
VPM

Fig. 3. VPA triggers demethylation of CMV-GFP but not global demethylation in HEK 293 cells. A. HEK 293 cells were transfected with methylated CMV-GFP and treated with either 20 mM VPA or left untreated (*control*). Purified DNA was subjected to bisulfite mapping analysis as previously described (Clark et al., 1994). The sequencing results are presented as a diagram, where each *line* represents an independent clone, *filled circles* represent methylated CG dinucleotides, and *empty circles* represent demethylated CG dinucleotides. The bases at which the CG sites are located are numbered according to Genbank™ accession no. U55763. **B.** HEK 293 cells were treated with either 20 mM VPA (72 h), 1 μM 5-aza-CdR (48 h), or were left untreated (72 h). DNA was then prepared and analyzed for global methylation levels using a radiolabeled [^{3}H]-dCTP extension assay as described in "materials and methods". The average of triplicate experiments is shown in the graph. The data is presented as percentage of demethylation (ratio of *Hpa*II/*Msp*I) +/- relative standard deviation.

A


control
clones
664
673
676
679
685
700
706
709
715
721
733
766
778
796-859
874-925
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2
3
4
5
6
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5
6
7
8
9
10
11
12
13

B

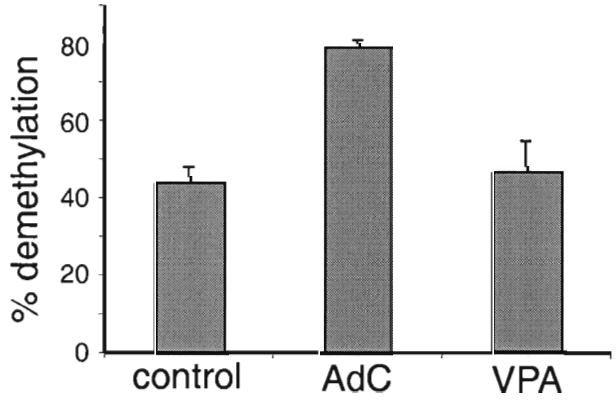
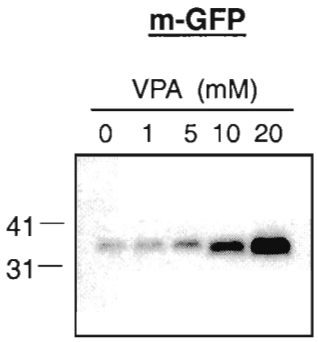

% demethylation
0
20
40
60
80
control
AdC
VPA

Fig. 4. The expression of methylated CMV-GFP is induced by VPA. A, C, E. HEK 293 cells were transiently transfected with either methylated CMV-GFP plasmid (*m-GFP*) (A, C) or unmethylated CMV-GFP (*GFP*) (E), and treated with the indicated concentrations of VPA or VPM. Total cell extracts were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to PDVF membrane and GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334). **B.** Experiments such as shown in (A) were performed in triplicate and GFP expression was quantified by densitometry and normalized to the amount of total protein per lane as determined by Amido black staining. Results are presented as fold induction relative to the level of GFP expression in the absence of VPA. **D and F.** Quantification of triplicate experiments as shown in (C) and (E) respectively; the averages +/- S. D. are presented in arbitrary units $*10^3$. **G.** Comparison of the GFP expression obtained upon treating either m-GFP or GFP transfected cells with 10 mM VPA; graph displays data combined from the experiments shown in (B) and (F).

A


m-GFP
VPA (mM)
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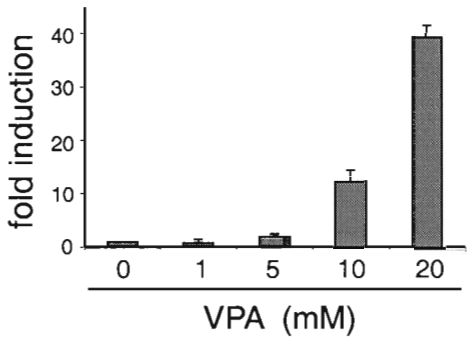
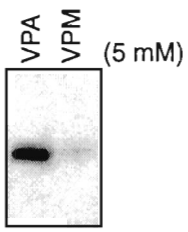
41
31

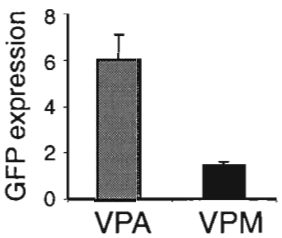
B


fold induction
40
30
20
10
0
0 1 5 10 20
VPA (mM)

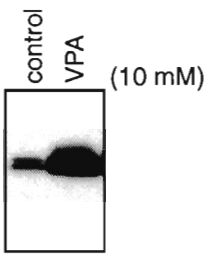
C


m-GFP
VPA
VPM
(5 mM)

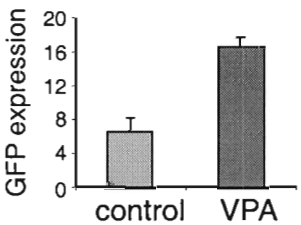
D


GFP expression
8
6
4
2
0
VPA
VPM

E


GFP
control
VPA
(10 mM)

F


GFP expression
20
16
12
8
4
0
control
VPA

G

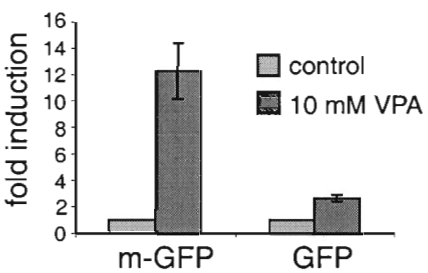
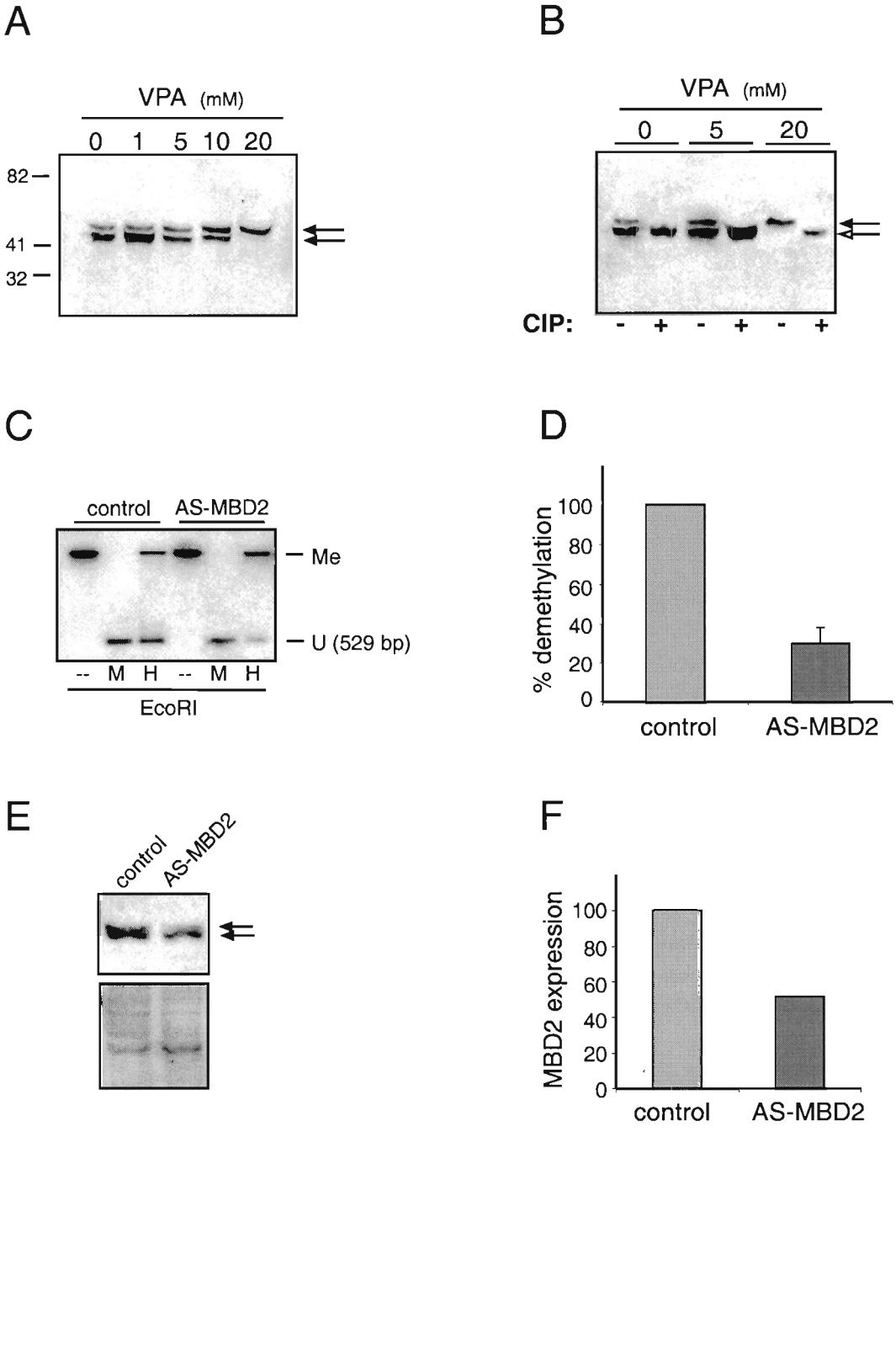

fold induction
16
14
12
10
8
6
4
2
0
control
10 mM VPA
m-GFP
GFP

Fig. 5. MBD2/dMTase is involved in the active demethylation induced by VPA.

A. HEK 293 cells were cultured in the presence of the indicated concentrations of VPA for 48 h and nuclear extracts were prepared and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, which was then probed for MBD2 protein using the monoclonal antibody IMG147 (Imgenex, San Diego, CA, sc-8334). Molecular weight markers in kilodaltons are indicated; *double arrows* indicate the two forms of MBD2 detected. **B.** Nuclear extracts from cells treated with 0, 5, or 10 mM VPA were subjected to alkaline phosphatase (CIP) treatment (+), or a mock treatment (-). Proteins were then resolved on SDS-PAGE and an MBD2 Western was performed as in (A). *Filled* and *open arrows* indicate phosphorylated and de-phosphorylated MBD2 respectively. **C.** HEK 293 cells were transfected with *in vitro* methylated CMV-GFP, together with either an AS-MBD2 expression plasmid or empty vector (*control*). Cells were harvested after 72 h and the methylation status of GFP DNA was determined as described in "materials and methods" and in Fig. 2. *Me*, methylated and *Hpa*II undigested GFP; *U*, unmethylated and *Hpa*II fully digested GFP (529bp); *M*, *Msp*I; *H*, *Hpa*II. **D.** The results of 3 independent experiments as shown in (C) were quantified by densitometry, and the average percent demethylation, [U/(U+Me)] *100, was calculated. Results are presented relative to the control, which was normalized to 100%. **E.** AS-MBD2 expression plasmid or empty vector (*control*) were transfected into HEK 293 cells; cells were harvested 72 h post-transfection and an MBD2 Western blot was performed as in (A). *Upper panel*, MBD2 Western; *lower panel*, Amido black stained membrane. **F.** Experiment in (E) was quantified and results are shown relative to the control, which was normalized to 100%.


A
VPA (mM)
0 1 5 10 20
82
41
32
B
VPA (mM)
0 5 20
CIP: - + - + - +
C
control AS-MBD2
Me
U (529 bp)
-- M H -- M H
EcoRI
D
% demethylation
100
80
60
40
20
0
control
AS-MBD2
E
control
AS-MBD2
F
MBD2 expression
100
80
60
40
20
0
control
AS-MBD2

General Discussion

The aims of this thesis were to determine: 1) The mechanism by which DNMT1 is regulated with cell growth and whether this control is involved in cellular transformation. 2) A potential mechanism by which regional hypermethylation and global hypomethylation co-exist in cancer. 3) The possibility of inhibiting demethylase activity by pharmacological agents with the goal of inhibiting tumorigenesis. 4) The potential to alter DNA methylation patterns even in somatic tissues in the absence of DNA replication. The findings described in the previous chapters provide intriguing alternative hypotheses to explain some of the unresolved issues of previously proposed models. The following discussion will aim to summarize the results of this thesis and show how they might potentially revise our basic concepts of DNA methylation. In addition, the potential implications of these data in the context of cancer and other therapeutics will be explored.

Cell cycle deregulation of DNMT1 in cancer

Over the last two decades, the findings that *DNMT1* is overexpressed in many cancers and is regulated by protooncogenic signaling pathways, together with the observations that *DNMT1* expression can lead to cellular transformation, have established DNMT1 as an important anticancer target. The generally accepted model is that a general increase in DNMT1 levels leads to the methylation of tumor suppressor genes and results in the stimulation of tumor growth. However, several findings appear to be in contradiction

with this model. First, since several studies have demonstrated that inactive chromatin and gene silencing often precede DNA methylation (Bachman et al., 2003; Lock et al., 1987; Szyf et al., 1990), it is possible that the methylation of tumor suppressors is not the primary reason for their inactivation. In addition, whereas ectopic expression of *DNMT1* leads to methylation of tumor suppressor genes (Vertino et al., 1996), this process is often slow and cannot explain the relatively rapid transformation observed (Bakin and Curran, 1999; Slack et al., 1999). Second, studies have shown that the increase in DNMT1 observed in tumor cells corresponds to the relative increase in DNA synthesis. The methylation capacity, which is defined as the amount of DNMT1 per replicating DNA unit, is therefore not increased in cancer cells (Eads et al., 1999; Lee et al., 1996; Sato et al., 2002). Third, studies that correlate the level of DNMT1 activity or expression and the state of methylation of tumor suppressor genes have failed to show a clear relationship (Eads et al., 1999; Oue et al., 2001; Saito et al., 2001; Sato et al., 2002). Furthermore, one group reported that although a *DNMT1 -/-* cancer cell line displayed a 90% reduction in methyltransferase activity, the tumor suppressor *p16* remained methylated (Rhee et al., 2000).

Because DNMT1 is a protein with other functions outside methylation, tumor suppressor methylation might be a result of other DNMT1 dependent processes, such as its interaction with repressor complexes. Moreover, DNMT1 was found to be regulated with the cell cycle, and studies have illustrated that the coordinated cell cycle regulation of DNMT1 is disrupted in colorectal cancer cells *in vivo* (De Marzo et al., 1999), as well as in estrogen receptor negative breast cancer cells (Nass et al., 1999). For these reasons,

we decided to test that hypothesis that deregulation of DNMT1 with the cell cycle, and not its general overexpression, is critical for cellular transformation. Chapter 1 demonstrates that the 3'UTR of the *DNMT1* mRNA, more specifically a 54-nucleotide highly conserved element, can confer a growth-dependent mRNA regulation at the posttranscriptional level by modulating mRNA turnover rates. In addition, the binding of a 40 kDa protein (p40) to the 3'UTR is increased in growth-arrested cells and is inversely correlated with *dnmt1* mRNA levels as cells are induced into the cell cycle. Most importantly, while ectopic expression of *DNMT1* lacking the 3'UTR can transform NIH-3T3 cells (Wu et al., 1993), inclusion of the 3'UTR prevented transformation. These results support the alternative hypothesis stated above, that deregulated expression of *DNMT1* with the cell cycle and not the total amount of DNMT1 is important for cellular transformation

One important question is how could ectopic expression of *DNMT1* in arrested cells cause transformation? It is possible that expression of *DNMT1* in arrested cells results in the *de novo* methylation and silencing of a critical tumor suppressor gene. An alternative hypothesis is that ectopic expression of *DNMT1* causes cell transformation by interfering with cell cycle regulatory circuits through DNMT1 protein- protein interactions. Since DNMT1 forms a complex with Rb and E2F (Robertson et al., 2000a) as well as HDACs 1 and 2 (Fuks et al., 2000; Rountree et al., 2000) it can inhibit the expression of tumor suppressors by a mechanism that does not involve DNA methylation, as has been previously shown (Milutinovic et al., 2000). In addition, since DNMT1 has been shown to bind to the replication protein PCNA at the same site as the cell cycle inhibitor p21,

DNMT1 could displace p21 from PCNA during the Go/G1 phase and allow replication to occur (Chuang et al., 1997). Thus, aberrant expression of *DNMT1* during the Go/G1 phase may override the silencing of tumor suppressors and eliminate normal arrest signals, leading to the uncontrolled growth that is observed in cancer cells. This can also explain why some studies (mentioned above) showed that *DNMT1* is not overexpressed in tumors when its expression is normalized to other cell cycle associated genes. We propose that ectopic expression of *DNMT1* at the wrong phase of the cell cycle leads to aberrant entry into S phase, which in turn stimulates the expression of other cell cycle associated genes.

It is also possible that both mechanisms, abnormal methylation and the elimination of growth arrest signals, occur. This is supported by studies in which inhibition of poly (ADP- ribosyl)ation was found to induce genomic hypermethylation, as well as increase the level of DNMT1 specifically at the G1/S border, which was accompanied by the premature formation of a DNMT1-PCNA complex (Zardo et al., 2002).

Several important questions still await answers, such as the mechanism by which the *DNMT1* 3'UTR and p40 affect mRNA stability during cell growth. One potential model is that the regulation of *DNMT1* expression with the cell cycle involves the degradation of the *DNMT1* mRNA in growth-arrested cells, through the interaction of p40 with the 3'UTR, either directly or via the targeting of RNase complexes. Although the causal role of p40 has not been determined as of yet, studies underway in our laboratory involving the purification and identification of p40 should result in the clarification of this

mechanism. In addition, it is important to determine whether defects involving the 3'UTR and p40 occur in various cancers, as these may represent potential diagnostic and therapeutic tools. For example, perhaps mutations within the DNMT1 3'UTR occur in cancers demonstrating abnormal DNMT1 cell cycle regulation, such as in the examples mentioned above. Although one recent study identified mutations within the *DNMT1* coding region in 7% of colorectal tumors (Kanai et al., 2003), the 3'UTR has yet to be investigated. It is also feasible that p40 expression or activity is deregulated in cancer, possibly through upstream oncogenic signaling pathways. For example, T-antigen was found to influence *dnmt1* at the posttranscriptional level by increasing the stability of the *dnmt1* mRNA (Slack et al., 1999). Although the exact mechanism involved in the T-antigen induced mRNA stability was not established, the possibility exists that it involves the same components as found in the cell cycle regulation of DNMT1, i.e. the 3'UTR and p40. One possible model is that Rb, a negative regulator of the cell cycle, acts upstream of p40 to downregulate the *DNMT1* mRNA in Go/G1 and thus prevent methylation in the absence of replication. Following entrance into S phase, or ectopic expression of T-antigen, Rb is inactivated, p40 levels are decreased, and *DNMT1* mRNA is stabilized. In the case of T-antigen, its constitutive expression would lead to increased DNMT1 throughout the cell cycle, thus promoting cellular transformation. However, all this is only speculative, and further studies defining the identity of p40 and its regulation should help to uncover the precise mechanism involved.

We propose that cell cycle regulatory functions and DNA methyltransferase activities have been assembled into one polypeptide to ensure that DNA replication will never

proceed in the absence of DNA methylation and vice versa. The hypothesis that DNMT1 evolved from an earlier replication-associated protein is supported by the discovery of a protein related to DNMT1 in *drosophila* (Hung et al., 1999). This DNMT1 related protein interacts with PCNA similarly to DNMT1 but does not possess catalytic methyltransferase activity.

According to the paradigm where a general overexpression of *DNMT1* leads to transformation by methylating tumor suppressors, the therapeutic goal is to inhibit DNA methylation so as to reactivate these genes and suppress tumor growth. However, this may inadvertently lead to detrimental effects via global hypomethylation as mentioned earlier. On the other hand, if the regulatory domains are responsible for promoting transformation following inappropriate *DNMT1* cell cycle expression, as we suggest, then other methods of therapy should be explored, such as agents targeted at these domains or at restoring proper *DNMT1* expression. Although our model argues that the methylation of tumor suppressors is not the primary cause of transformation by DNMT1, the common occurrence of hypermethylation in cancer indicates that it is important. Thus, another promising option is the use of agents that knockdown DNMT1, since they have been found to inhibit DNA replication and cell growth (Knox et al., 2000; Milutinovic et al., 2003) and thus protect the genome from global hypomethylation, and also indirectly bring about the induction and demethylation of tumor suppressor genes (Fournel et al., 1999).

A multifunctional MBD2/dMTase

A fundamental question that has puzzled us when we try to comprehend the role of DNA methylation in cancer is the co-existence of regional hypermethylation and global hypomethylation. It is critical to unravel the mechanisms responsible for this phenomenon in order to design correct therapeutic strategies targeting the DNA methylation machinery. Several possible explanations were discussed in the introduction, such as the targeting of DNMTs and demethylases to specific regions through their interactions with other proteins (Di Croce et al., 2002; Robertson et al., 2000a; Rountree et al., 2000), the protection of specific sequences from methylation/demethylation via the binding of other proteins (Brandeis et al., 1994; Han et al., 2001; Lin and Hsieh, 2001; Macleod et al., 1994), and the determination of DNA methylation patterns by local changes in chromatin structure (Cervoni and Szyf, 2001; Dennis et al., 2001; Gibbons et al., 2000; Jeddeloh et al., 1999; Selker, 1998).

In addition, since the protein MBD2 has been shown to be both a repressor of methylated DNA and a DNA demethylase, the possibility exists that global hypomethylation and local repression are catalyzed by the same multifunctional protein. We hypothesized that different promoter interactions can direct the opposite activities of MBD2, and thus even its overexpression will not result in the demethylation and activation of all genes. This would also resolve the apparently contradictory functions of MBD2 reported by various groups. Results presented in chapter 2 demonstrate that ectopic expression of MBD2/dMTase differentially activated some but not all promoters in a time- and

concentration-dependent manner. We also found that MBD2/dMTase expression induced demethylation within the SV40 promoter concurrent with its activation. These data support our hypothesis that the complex functional role of this protein depends on the promoter context, and also suggests that MBD2/dMTase could be involved in maintaining both the DNA hyper and hypomethylation observed in cancer.

Further studies are necessary to uncover the promoter elements that are implicated in determining the role of MBD2/dMTase in transcription, however it is highly likely that both chromatin structure and interactions between MBD2 and other proteins are also involved. It was demonstrated that when an identical reporter gene was placed under different methylated promoters, either active or inactive, the reporter sequence was demethylated only when it was associated with an active promoter; this was found to depend upon the presence of histone acetylation (Cervoni and Szyf, 2001). Thus it may not be particular elements within the promoter that determine whether MBD2/dMTase acts as a repressor or activator, but rather the chromatin structure the DNA adopts in the cell. On the other hand, specific promoter sequences may be involved in attracting certain activators or repressors that could potentially interact with MBD2/dMTase and affect its function.

One recently identified protein, MBDin, was shown to relieve MBD2 mediated transcriptional repression from methylated DNA (Lembo et al., 2003). Although demethylation was not observed in this study, this aspect may have been assessed too soon after transfection, as we also did not observe demethylation after a similar length of

time. Perhaps only a transient chromatin activation by MBDin was achieved in their study, and possibly the continued presence of MBDin is necessary to cause a constant change in chromatin structure that would eventually lead to demethylation and stabilization of the active state. MBD2/dMTase protein-protein interactions may also be influenced by other factors, which is supported by the observation that different MeCP1 complexes, some of which do not contain MBD2, are formed in diverse cell types (Hendrich et al., 2001). One interesting prospect stems from data presented in chapter 4 illustrating that VPA induces active DNA demethylation and the phosphorylation of MBD2 in parallel. Since this posttranslational modification has been shown to regulate the activity of other proteins, it may well play a role in determining which of the two functions MBD2/dMTase assumes.

Data presented in chapter 2 is consistent with our hypothesis that MBD2/dMTase can be involved in mediating both regional hypermethylation and global hypomethylation in cancer. We suggest that cancer cells take advantage of this protein to coordinate a program that requires suppression of some methylated genes, such as tumor suppressor genes, and activation of others, for instance genes involved in invasion and metastasis. If this is correct, then MBD2 truly represents an ideal anticancer target since its inhibition would result in both repression and activation of the desired genes. Although still speculative, future studies identifying the promoter elements and their interacting proteins, and whether these differ between the various classes of genes involved in cancer, should help substantiate our hypothesis.

Therapeutic implications of inhibiting MBD2/dMTase activity

It has long been believed that DNA methylation is a unidirectional and irreversible reaction, which is catalyzed by DNMT exclusively. If this were true, then the only potential target for modulating DNA methylation would be DNMTs. However, there is sufficient data to support the hypothesis that DNA methylation *in vivo* is also fashioned by active demethylation (Lucarelli et al., 2000; Oswald et al., 2000; Szyf et al., 1985);Bruniquel, 2003}. This is further substantiated by the purification of a DNA demethylase activity from human lung carcinoma A549 cells (Ramchandani et al., 1999) and by the discovery that the protein MBD2 can also function as a DNA demethylase (Bhattacharya et al., 1999). As a result, we need to modify our classical view of DNA methylation from a unidirectional irreversible reaction to a reversible reaction mediated by both DNMT and demethylase activities. Although the concept of reversible biological signals is accepted for other modifications, such as acetylation and phosphorylation, it has not been considered as a possible mechanism for the modulation of DNA methylation by physiological and pharmacological agents. However, if true, we are provided with another target in addition to DNMTs with which to correct aberrant DNA methylation and gene expression in pathological conditions. In this thesis, a potential inhibitor of this target was identified as AdoMet.

AdoMet has been used for many years as a therapeutic and a research agent to increase DNA methylation *in vivo* and *in vitro*, and it is also deemed an important physiological, nutritional, and pathological regulator of DNA methylation patterns. However the prevailing model explaining the mechanism of action of AdoMet has been that increased

AdoMet levels stimulate DNMT activity thus triggering hypermethylation and protecting the genome against global hypomethylation. As mentioned in chapter 3, there are several unresolved issues with this paradigm. Furthermore, since one study demonstrated that AdoMet inhibited the rapid demethylation of a CG site in the 5' region of the *myogenin* gene during C2C12 differentiation (Fuso et al., 2001), this suggested to us that AdoMet might affect active demethylation. In chapter 3, we take into account the demethylase side of the methylation reaction, and propose the alternative hypothesis that AdoMet prevents global hypomethylation by inhibiting active demethylases. Our data demonstrate that AdoMet inhibits active demethylation and expression of methylated DNA in living cells, and also inhibits MBD2/dMTase, as well as endogenous demethylase activity extracted from HEK 293 cells, *in vitro*. Our hypothesis is further substantiated by the fact that AdoMet does not stimulate *de novo* methylation of unmethylated DNA. Most importantly, our finding that AdoMet inhibits anchorage independent growth of HEK 293 and A549 cells at the same concentration that inhibits demethylase activity is consistent with the premise that hypomethylation mediated by DNA demethylases is critical for tumorigenesis.

This study identifies the first inhibitor of demethylase *in vitro* and in living cells, and illustrates the dynamic nature of the DNA methylation pattern and how it can be modified by important metabolic regulators. Since intracellular AdoMet levels can be altered by folate supplementation (Miller et al., 1994; Poirier et al., 2001), this raises the exciting possibility that AdoMet may also be beneficial in cancer prevention simply through dietary changes. To confirm our hypothesis that hypomethylation catalyzed by

demethylases is important for cancer, future experiments are required to identify the genes involved in invasion and metastasis that are upregulated by demethylases and suppressed by AdoMet. Unpublished data indicate that uPA might be a potential candidate, since treatment of MDA-231 breast cancer cells with AdoMet or with an MBD2 antisense oligonucleotide resulted in the inhibition of uPA mRNA expression and a decrease in tumor cell invasive capacity (Pakneshan et al, unpublished data). In addition, the mechanism by which AdoMet inhibits MBD2/dMTase and HEK 293 demethylase activities, and whether these are one and the same enzyme, remains to be determined. To fully understand the therapeutic potential of AdoMet, it is critical to identify how many demethylases are present in various types of cancer cells, which ones are inhibited by this agent, and whether they differentially regulate genes that contribute to cancer progression.

The combined data in chapters 1 and 3, together with other published data on the involvement of DNA methylation and its machinery in cancer, evoke a model whereby the changes in DNA methylation required for tumorigenesis involve two separate steps. The first step involves an increase in DNMT1. This step leads to the circumvention of tumor growth signals, through protein-protein interactions with cell cycle machinery, and through the inhibition of tumor suppressors by methylation independent mechanisms and possibly through hypermethylation. Once uncontrolled growth is achieved, the tumor must then be able to invade and metastasize to surrounding tissues. Overexpression of MBD2 and other demethylases is thus critical to achieve the hypomethylation that is required for the activation of growth invasive genes involved in this process. A

combination of inhibitors that knock down DNMT1 or affect its protein interactions, together with demethylase inhibitors such as AdoMet, may thus represent the ideal therapeutic strategy for cancer.

Therapeutic implications of promoting MBD2/dMTase activity

Another intriguing opportunity that arises from the fact that DNA methylation is dynamic and reversible is that epigenomic states might be manipulated therapeutically in postmitotic tissues. If DNA methylation were only mediated by DNMT activities, as the original model proposes, the only possible means of demethylating and reactivating genes would be to induce passive demethylation by inhibiting DNMT1 during DNA replication. Such DNMT1 inhibitors are extremely effective on dividing cells but are useless for post mitotic cells that do not divide. However, if we can identify agents that can pharmacologically stimulate active demethylation by endogenous demethylases, then we might have a chance to alter DNA methylation patterns in adult non-dividing tissue, such as the brain. Previous findings illustrating that VPA can activate methylated genes in non-dividing cells (Manev and Uz, 2002; Tremolizzo et al., 2002) led us to the hypothesis VPA does so by stimulating active DNA demethylation. Data presented in chapter 4 demonstrate that this drug can stimulate active replication independent demethylation and activation of a methylated reporter construct, while global methylation levels are unaffected. We also show that the mechanism of demethylation by VPA involves histone acetylation, further confirming previous observations that demethylase activity is determined by chromatin structure (Cervoni and Szyf, 2001). Antisense

knockdown indicates that MBD2/dMTase is involved in the process, and experiments showing that VPA induces the phosphorylation of MBD2/dMTase suggest that this modification may regulate the activity of this enzyme.

Thus, we provide the first demonstration that a well-established drug can erase DNA methylation patterns in absence of DNA replication. VPA is widely used as an anticonvulsant, in psychiatric disease such as bipolar disorder, and also as an adjunct therapy in schizophrenia. Although VPA has been shown to function through several mechanisms such as increasing levels of GABA (Loscher, 2002), inhibiting the protein kinase C pathway (Chen et al., 1994), and by activating AP-1 dependent transcription (Asghari et al., 1998), our data raise the possibility that VPA's therapeutic effects might also be a result of the demethylation and reexpression of methylated genes. As mentioned in chapter 4, this newly found mechanism of action of VPA might also provide a new therapy for other conditions where the underlying problem is the silencing of essential genes by methylation. Importantly, our data indicate that VPA might be able to achieve the activation of critical genes without the deleterious effect of inducing global demethylation and possibly promoting cancer. Although it remains to be determined what distinguishes genes that are demethylated by VPA and those that are not, it is highly possible that both chromatin structure, as well as the previously mentioned factors determining the role of MBD2/dMTase, are involved.

Further studies are obviously required to confirm the therapeutic relevance of our findings. Several possible candidates for VPA induced demethylation already exist, such

as *reelin, gad67, FMR1*, and *NF2*. *Reelin* and *gad67* have already been shown to be upregulated by VPA in the brain (Tremolizzo et al., 2002), and future studies revealing whether they also become demethylated should confirm our hypothesis. In addition, *FMR1* (El-Osta, 2002), and *NF2* (Kino et al., 2001), which are pathologically downregulated by methylation, should be examined for induction and demethylation by VPA. Focus should also be placed on identifying novel endogenous genes that are upregulated by VPA, most importantly in post mitotic tissues, and whether these genes are regulated by methylation. Furthermore, it is critical to determine whether hypermethylation of such genes occurs during the development of disease conditions, as these will be ideal targets for VPA. The recent development of microarray-based DNA methylation analysis may be a useful tool for such a study (Adorjan et al., 2002).

The future discovery of other VPA-activated demethylases expressed in post-mitotic tissues, as well as of the role of phosphorylation on their activity, should further clarify the effects of VPA. Subsequent investigations into the expression levels and phosphorylation status of demethylases in normal versus pathogenic conditions may then be carried out in order to further confirm both our hypothesis and the therapeutic use of VPA.

Conclusion

In summary, the studies undertaken in this thesis provide alternative models to explain some of the unresolved issues involving the mechanisms and role of *DNMT1* overexpression, regional hypermethylation and global hypomethylation in cancer, and

therapeutic approaches for modulating DNA methylation patterns in cancer and other diseases. The data that are presented support a unifying hypothesis in which the DNA methylation pattern is maintained in a steady-state by an equilibrium of methylation and demethylation reactions that are determined by chromatin structure. Environmental cues that alter either chromatin structure or the regulation of the DNA methylation machinery will cause a shift in the equilibrium and therefore a change in gene expression. When these signals lead to aberrant DNA methylation and expression, pathogenic conditions result. Thus, one might take advantage of the reversibility of DNA methylation patterns to cause either hypo or hypermethylation in different contexts, with the aim of restoring proper gene expression. The advantage of modulating demethylase activity is that it is independent of DNA replication and is therefore applicable to postmitotic tissues. With regards to cancer, regional hypermethylation might not be as important as previously assumed, and focus should rather be placed on the deregulation of *DNMT1* expression and the consequences of its ensuing protein interactions. On the other hand, the finding that AdoMet can inhibit active demethylases and cellular transformation suggests that global hypomethylation should be given a higher priority. These discoveries should guide us in understanding which functions of the DNA methylation machinery are critical for tumorigenesis, and also in developing more effective therapies targeting the DNA methylation machinery for the treatment of cancer and other pathologies.

REFERENCES

Aagaard, L., Laible, G., Selenko, P., Schmid, M., Dorn, R., Schotta, G., Kuhfittig, S., Wolf, A., Lebersorger, A., Singh, P. B., *et al.* (1999). Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. Embo J *18*, 1923-1938.

Aaronson, S. A., and Todaro, G. J. (1968). SV40 T antigen induction and transformation in human fibroblast cell strains. Virology *36*, 254-261.

Adorjan, P., Distler, J., Lipscher, E., Model, F., Muller, J., Pelet, C., Braun, A., Florl, A. R., Gutig, D., Grabs, G., *et al.* (2002). Tumour class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acids Res *30*, e21.

Ahuja, N., Li, Q., Mohan, A. L., Baylin, S. B., and Issa, J. P. (1998). Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res *58*, 5489-5494.

Aletta, J. M., Cimato, T. R., and Ettinger, M. J. (1998). Protein methylation: a signal event in post-translational modification. Trends Biochem Sci *23*, 89-91.

Alonso-Aperte, E., Ubeda, N., Achon, M., Perez-Miguelsanz, J., and Varela-Moreiras, G. (1999). Impaired methionine synthesis and hypomethylation in rats exposed to valproate during gestation. Neurology *52*, 750-756.

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2 [see comments]. Nat Genet *23*, 185-188.

Araujo, F. D., Croteau, S., Slack, A. D., Milutinovic, S., Bigey, P., Price, G. B., Zannis-Hadjopoulos, M., and Szyf, M. (2000). The DNMT1 target recognition domain resides in the N-terminus. J Biol Chem *276*, 6930-6936.

Araujo, F. D., Knox, J. D., Szyf, M., Price, G. B., and Zannis-Hadjopoulos, M. (1998). Concurrent replication and methylation at mammalian origins of replication [published erratum appears in Mol Cell Biol 1999 Jun;19(6):4546]. Mol Cell Biol *18*, 3475-3482.

Arney, K. L., Bao, S., Bannister, A. J., Kouzarides, T., and Surani, M. A. (2002). Histone methylation defines epigenetic asymmetry in the mouse zygote. Int J Dev Biol *46*, 317-320.

Asghari, V., Wang, J. F., Reiach, J. S., and Young, L. T. (1998). Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. Brain Res Mol Brain Res *58*, 95-102.

Asson-Batres, M. A., Spurgeon, S. L., Diaz, J., DeLoughery, T. G., and Bagby, G. C. (1994). Evolutionary conservation of the AU-rich 3' untranslated region of messenger RNA. Proc Natl Acad Sci U S A *91*, 1318-1322.

Atasoy, U., Watson, J., Patel, D., and Keene, J. D. (1998). ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. J Cell Sci *111*, 3145-3156.

Ausubel, F. M., Brent, R., Kingston R.E., Moore, D.D.,Smith, J.A., Seidman, J.G., and Stairwell, K. (1988). Current Protocols in Molecular Biology (New York, John Wiley and Sons).

Axelrod, J. T., R. (1958). Enzymatic O-methylation of epinephrine and other catechols. Journal of Biological Chemistry *233*, 702-705.

Bachman, K. E., Park, B. H., Rhee, I., Rajagopalan, H., Herman, J. G., Baylin, S. B., Kinzler, K. W., and Vogelstein, B. (2003). Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell *3*, 89-95.

Bachman, K. E., Rountree, M. R., and Baylin, S. B. (2001). Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. J Biol Chem *276*, 32282-32287.

Bacolla, A., Pradhan, S., Larson, J., Roberts, R., and Wells, R. (2001). Recombinant human DNA (cytosine-5) methyltransferase. III. Allosteric control, reaction order, and influence of plasmid topology and triplet repeat length on methylation of the fragile X CGG.CCG sequence. J Biol Chem *276*, 18605-18613.

Baker, D. M., Wang, S. L., Bell, D. J., Drevon, C. A., and Davis, R. A. (2000). One or more labile proteins regulate the stability of chimeric mRNAs containing the 3'-untranslated region of cholesterol-7alpha - hydroxylase mRNA. J Biol Chem *275*, 19985-19991.

Bakin, A. V., and Curran, T. (1999). Role of DNA 5-methylcytosine transferase in cell transformation by fos. Science *283*, 387-390.

Bakker, A., and Smith, D. W. (1989). Methylation of GATC sites is required for precise timing between rounds of DNA replication in Escherichia coli. J Bacteriol *171*, 5738-5742.

Ballestar, E., and Wolffe, A. P. (2001). Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem *268*, 1-6.

Bannister, A. J., Schneider, R., and Kouzarides, T. (2002). Histone methylation: dynamic or static? Cell *109*, 801-806.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120-124.

Bauer, U. M., Daujat, S., Nielsen, S. J., Nightingale, K., and Kouzarides, T. (2002). Methylation at arginine 17 of histone H3 is linked to gene activation. EMBO Rep *3*, 39-44.

Baylin, S. B. (1992). Abnormal regional hypermethylation in cancer cells. AIDS Res Hum Retroviruses *8*, 811-820.

Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res *72*, 141-196.

Baylin, S. B., Hoppener, J. W., de Bustros, A., Steenbergh, P. H., Lips, C. J., and Nelkin, B. D. (1986). DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. Cancer Res *46*, 2917-2922.

Beaulieu, N., Morin, S., Chute, I., Robert, M., Nguyen, H., and MacLeod, A. (2002). An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J Biol Chem *277*, 28176-28181.

Bedford, M. T., and van Helden, P. D. (1987). Hypomethylation of DNA in pathological conditions of the human prostate. Cancer Res *47*, 5274-5276.

Belinsky, S. A., Nikula, K. J., Baylin, S. B., and Issa, J. P. (1996). Increased cytosine DNA-methyltransferase activity is target-cell- specific and an early event in lung cancer. Proc Natl Acad Sci U S A *93*, 4045-4050.

Belinsky, S. A., Nikula, K. J., Palmisano, W. A., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S. B., and Herman, J. G. (1998). Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A *95*, 11891-11896.

Bentley, R., and Chasteen, T. G. (2002). Microbial methylation of metalloids: arsenic, antimony, and bismuth. Microbiol Mol Biol Rev *66*, 250-271.

Bestor, T. H. (1988). Cloning of a mammalian DNA methyltransferase. Gene *74*, 9-12.

Bestor, T. H. (2000). The DNA methyltransferases of mammals. Hum Mol Genet *9*, 2395-2402.

Bhattacharya, S. K., Ramchandani, S., Cervoni, N., and Szyf, M. (1999). A mammalian protein with specific demethylase activity for mCpG DNA. Nature *397*, 579-583.

Bigey, P., Knox, J. D., Croteau, S., Bhattacharya, S. K., Theberge, J., and Szyf, M. (1999). Modified oligonucleotides as bona fide antagonists of proteins interacting with DNA. Hairpin antagonists of the human DNA methyltransferase. J Biol Chem *274*, 4594-4606.

Bigey, P., Ramchandani, S., Theberge, J., Araujo, F. D., and Szyf, M. (2000). Transcriptional regulation of the human DNA Methyltransferase (dnmt1) gene. Gene *242*, 407-418.

Billard, L. M., Magdinier, F., Lenoir, G. M., Frappart, L., and Dante, R. (2002). MeCP2 and MBD2 expression during normal and pathological growth of the human mammary gland. Oncogene *21*, 2704-2712.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. Nature *321*, 209-213.

Black, A. R., Black, J. D., and Azizkhan-Clifford, J. (2001). Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. J Cell Physiol *188*, 143-160.

Boeke, J., Ammerpohl, O., Kegel, S., Moehren, U., and Renkawitz, R. (2000). The minimal repression domain of MBD2b overlaps with the methyl-CpG- binding domain and binds directly to Sin3A. J Biol Chem *275*, 34963-34967.

Bonfils, C., Beaulieu, N., Chan, E., Cotton-Montpetit, J., and MacLeod, A. R. (2000). Characterization of the human DNA methyltransferase splice variant Dnmt1b. J Biol Chem *275*, 10754-10760.

Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994). Sp1 elements protect a CpG island from de novo methylation. Nature *371*, 435-438.

Bruniquel, D., and Schwartz, R. H. (2003). Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol *4*, 235-240.

Buzby, J. S., Brewer, G., and Nugent, D. J. (1999). Developmental regulation of RNA transcript destabilization by A + U- rich elements is AUF1-dependent. J Biol Chem *274*, 33973-33978.

Buzby, J. S., Lee, S. M., Van Winkle, P., DeMaria, C. T., Brewer, G., and Cairo, M. S. (1996). Increased granulocyte-macrophage colony-stimulating factor mRNA instability in cord versus adult mononuclear cells is translation- dependent and associated with increased levels of A + U-rich element binding factor. Blood *88*, 2889-2897.

Byvoet, P. (1972). In vivo turnover and distribution of radio-N-methyl in arginine-rich histones from rat tissues. Arch Biochem Biophys *152*, 887-888.

Byvoet, P., Shepherd, G. R., Hardin, J. M., and Noland, B. J. (1972). The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. Arch Biochem Biophys *148*, 558-567.

Cai, J., Mao, Z., Hwang, J. J., and Lu, S. C. (1998). Differential expression of methionine adenosyltransferase genes influences the rate of growth of human hepatocellular carcinoma cells. Cancer Res *58*, 1444-1450.

Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re- expression of genes silenced in cancer. Nat Genet *21*, 103-107.

Campbell, P. M., and Szyf, M. (2003). Human DNA methyltransferase gene DNMT1 is regulated by the APC pathway. Carcinogenesis *24*, 17-24.

Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell *14*, 105-113.

Carlson, L. L., Page, A. W., and Bestor, T. H. (1992). Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. Genes Dev *6*, 2536-2541.

Caudill, M., Wang, J., Melnyk, S., Pogribny, I., Jernigan, S., Collins, M., Santos-Guzman, J., Swendseid, M., Cogger, E., and James, S. (2001). Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr *131*, 2811-2818.

Cedar, H., Stein, R., Gruenbaum, Y., Naveh-Many, T., Sciaky-Gallili, N., and Razin, A. (1983). Effect of DNA methylation on gene expression. Cold Spring Harb Symp Quant Biol *47*, 605-609.

Cedar, H., and Verdine, G. L. (1999). Gene expression. The amazing demethylase. Nature *397*, 568-569.

Cervoni, N., Bhattacharya, S., and Szyf, M. (1999). DNA demethylase is a processive enzyme. J Biol Chem *274*, 8363-8366.

Cervoni, N., Detich, N., Seo, S., Chakravarti, D., and Szyf, M. (2002). The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem *277*, 25026-25031.

Cervoni, N., and Szyf, M. (2001). Demethylase activity is directed by histone acetylation. J Biol Chem *276*, 40778-40787.

Chedin, F., Lieber, M. R., and Hsieh, C. L. (2002). The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc Natl Acad Sci U S A *99*, 16916-16921.

Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999a). Regulation of transcription by a protein methyltransferase. Science *284*, 2174-2177.

Chen, G., Huang, L. D., Jiang, Y. M., and Manji, H. K. (1999b). The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. J Neurochem *72*, 1327-1330.

Chen, G., Manji, H. K., Hawver, D. B., Wright, C. B., and Potter, W. Z. (1994). Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon in vitro. J Neurochem *63*, 2361-2364.

Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L., and Jaenisch, R. (1998). DNA hypomethylation leads to elevated mutation rates. Nature *395*, 89-93.

Chen, Y., Sharma, R. P., Costa, R. H., Costa, E., and Grayson, D. R. (2002). On the epigenetic regulation of the human reelin promoter. Nucleic Acids Res *30*, 2930-2939.

Cheng, J. C., Matsen, C. B., Gonzales, F. A., Ye, W., Greer, S., Marquez, V. E., Jones, P. A., and Selker, E. U. (2003). Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst *95*, 399-409.

Cheng, X., Kumar, S., Posfai, J., Pflugrath, J. W., and Roberts, R. J. (1993). Crystal structure of the HhaI DNA methyltransferase complexed with S-adenosyl-L-methionine. Cell *74*, 299-307.

Cheng, X., and Roberts, R. J. (2001). AdoMet-dependent methylation, DNA methyltransferases and base flipping. Nucleic Acids Res *29*, 3784-3795.

Chiang, P. K. (1998). Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. Pharmacol Ther *77*, 115-134.

Chiang, P. K., Gordon, R. K., Tal, J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., and McCann, P. P. (1996). S-Adenosylmethionine and methylation. Faseb J *10*, 471-480.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294-5299.

Choi, M. S., Shim, Y. H., Hwa, J. Y., Lee, S. K., Ro, J. Y., Kim, J. S., and Yu, E. (2003). Expression of DNA methyltransferases in multistep hepatocarcinogenesis. Hum Pathol *34*, 11-17.

Christman, J. K. (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene *21*, 5483-5495.

Chuang, L. S., Ian, H. I., Koh, T. W., Ng, H. H., Xu, G., and Li, B. F. (1997). Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. Science *277*, 1996-2000.

Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994). High sensitivity mapping of methylated cytosines. Nucleic Acids Res *22*, 2990-2997.

Clevers, H., and van de Wetering, M. (1997). TCF/LEF factor earn their wings. Trends Genet *13*, 485-489.

Comb, M., and Goodman, H. M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res *18*, 3975-3982.

Conne, B., Stutz, A., and Vassalli, J. D. (2000). The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? Nat Med *6*, 637-641.

Cooper, D. N., Taggart, M. H., and Bird, A. P. (1983). Unmethylated domains in vertebrate DNA. Nucleic Acids Res *11*, 647-658.

Cornacchia, E., Golbus, J., Maybaum, J., Strahler, J., Hanash, S., and Richardson, B. (1988). Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. J Immunol *140*, 2197-2200.

Cravo, M., Pinto, R., Fidalgo, P., Chaves, P., Gloria, L., Nobre-Leitao, C., and Costa Mira, F. (1996). Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. Gut *39*, 434-438.

Cross, S. H., Meehan, R. R., Nan, X., and Bird, A. (1997). A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. Nat Genet *16*, 256-259.

Daskalakis, M., Nguyen, T. T., Nguyen, C., Guldberg, P., Kohler, G., Wijermans, P., Jones, P. A., and Lubbert, M. (2002). Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood *100*, 2957-2964.

Datta, J., Ghoshal, K., Sharma, S. M., Tajima, S., and Jacob, S. T. (2003). Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J Cell Biochem *88*, 855-864.

Datto, M. B., Yu, Y., and Wang, X. F. (1995). Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem *270*, 28623-28628.

de Bustros, A., Nelkin, B. D., Silverman, A., Ehrlich, G., Poiesz, B., and Baylin, S. B. (1988). The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. Proc Natl Acad Sci U S A *85*, 5693-5697.

De Capoa, A., Musolino, A., Della Rosa, S., Caiafa, P., Mariani, L., Del Nonno, F., Vocaturo, A., Perrone Donnorso, R., Niveleau, A., and Grappelli, C. (2003). DNA demethylation is directly related to tumour progression: Evidence in normal, pre-malignant and malignant cells from uterine cervix samples. Oncol Rep *10*, 545-549.

De Marzo, A. M., Marchi, V. L., Yang, E. S., Veeraswamy, R., Lin, X., and Nelson, W. G. (1999). Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res *59*, 3855-3860.

De Sarno, P., Li, X., and Jope, R. S. (2002). Regulation of Akt and glycogen synthase kinase-3beta phosphorylation by sodium valproate and lithium. Neuropharmacology *43*, 1158-1164.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell *54*, 275-283.

Decatur, W. A., and Fournier, M. J. (2003). RNA-guided nucleotide modification of ribosomal and other RNAs. J Biol Chem *278*, 695-698.

DeMaria, C. T., and Brewer, G. (1996). AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. J Biol Chem *271*, 12179-12184.

Deng, C., Kaplan, M. J., Yang, J., Ray, D., Zhang, Z., McCune, W. J., Hanash, S. M., and Richardson, B. C. (2001). Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. Arthritis Rheum *44*, 397-407.

Deng, C., Yang, J., Scott, J., Hanash, S., and Richardson, B. C. (1998). Role of the ras-MAPK signaling pathway in the DNA methyltransferase response to DNA hypomethylation. Biol Chem *379*, 1113-1120.

Deng, J., and Szyf, M. (1998). Multiple isoforms of DNA methyltransferase are encoded by the vertebrate cytosine DNA methyltransferase gene. J Biol Chem *273*, 22869-22872.

Deng, J., and Szyf, M. (1999). Downregulation of DNA (cytosine-5-)methyltransferase is a late event in NGF-induced PC12 cell differentiation. Brain Res Mol Brain Res *71*, 23-31.

Dennis, K., Fan, T., Geiman, T., Yan, Q., and Muegge, K. (2001). Lsh, a member of the SNF2 family, is required for genome-wide methylation. Genes Dev *15*, 2940-2944.

Deplus, R., Brenner, C., Burgers, W. A., Putmans, P., Kouzarides, T., de Launoit, Y., and Fuks, F. (2002). Dnmt3L is a transcriptional repressor that recruits histone deacetylase. Nucleic Acids Res *30*, 3831-3838.

Desrosiers, R., and Tanguay, R. M. (1988). Methylation of Drosophila histones at proline, lysine, and arginine residues during heat shock. J Biol Chem *263*, 4686-4692.

Detich, N., Ramchandani, S., and Szyf, M. (2001). A conserved 3'-untranslated element mediates growth regulation of dna methyltransferase 1 and inhibits its transforming activity. J Biol Chem *276*, 24881-24890.

Detich, N., Theberge, J., and Szyf, M. (2002). Promoter-specific Activation and Demethylation by MBD2/Demethylase. J Biol Chem *277*, 35791-35794.

Di Croce, L., Raker, V. A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., *et al.* (2002). Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science *295*, 1079-1082.

Dillon, N., and Festenstein, R. (2002). Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. Trends Genet *18*, 252-258.

Doherty, A. S., Bartolomei, M. S., and Schultz, R. M. (2002). Regulation of stage-specific nuclear translocation of Dnmt1o during preimplantation mouse development. Dev Biol *242*, 255-266.

Dover, J., Schneider, J., Tawiah-Boateng, M. A., Wood, A., Dean, K., Johnston, M., and Shilatifard, A. (2002). Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J Biol Chem *277*, 28368-28371.

Drewell, R. A., Goddard, C. J., Thomas, J. O., and Surani, M. A. (2002). Methylation-dependent silencing at the H19 imprinting control region by MeCP2. Nucleic Acids Res *30*, 1139-1144.

Eads, C. A., Danenberg, K. D., Kawakami, K., Saltz, L. B., Danenberg, P. V., and Laird, P. W. (1999). CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression [published erratum appears in Cancer Res 1999 Nov 15;59(22):5860]. Cancer Res *59*, 2302-2306.

Ehrlich, M. (2002b). DNA hypomethylation, cancer, the immunodeficiency, centromeric region instability, facial anomalies syndrome and chromosomal rearrangements. J Nutr *132*, 2424S-2429S.

Ehrlich, M. (2002a). DNA methylation in cancer: too much, but also too little. Oncogene *21*, 5400-5413.

el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S. R., and Baylin, S. B. (1991). High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. Proc Natl Acad Sci U S A *88*, 3470-3474.

El-Osta, A. (2002). FMR1 silencing and the signals to chromatin: a unified model of transcriptional regulation. Biochem Biophys Res Commun *295*, 575-581.

Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene *21*, 5427-5440.

Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B., and Herman, J. G. (1999). Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res *59*, 793-797.

Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C., Repasky, E. A., *et al.* (2000). Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors [see comments]. J Natl Cancer Inst *92*, 564-569.

Fatemi, M., Hermann, A., Gowher, H., and Jeltsch, A. (2002). Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. Eur J Biochem *269*, 4981-4984.

Feinberg, A. P., Gehrke, C. W., Kuo, K. C., and Ehrlich, M. (1988). Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res *48*, 1159-1161.

Feinberg, A. P., and Vogelstein, B. (1983a). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature *301*, 89-92.

Feinberg, A. P., and Vogelstein, B. (1983b). Hypomethylation of ras oncogenes in primary human cancers. Biochem Biophys Res Commun *111*, 47-54.

Feng, Q., and Zhang, Y. (2001). The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. Genes Dev *15*, 827-832.

Finkelstein, J. D. (2000). Pathways and regulation of homocysteine metabolism in mammals. Semin Thromb Hemost *26*, 219-225.

Firestein, R., Cui, X., Huie, P., and Cleary, M. L. (2000). Set domain-dependent regulation of transcriptional silencing and growth control by SUV39H1, a mammalian ortholog of Drosophila Su(var)3-9. Mol Cell Biol *20*, 4900-4909.

Ford, L. P., Watson, J., Keene, J. D., and Wilusz, J. (1999). ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. Genes Dev *13*, 188-201.

Fournel, M., Sapieha, P., Beaulieu, N., Besterman, J. M., and MacLeod, A. R. (1999). Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. J Biol Chem *274*, 24250-24256.

Francis, J., Jung, B., Zhang, G., Cheng, J., Ho, W., Burnham, W. M., and Eubanks, J. H. (2002). Kindling induces the mRNA expression of methyl DNA-binding factors in the adult rat hippocampus. Neuroscience *113*, 79-87.

Freedman, V. H., and Shin, S. I. (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell *3*, 355-359.

Friso, S., Choi, S., Girelli, D., Mason, J., Dolnikowski, G., Bagley, P., Olivieri, O., Jacques, P., Rosenberg, I., Corrocher, R., and Selhub, J. (2002). A common mutation in

the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A *99*, 5606-5611.

Fujita, H., Fujii, R., Aratani, S., Amano, T., Fukamizu, A., and Nakajima, T. (2003). Antithetic effects of MBD2a on gene regulation. Mol Cell Biol *23*, 2645-2657.

Fujita, N., Shimotake, N., Ohki, I., Chiba, T., Saya, H., Shirakawa, M., and Nakao, M. (2000). Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1. Mol Cell Biol *20*, 5107-5118.

Fujita, N., Takebayashi, S., Okumura, K., Kudo, S., Chiba, T., Saya, H., and Nakao, M. (1999). Methylation-mediated transcriptional silencing in euchromatin by methyl- CpG binding protein MBD1 isoforms. Mol Cell Biol *19*, 6415-6426.

Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000). DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet *24*, 88-91.

Fuks, F., Burgers, W. A., Godin, N., Kasai, M., and Kouzarides, T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. Embo J *20*, 2536-2544.

Fuks, F., Hurd, P. J., Wolf, D., Nan, X., Bird, A. P., and Kouzarides, T. (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem *278*, 4035-4040.

Fuso, A., Cavallaro, R., Orru, L., Buttarelli, F., and Scarpa, S. (2001). Gene silencing by S-adenosylmethionine in muscle differentiation. FEBS Lett *508*, 337-340.

Garcea, R., Daino, L., Pascale, R., Simile, M. M., Puddu, M., Ruggiu, M. E., Seddaiu, M. A., Satta, G., Sequenza, M. J., and Feo, F. (1989). Protooncogene methylation and expression in regenerating liver and preneoplastic liver nodules induced in the rat by diethylnitrosamine: effect of variations of S-adenosylmethionine:S-adenosylhomocysteine ratio. Carcinogenesis *10*, 1183-1192.

Gary, J. D., and Clarke, S. (1998). RNA and protein interactions modulated by protein arginine methylation. Prog Nucleic Acid Res Mol Biol *61*, 65-131.

Ghoshal, K., Datta, J., Majumder, S., Bai, S., Dong, X., Parthun, M., and Jacob, S. T. (2002). Inhibitors of histone deacetylase and DNA methyltransferase synergistically activate the methylated metallothionein I promoter by activating the transcription factor MTF-1 and forming an open chromatin structure. Mol Cell Biol *22*, 8302-8319.

Gibbons, R., McDowell, T., Raman, S., O'Rourke, D., Garrick, D., Ayyub, H., and Higgs, D. (2000). Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. Nat Genet *24*, 368-371.

Goel, A., Mathupala, S., and Pedersen, P. L. (2003). Glucose metabolism in cancer: Evidence that demethylation events play a role in activating type II hexokinase gene expression. J Biol Chem *278*, 15333-15340.

Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzel, T. (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. Embo J *20*, 6969-6978.

Gowher, H., and Jeltsch, A. (2001). Enzymatic properties of recombinant Dnmt3a DNA methyltransferase from mouse: the enzyme modifies DNA in a non-processive manner and also methylates non-CpG [correction of non-CpA] sites. J Mol Biol *309*, 1201-1208.

Gowher, H., Leismann, O., and Jeltsch, A. (2000). DNA of Drosophila melanogaster contains 5-methylcytosine. Embo J *19*, 6918-6923.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., *et al.* (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391*, 815-818.

Grigorian, M., Ambartsumian, N., Lykkesfeldt, A. E., Bastholm, L., Elling, F., Georgiev, G., and Lukanidin, E. (1996). Effect of mts1 (S100A4) expression on the progression of human breast cancer cells. Int J Cancer *67*, 831-841.

Grigorian, M. S., Tulchinsky, E. M., Zain, S., Ebralidze, A. K., Kramerov, D. A., Kriajevska, M. V., Georgiev, G. P., and Lukanidin, E. M. (1993). The mts1 gene and control of tumor metastasis. Gene *135*, 229-238.

Gruenbaum, Y., Cedar, H., and Razin, A. (1982). Substrate and sequence specificity of a eukaryotic DNA methylase. Nature *295*, 620-622.

Guan, R. J., Fu, Y., Holt, P. R., and Pardee, A. B. (1999). Association of K-ras mutations with p16 methylation in human colon cancer. Gastroenterology *116*, 1063-1071.

Gunthert, U., Schweiger, M., Stupp, M., and Doerfler, W. (1976). DNA methylation in adenovirus, adenovirus-transformed cells, and host cells. Proc Natl Acad Sci U S A *73*, 3923-3927.

Guo, Y., Pakneshan, P., Gladu, J., Slack, A., Szyf, M., and Rabbani, S. (2002). Regulation of DNA Methylation in Human Breast Cancer. EFFECT ON THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR GENE PRODUCTION AND TUMOR INVASION. J Biol Chem *277*, 41571-41579.

Habets, G. G., van der Kammen, R. A., Scholtes, E. H., and Collard, J. G. (1990). Induction of invasive and metastatic potential in mouse T-lymphoma cells (BW5147) by treatment with 5-azacytidine. Clin Exp Metastasis *8*, 567-577.

Hai, T., and Hartman, M. G. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene *273*, 1-11.

Han, L., Lin, I. G., and Hsieh, C. L. (2001). Protein binding protects sites on stable episomes and in the chromosome from de novo methylation. Mol Cell Biol *21*, 3416-3424.

Hansen, R. S., Wijmenga, C., Luo, P., Stanek, A. M., Canfield, T. K., Weemaes, C. M., and Gartler, S. M. (1999). The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc Natl Acad Sci U S A *96*, 14412-14417.

Harrington, M. A., Jones, P. A., Imagawa, M., and Karin, M. (1988). Cytosine methylation does not affect binding of transcription factor Sp1. Proc Natl Acad Sci U S A *85*, 2066-2070.

Hata, K., Okano, M., Lei, H., and Li, E. (2002). Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development *129*, 1983-1993.

Hattori, M., Sakamoto, H., Satoh, K., and Yamamoto, T. (2001). DNA demethylase is expressed in ovarian cancers and the expression correlates with demethylation of CpG sites in the promoter region of c- erbB-2 and survivin genes. Cancer Lett *169*, 155-164.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998a). Identification of c-MYC as a target of the APC pathway [see comments]. Science *281*, 1509-1512.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998b). A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A *95*, 2509-2514.

Heaton, J. H., Dlakic, W. M., Dlakic, M., and Gelehrter, T. D. (2000). Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide responsive sequence in the type-1 plasminogen activator inhibitor mRNA. J Biol Chem *276*, 3341-3347.

Hendrich, B., and Bird, A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol *18*, 6538-6547.

Hendrich, B., Guy, J., Ramsahoye, B., Wilson, V. A., and Bird, A. (2001). Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev *15*, 710-723.

Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D. S., Gnarra, J. R., Linehan, W. M., and et al. (1994). Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci U S A *91*, 9700-9704.

Hew, Y., Grzelczak, Z., Lau, C., and Keeley, F. W. (1999). Identification of a large region of secondary structure in the 3'- untranslated region of chicken elastin mRNA with implications for the regulation of mRNA stability. J Biol Chem *274*, 14415-14421.

His, W. (1887). Ueber das Stoffwechselproduct des Pyridins. Arch Exp Pathol Pharmakol *22*, 253-260.

Hockly, E., Richon, V. M., Woodman, B., Smith, D. L., Zhou, X., Rosa, E., Sathasivam, K., Ghazi-Noori, S., Mahal, A., Lowden, P. A., *et al.* (2003). Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Natl Acad Sci U S A *100*, 2041-2046.

Hodge, D. R., Xiao, W., Clausen, P. A., Heidecker, G., Szyf, M., and Farrar, W. L. (2001). Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. J Biol Chem *276*, 39508-39511.

Holler, M., Westin, G., Jiricny, J., and Schaffner, W. (1988). Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev *2*, 1127-1135.

Hotchkiss, R. D. (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. Journal of Biological Chemistry *175*, 315-332.

Hsieh, C. L. (1999). Evidence that protein binding specifies sites of DNA demethylation. Mol Cell Biol *19*, 46-56.

Hung, M. S., Karthikeyan, N., Huang, B., Koo, H. C., Kiger, J., and Shen, C. J. (1999). Drosophila proteins related to vertebrate DNA (5-cytosine) methyltransferases. Proc Natl Acad Sci U S A *96*, 11940-11945.

Iida, T., Suetake, I., Tajima, S., Morioka, H., Ohta, S., Obuse, C., and Tsurimoto, T. (2002). PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA. Genes Cells *7*, 997-1007.

Issa, J. P. (1999). Aging, DNA methylation and cancer. Crit Rev Oncol Hematol *32*, 31-43.

Issa, J. P., Vertino, P. M., Wu, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R., and Baylin, S. B. (1993). Increased cytosine DNA-methyltransferase activity during colon cancer progression. J Natl Cancer Inst *85*, 1235-1240.

Jackson, J., Lindroth, A., Cao, X., and Jacobsen, S. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556-560.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E., and Jaenisch, R. (2001). Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet *27*, 31-39.

Jahner, D., and Jaenisch, R. (1985). Retrovirus-induced de novo methylation of flanking host sequences correlates with gene inactivity. Nature *315*, 594-597.

Jang, B. C., Sanchez, T., Schaefers, H. J., Trifan, O. C., Liu, C. H., Creminon, C., Huang, C. K., and Hla, T. (2000). Serum withdrawal-induced post-transcriptional stabilization of cyclooxygenase-2 mRNA in MDA-MB-231 mammary carcinoma cells requires the activity of the p38 stress-activated protein kinase. J Biol Chem *275*, 39507-39515.

Jason, L. J., Moore, S. C., Lewis, J. D., Lindsey, G., and Ausio, J. (2002). Histone ubiquitination: a tagging tail unfolds? Bioessays *24*, 166-174.

Jeddeloh, J., Stokes, T., and Richards, E. (1999). Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat Genet *22*, 94-97.

Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem *3*, 274-293.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Ji, W., Hernandez, R., Zhang, X. Y., Qu, G. Z., Frady, A., Varela, M., and Ehrlich, M. (1997). DNA demethylation and pericentromeric rearrangements of chromosome 1. Mutat Res *379*, 33-41.

Johnstone, R. W. (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov *1*, 287-299.

Jones, P. A. (1985). Altering gene expression with 5-azacytidine. Cell *40*, 485-486.

Jones, P. A. (1996). DNA methylation errors and cancer. Cancer Res *56*, 2463-2467.

Jones, P. A., and Taylor, S. M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. Cell *20*, 85-93.

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet *19*, 187-191.

Jones, T. R., and Cole, M. D. (1987). Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. Mol Cell Biol *7*, 4513-4521.

Joseph, B., Orlian, M., and Furneaux, H. (1998). p21(waf1) mRNA contains a conserved element in its 3'-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins. J Biol Chem *273*, 20511-20516.

Jost, J., Oakeley, E., Zhu, B., Benjamin, D., Thiry, S., Siegmann, M., and Jost, Y. (2001). 5-Methylcytosine DNA glycosylase participates in the genome-wide loss of DNA methylation occurring during mouse myoblast differentiation. Nucleic Acids Res *29*, 4452-4461.

Jost, J. P., and Jost, Y. C. (1994). Transient DNA demethylation in differentiating mouse myoblasts correlates with higher activity of 5-methyldeoxycytidine excision repair. J Biol Chem *269*, 10040-10043.

Jost, J. P., Siegmann, M., Sun, L., and Leung, R. (1995). Mechanisms of DNA demethylation in chicken embryos. Purification and properties of a 5-methylcytosine-DNA glycosylase. J Biol Chem *270*, 9734-9739.

Jung, B. P., Zhang, G., Ho, W., Francis, J., and Eubanks, J. H. (2002). Transient forebrain ischemia alters the mRNA expression of methyl DNA-binding factors in the adult rat hippocampus. Neuroscience *115*, 515-524.

Jurgens, B., Schmitz-Drager, B. J., and Schulz, W. A. (1996). Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. Cancer Res *56*, 5698-5703.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., and Razin, A. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev *6*, 705-714.

Kafri, T., Gao, X., and Razin, A. (1993). Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo. Proc Natl Acad Sci U S A *90*, 10558-10562.

Kanai, Y., Ushijima, S., Nakanishi, Y., Sakamoto, M., and Hirohashi, S. (2003). Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett *192*, 75-82.

Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C. F., Wolffe, A., Ohlsson, R., and Lobanenkov, V. V. (2000). Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive [In Process Citation]. Curr Biol *10*, 853-856.

Kaneko, Y., Shibuya, M., Nakayama, T., Hayashida, N., Toda, G., Endo, Y., Oka, H., and Oda, T. (1985). Hypomethylation of c-myc and epidermal growth factor receptor genes in human hepatocellular carcinoma and fetal liver. Jpn J Cancer Res *76*, 1136-1140.

Kautiainen, T. L., and Jones, P. A. (1986). DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. J Biol Chem *261*, 1594-1598.

Kessler, O., and Chasin, L. A. (1996). Effects of nonsense mutations on nuclear and cytoplasmic adenine phosphoribosyltransferase RNA. Mol Cell Biol *16*, 4426-4435.

Kim, G. D., Ni, J., Kelesoglu, N., Roberts, R. J., and Pradhan, S. (2002). Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. Embo J *21*, 4183-4195.

Kim, Y. I., Giuliano, A., Hatch, K. D., Schneider, A., Nour, M. A., Dallal, G. E., Selhub, J., and Mason, J. B. (1994). Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. Cancer *74*, 893-899.

Kimura, H., and Shiota, K. (2002). Methyl-CpG binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem *278*,4806-4812.

Kino, T., Takeshima, H., Nakao, M., Nishi, T., Yamamoto, K., Kimura, T., Saito, Y., Kochi, M., Kuratsu, J., Saya, H., and Ushio, Y. (2001). Identification of the cis-acting region in the NF2 gene promoter as a potential target for mutation and methylation-dependent silencing in schwannoma. Genes Cells *6*, 441-454.

Kirkwood, L. C., Nation, R. L., and Somogyi, A. A. (1997). Characterization of the human cytochrome P450 enzymes involved in the metabolism of dihydrocodeine. Br J Clin Pharmacol *44*, 549-555.

Kishikawa, S., Murata, T., Kimura, H., Shiota, K., and Yokoyama, K. K. (2002). Regulation of transcription of the Dnmt1 gene by Sp1 and Sp3 zinc finger proteins. Eur J Biochem *269*, 2961-2970.

Klein, G., Giovanella, B. C., Lindahl, T., Fialkow, P. J., Singh, S., and Stehlin, J. S. (1974). Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. Proc Natl Acad Sci U S A *71*, 4737-4741.

Knox, J. D., Araujo, F. D., Bigey, P., Slack, A. D., Price, G. B., Zannis-Hadjopoulos, M., and Szyf, M. (2000). Inhibition of DNA methyltransferase inhibits DNA replication. J Biol Chem *275*, 17986-17990.

Kondo, Y., Shen, L., and Issa, J. P. (2003). Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. Mol Cell Biol *23*, 206-215.

Kopin, I. J. (1985). Catecholamine metabolism: basic aspects and clinical significance. Pharmacol Rev *37*, 333-364.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-

catenin-Tcf complex in APC-/- colon carcinoma [see comments]. Science *275*, 1784-1787.

Kouzarides, T. (2002). Histone methylation in transcriptional control. Curr Opin Genet Dev *12*, 198-209.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. Nucleic Acids Res *22*, 1-10.

Kuo, M. H., and Allis, C. D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays *20*, 615-626.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A., and Jaenisch, R. (1995). Suppression of intestinal neoplasia by DNA hypomethylation. Cell *81*, 197-205.

Landoulsi, A., Malki, A., Kern, R., Kohiyama, M., and Hughes, P. (1990). The E. coli cell surface specifically prevents the initiation of DNA replication at oriC on hemimethylated DNA templates. Cell *63*, 1053-1060.

Lee, P. J., Washer, L. L., Law, D. J., Boland, C. R., Horon, I. L., and Feinberg, A. P. (1996). Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. Proc Natl Acad Sci U S A *93*, 10366-10370.

Lembo, F., Pero, R., Angrisano, T., Vitiello, C., Iuliano, R., Bruni, C. B., and Chiariotti, L. (2003). MBDin, a novel MBD2-interacting protein, relieves MBD2 repression

potential and reactivates transcription from methylated promoters. Mol Cell Biol *23*, 1656-1665.

Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell *71*, 865-873.

Lew, J. (2003). MAP Kinases and CDKs: Kinetic Basis for Catalytic Activation. Biochemistry *42*, 849-856.

Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F., and Bird, A. (1992). Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell *69*, 905-914.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-926.

Liang, G., Chan, M., Tomigahara, Y., Tsai, Y., Gonzales, F., Li, E., Laird, P., and Jones, P. (2002). Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol *22*, 480-491.

Lin, I. G., and Hsieh, C. L. (2001). Chromosomal DNA demethylation specified by protein binding. EMBO Rep *2*, 108-112.

Lin, I. G., Tomzynski, T. J., Ou, Q., and Hsieh, C. L. (2000). Modulation of DNA binding protein affinity directly affects target site demethylation. Mol Cell Biol *20*, 2343-2349.

Lipsanen, V., Leinonen, P., Alhonen, L., and Janne, J. (1988). Hypomethylation of ornithine decarboxylase gene and erb-A1 oncogene in human chronic lymphatic leukemia. Blood *72*, 2042-2044.

Liteplo, R. G., and Kerbel, R. S. (1987). Reduced levels of DNA 5-methylcytosine in metastatic variants of the human melanoma cell line MeWo. Cancer Res *47*, 2264-2267.

Litt, M. D., Simpson, M., Gaszner, M., Allis, C. D., and Felsenfeld, G. (2001). Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. Science *293*, 2453-2455.

Liu, K., Wang, Y. F., Cantemir, C., and Muller, M. T. (2003). Endogenous Assays of DNA Methyltransferases: Evidence for Differential Activities of DNMT1, DNMT2, and DNMT3 in Mammalian Cells In Vivo. Mol Cell Biol *23*, 2709-2719.

Liu, Y., Sun, L., and Jost, J. P. (1996). In differentiating mouse myoblasts DNA methyltransferase is posttranscriptionally and posttranslationally regulated. Nucleic Acids Res *24*, 2718-2722.

Lock, L. F., Takagi, N., and Martin, G. R. (1987). Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. Cell *48*, 39-46.

Loscher, W. (2002). Basic pharmacology of valproate: a review after 35 years of clinical use for the treatment of epilepsy. CNS Drugs *16*, 669-694.

Lu, L. J., Randerath, E., and Randerath, K. (1983). DNA hypomethylation in Morris hepatomas. Cancer Lett *19*, 231-239.

Lu, S. C. (2000). S-Adenosylmethionine. Int J Biochem Cell Biol *32*, 391-395.

Lucarelli, M., Fuso, A., Strom, R., and Scarpa, S. (2000). The dynamics of myogenin site-specific demethylation is strongly correlated with its expression and with muscle differentiation. J Biol Chem *276*, 7500-7506.

Lusser, A. (2002). Acetylated, methylated, remodeled: chromatin states for gene regulation. Curr Opin Plant Biol *5*, 437-443.

Lyko, F., Ramsahoye, B. H., and Jaenisch, R. (2000). DNA methylation in Drosophila melanogaster. Nature *408*, 538-540.

Ma, H., Baumann, C. T., Li, H., Strahl, B. D., Rice, R., Jelinek, M. A., Aswad, D. W., Allis, C. D., Hager, G. L., and Stallcup, M. R. (2001). Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. Curr Biol *11*, 1981-1985.

MacLeod, A. R., Rouleau, J., and Szyf, M. (1995). Regulation of DNA methylation by the Ras signaling pathway. J Biol Chem *270*, 11327-11337.

MacLeod, A. R., and Szyf, M. (1995). Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. J Biol Chem *270*, 8037-8043.

Macleod, D., Charlton, J., Mullins, J., and Bird, A. P. (1994). Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev *8*, 2282-2292.

Maden, B. E. (1990). The numerous modified nucleotides in eukaryotic ribosomal RNA. Prog Nucleic Acid Res Mol Biol *39*, 241-303.

Magdinier, F., Billard, L. M., Wittmann, G., Frappart, L., Benchaib, M., Lenoir, G. M., Guerin, J. F., and Dante, R. (2000). Regional methylation of the 5' end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. Faseb J *14*, 1585-1594.

Maity, A., McKenna, W. G., and Muschel, R. J. (1997). Cyclin A message stability varies with the cell cycle. Cell Growth Differ *8*, 311-318.

Malter, J. S., and Hong, Y. (1991). A redox switch and phosphorylation are involved in the post- translational up-regulation of the adenosine-uridine binding factor by phorbol ester and ionophore. J Biol Chem *266*, 3167-3171.

Manev, H., and Uz, T. (2002). DNA hypomethylating agents 5-aza-2'-deoxycytidine and valproate increase neuronal 5-lipoxygenase mRNA. Eur J Pharmacol *445*, 149-150.

Marhold, J., Zbylut, M., Lankenau, D. H., Li, M., Gerlich, D., Ballestar, E., Mechler, B. M., and Lyko, F. (2002). Stage-specific chromosomal association of Drosophila dMBD2/3 during genome activation. Chromosoma *111*, 13-21.

Martienssen, R. A., and Colot, V. (2001). DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science *293*, 1070-1074.

Martinez-Chantar, M. L., Corrales, F. J., Martinez-Cruz, L. A., Garcia-Trevijano, E. R., Huang, Z. Z., Chen, L., Kanel, G., Avila, M. A., Mato, J. M., and Lu, S. C. (2002). Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. Faseb J *16*, 1292-1294.

Masucci, M. G., Contreras-Salazar, B., Ragnar, E., Falk, K., Minarovits, J., Ernberg, I., and Klein, G. (1989). 5-Azacytidine up regulates the expression of Epstein-Barr virus

nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt's lymphoma line rael. J Virol *63*, 3135-3141.

Mato, J., Alvarez, L., Ortiz, P., and Pajares, M. (1997). S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. Pharmacol Ther *73*, 265-280.

Meehan, R. R., Lewis, J. D., and Bird, A. P. (1992). Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Res *20*, 5085-5092.

Meehan, R. R., Lewis, J. D., McKay, S., Kleiner, E. L., and Bird, A. P. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell *58*, 499-507.

Melamed, M. R., Mullaney,P.F, and Mendelsohn,M.L. (1990). Flow Cytometry and Sorting (New York, Wiley-Liss).

Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med *1*, 686-692.

Mertineit, C., Yoder, J. A., Taketo, T., Laird, D. W., Trasler, J. M., and Bestor, T. H. (1998). Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development *125*, 889-897.

Mikovits, J. A., Young, H. A., Vertino, P., Issa, J. P., Pitha, P. M., Turcoski-Corrales, S., Taub, D. D., Petrow, C. L., Baylin, S. B., and Ruscetti, F. W. (1998). Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in

de novo methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. Mol Cell Biol *18*, 5166-5177.

Miller, J. W., Nadeau, M. R., Smith, J., Smith, D., and Selhub, J. (1994). Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. Biochem J *298 (Pt 2)*, 415-419.

Milutinovic, S., Knox, J. D., and Szyf, M. (2000). DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21(WAF1/CIP1/sdi1). J Biol Chem *275*, 6353-6359.

Milutinovic, S., Zhuang, Q., Niveleau, A., and Szyf, M. (2003). Epigenomic stress response: Knock-down of DNA methyltransferase 1 triggers an intra S-phase arrest of DNA replication and induction of stress response genes. J Biol Chem *278*, 14985-14995.

Ming, X. F., Kaiser, M., and Moroni, C. (1998). c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells. Embo J *17*, 6039-6048.

Mizuno, S., Chijiwa, T., Okamura, T., Akashi, K., Fukumaki, Y., Niho, Y., and Sasaki, H. (2001). Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood *97*, 1172-1179.

Moens, U., Subramaniam, N., Johansen, B., and Aarbakke, J. (1993). The c-fos cAMP-response element: regulation of gene expression by a beta 2-adrenergic agonist, serum and DNA methylation. Biochim Biophys Acta *1173*, 63-70.

Moisan, S., Demers, M., Mercier, J., Magnaldo, T., Potworowski, E. F., and St-Pierre, Y. (2003). Upregulation of galectin-7 in murine lymphoma cells is associated with progression toward an aggressive phenotype. Leukemia *17*, 751-759.

Morgenstern, J. P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res *18*, 3587-3596.

Muller, H. (1930). Types of visible variations induced by X-rays in *Drosophila*. J Genet *22*, 299-334.

Muller, H., and Helin, K. (2000). The E2F transcription factors: key regulators of cell proliferation. Biochim Biophys Acta *1470*, M1-12.

Murray, K. (1964). The occurrence of e-N-methyllysine in histones. Biochemistry *3*, 10-15.

Najbauer, J., Johnson, B. A., Young, A. L., and Aswad, D. W. (1993). Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. J Biol Chem *268*, 10501-10509.

Najbauer, J., Orpiszewski, J., and Aswad, D. W. (1996). Molecular aging of tubulin: accumulation of isoaspartyl sites in vitro and in vivo. Biochemistry *35*, 5183-5190.

Nakamura, N., and Takenaga, K. (1998). Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. Clin Exp Metastasis *16*, 471-479.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Nambu, S., Inoue, K., and Saski, H. (1987). Site-specific hypomethylation of the c-myc oncogene in human hepatocellular carcinoma. Jpn J Cancer Res *78*, 695-704.

Nan, X., Meehan, R. R., and Bird, A. (1993). Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res *21*, 4886-4892.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-389.

Nass, S. J., Ferguson, A. T., El-Ashry, D., Nelson, W. G., and Davidson, N. E. (1999). Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells. Oncogene *18*, 7453-7461.

Ng, H. H., and Bird, A. (2000). Histone deacetylases: silencers for hire. Trends Biochem Sci *25*, 121-126.

Ng, H. H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y., and Struhl, K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev *16*, 1518-1527.

Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet *23*, 58-61.

Nguyen, C. T., Weisenberger, D. J., Velicescu, M., Gonzales, F. A., Lin, J. C., Liang, G., and Jones, P. A. (2002). Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. Cancer Res *62*, 6456-6461.

Nielsen, S. J., Schneider, R., Bauer, U. M., Bannister, A. J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R. E., and Kouzarides, T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Noe, V., Ciudad, C. J., and Chasin, L. A. (1999). Effect of differential polyadenylation and cell growth phase on dihydrofolate reductase mRNA stability. J Biol Chem *274*, 27807-27814.

Noma, K., Allis, C. D., and Grewal, S. I. (2001). Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science *293*, 1150-1155.

Ohtani-Fujita, N., Dryja, T. P., Rapaport, J. M., Fujita, T., Matsumura, S., Ozasa, K., Watanabe, Y., Hayashi, K., Maeda, K., Kinoshita, S., *et al.* (1997). Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet *98*, 43-49.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Okano, M., Xie, S., and Li, E. (1998a). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet *19*, 219-220.

Okano, M., Xie, S., and Li, E. (1998b). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res *26*, 2536-2540.

Oshima, T., Wada, C., Kawagoe, Y., Ara, T., Maeda, M., Masuda, Y., Hiraga, S., and Mori, H. (2002). Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in Escherichia coli. Mol Microbiol *45*, 673-695.

Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994). Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. Embo J *13*, 1476-1481.

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W., and Walter, J. (2000). Active demethylation of the paternal genome in the mouse zygote. Curr Biol *10*, 475-448.

Otterson, G. A., Khleif, S. N., Chen, W., Coxon, A. B., and Kaye, F. J. (1995). CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza 2'deoxycytidine. Oncogene *11*, 1211-1216.

Oue, N., Kuraoka, K., Kuniyasu, H., Yokozaki, H., Wakikawa, A., Matsusaki, K., and Yasui, W. (2001). DNA methylation status of hMLH1, p16(INK4a), and CDH1 is not associated with mRNA expression levels of DNA methyltransferase and DNA demethylase in gastric carcinomas. Oncol Rep *8*, 1085-1089.

Papavassiliou, A. G. (1993). c-Jun phosphorylation in signal transduction and gene regulation. Anticancer Res *13*, 2213-2220.

Park, J. G., and Chapman, V. M. (1994). CpG island promoter region methylation patterns of the inactive-X-chromosome hypoxanthine phosphoribosyltransferase (Hprt) gene. Mol Cell Biol *14*, 7975-7983.

Paroush, Z., Keshet, I., Yisraeli, J., and Cedar, H. (1990). Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. Cell *63*, 1229-1237.

Pascale, R., Simile, M., De, M. M., and Feo, F. (2002). Chemoprevention of hepatocarcinogenesis. S-adenosyl-L-methionine. Alcohol *27*, 193.

Pascale, R., Simile, M. M., Ruggiu, M. E., Seddaiu, M. A., Satta, G., Sequenza, M. J., Daino, L., Vannini, M. G., Lai, P., and Feo, F. (1991). Reversal by 5-azacytidine of the S-adenosyl-L-methionine-induced inhibition of the development of putative preneoplastic foci in rat liver carcinogenesis. Cancer Lett *56*, 259-265.

Pende, A., Tremmel, K. D., DeMaria, C. T., Blaxall, B. C., Minobe, W. A., Sherman, J. A., Bisognano, J. D., Bristow, M. R., Brewer, G., and Port, J. (1996). Regulation of the mRNA-binding protein AUF1 by activation of the beta- adrenergic receptor signal transduction pathway. J Biol Chem *271*, 8493-8501.

Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., and Klein, P. S. (2001). Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem *276*, 36734-36741.

Plumb, J. A., Strathdee, G., Sludden, J., Kaye, S. B., and Brown, R. (2000). Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5- azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res *60*, 6039-6044.

Pogribny, I., Yi, P., and James, S. J. (1999). A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands. Biochem Biophys Res Commun *262*, 624-628.

Pogribny, I. P., Poirier, L. A., and James, S. J. (1995). Differential sensitivity to loss of cytosine methyl groups within the hepatic p53 gene of folate/methyl deficient rats. Carcinogenesis *16*, 2863-2867.

Poirier, L. A. (2002). The effects of diet, genetics and chemicals on toxicity and aberrant DNA methylation: an introduction. J Nutr *132*, 2336S-2339S.

Poirier, L. A., Wise, C. K., Delongchamp, R. R., and Sinha, R. (2001). Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer Epidemiol Biomarkers Prev *10*, 649-655.

Polychronakos, C. (1993). Parental imprinting of the genes for IGF-II and its receptor. Adv Exp Med Biol *343*, 189-203.

Potter, J. (2002). Methyl supply, methyl metabolizing enzymes and colorectal neoplasia. J Nutr *132*, 2410S-2412S.

Pradhan, S., and Kim, G. D. (2002). The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. Embo J *21*, 779-788.

Prendergast, G. C., and Ziff, E. B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science *251*, 186-189.

Quinn, P. G. (2002). Mechanisms of basal and kinase-inducible transcription activation by CREB. Prog Nucleic Acid Res Mol Biol *72*, 269-305.

Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999). DNA methylation is a reversible biological signal [see comments]. Proc Natl Acad Sci U S A *96*, 6107-6112.

Ramchandani, S., MacLeod, A. R., Pinard, M., von Hofe, E., and Szyf, M. (1997). Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. Proc Natl Acad Sci U S A *94*, 684-689.

Ramsahoye, B. H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A. P., and Jaenisch, R. (2000). Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci U S A *97*, 5237-5242.

Rasmussen, B. B., Nielsen, T. L., and Brosen, K. (1998). Fluvoxamine is a potent inhibitor of the metabolism of caffeine in vitro. Pharmacol Toxicol *83*, 240-245.

Razin, A. (1998). CpG methylation, chromatin structure and gene silencing-a three-way connection. Embo J *17*, 4905-4908.

Razin, A., and Cedar, H. (1977). Distribution of 5-methylcytosine in chromatin. Proc Natl Acad Sci U S A *74*, 2725-2728.

Razin, A., and Riggs, A. D. (1980). DNA methylation and gene function. Science *210*, 604-610.

Razin, A., and Shemer, R. (1995). DNA methylation in early development. Hum Mol Genet *4 Spec No*, 1751-1755.

Razin, A., and Szyf, M. (1984). DNA methylation patterns. Formation and function. Biochim Biophys Acta *782*, 331-342.

Razin, A., Szyf, M., Kafri, T., Roll, M., Giloh, H., Scarpa, S., Carotti, D., and Cantoni, G. L. (1986). Replacement of 5-methylcytosine by cytosine: a possible mechanism for transient DNA demethylation during differentiation. Proc Natl Acad Sci U S A *83*, 2827-2831.

Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N., and Cedar, H. (1984). Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. Proc Natl Acad Sci U S A *81*, 2275-2279.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Reid, G. K., Besterman, J. M., and MacLeod, A. R. (2002). Selective inhibition of DNA methyltransferase enzymes as a novel strategy for cancer treatment. Curr Opin Mol Ther *4*, 130-137.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science *293*, 1089-1093.

Rhee, I., Bachman, K. E., Park, B. H., Jair, K. W., Yen, R. W., Schuebel, K. E., Cui, H., Feinberg, A. P., Lengauer, C., Kinzler, K. W., *et al.* (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature *416*, 552-556.

Rhee, I., Jair, K. W., Yen, R. W., Lengauer, C., Herman, J. G., Kinzler, K. W., Vogelstein, B., Baylin, S. B., and Schuebel, K. E. (2000). CpG methylation is maintained in human cancer cells lacking DNMT1. Nature *404*, 1003-1007.

Richards, E. J., and Elgin, S. C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell *108*, 489-500.

Richardson, B., Powers, D., Hooper, F., Yung, R. L., and O'Rourke, K. (1994). Lymphocyte function-associated antigen 1 overexpression and T cell autoreactivity. Arthritis Rheum *37*, 1363-1372.

Roberts, R. J., and Macelis, D. (2000). REBASE - restriction enzymes and methylases. Nucleic Acids Res *28*, 306-307.

Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. (2000a). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet *25*, 338-342.

Robertson, K. D., Keyomarsi, K., Gonzales, F. A., Velicescu, M., and Jones, P. A. (2000b). Differential mRNA expression of the human DNA methyltransferases (DNMTs) 1, 3a and 3b during the G(0)/G(1) to S phase transition in normal and tumor cells. Nucleic Acids Res *28*, 2108-2113.

Robertson, K. D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F. A., and Jones, P. A. (1999). The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res *27*, 2291-2298.

Rosty, C., Ueki, T., Argani, P., Jansen, M., Yeo, C. J., Cameron, J. L., Hruban, R. H., and Goggins, M. (2002). Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. Am J Pathol *160*, 45-50.

Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone acetyltransferases. Annu Rev Biochem *70*, 81-120.

Rouleau, J., MacLeod, A. R., and Szyf, M. (1995). Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. J Biol Chem *270*, 1595-1601.

Rouleau, J., Tanigawa, G., and Szyf, M. (1992). The mouse DNA methyltransferase 5'-region. A unique housekeeping gene promoter. J Biol Chem *267*, 7368-7377.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet *25*, 269-277.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science *275*, 1790-1792.

Saito, M., and Ishikawa, F. (2002). The mCpG-binding domain of human MBD3 does not bind to mCpG but interacts with NuRD/Mi2 components HDAC1 and MTA2. J Biol Chem *277*, 35434-35439.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. (2001). Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology *33*, 561-568.

Sato, M., Horio, Y., Sekido, Y., Minna, J. D., Shimokata, K., and Hasegawa, Y. (2002). The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines. Oncogene *21*, 4822-4829.

Sato, N., Maehara, N., Su, G. H., and Goggins, M. (2003). Effects of 5-aza-2'-deoxycytidine on matrix metalloproteinase expression and pancreatic cancer cell invasiveness. J Natl Cancer Inst *95*, 327-330.

Scanlan, M. J., Welt, S., Gordon, C. M., Chen, Y. T., Gure, A. O., Stockert, E., Jungbluth, A. A., Ritter, G., Jager, D., Jager, E., *et al.* (2002). Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. Cancer Res *62*, 4041-4047.

Scheper, W., Holthuizen, P. E., and Sussenbach, J. S. (1996). Growth-condition-dependent regulation of insulin-like growth factor II mRNA stability. Biochem J *318*, 195-201.

Schlegel, J., Guneysu, S., and Mennel, H. D. (2002). Expression of the genes of methyl-binding domain proteins in human gliomas. Oncol Rep *9*, 393-395.

Schuffenhauer, S., Bartsch, O., Stumm, M., Buchholz, T., Petropoulou, T., Kraft, S., Belohradsky, B., Hinkel, G. K., Meitinger, T., and Wegner, R. D. (1995). DNA, FISH and complementation studies in ICF syndrome: DNA hypomethylation of repetitive and single copy loci and evidence for a trans acting factor. Hum Genet *96*, 562-571.

Searles, C. D., Miwa, Y., Harrison, D. G., and Ramasamy, S. (1999). Posttranscriptional regulation of endothelial nitric oxide synthase during cell growth. Circ Res *85*, 588-595.

Sekimata, M., Takahashi, A., Murakami-Sekimata, A., and Homma, Y. (2001). Involvement of a novel zinc finger protein, MIZF, in transcriptional repression by interacting with a methyl-CpG-binding protein, MBD2. J Biol Chem *276*, 42632-42638.

Sela-Brown, A., Silver, J., Brewer, G., and Naveh-Many, T. (2000). Identification of AUF1 as a parathyroid hormone mRNA 3'-untranslated region-binding protein that determines parathyroid hormone mRNA stability. J Biol Chem *275*, 7424-7429.

Selker, E. U. (1998). Trichostatin A causes selective loss of DNA methylation in Neurospora. Proc Natl Acad Sci U S A *95*, 9430-9435.

Seo, S. B., Macfarlan, T., McNamara, P., Hong, R., Mukai, Y., Heo, S., and Chakravarti, D. (2002). Regulation of histone acetylation and transcription by nuclear protein pp32, a subunit of the INHAT complex. J Biol Chem *277*, 14005-14010.

Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001). Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell *104*, 119-130.

Sharrard, R. M., Royds, J. A., Rogers, S., and Shorthouse, A. J. (1992). Patterns of methylation of the c-myc gene in human colorectal cancer progression. Br J Cancer *65*, 667-672.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659-667.

Shearer, G. M., and Clerici, M. (1991). Early T-helper cell defects in HIV infection. Aids *5*, 245-253.

Shen, L., Fang, J., Qiu, D., Zhang, T., Yang, J., Chen, S., and Xiao, S. (1998). Correlation between DNA methylation and pathological changes in human hepatocellular carcinoma. Hepatogastroenterology *45*, 1753-1759.

Shi, H., Yan, P. S., Chen, C. M., Rahmatpanah, F., Lofton-Day, C., Caldwell, C. W., and Huang, T. H. (2002). Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells. Cancer Res *62*, 3214-3220.

Shibata, D., and Weiss, L. M. (1992). Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol *140*, 769-774.

Sibani, S., Melnyk, S., Pogribny, I. P., Wang, W., Hiou-Tim, F., Deng, L., Trasler, J., James, S. J., and Rozen, R. (2002). Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice. Carcinogenesis *23*, 61-65.

Silva, A. J., Ward, K., and White, R. (1993). Mosaic methylation in clonal tissue. Dev Biol *156*, 391-398.

Simizu, S., Ishida, K., Wierzba, M. K., Sato, T. A., and Osada, H. (2003). Expression of heparanase in human tumor cell lines and human head and neck tumors. Cancer Lett *193*, 83-89.

Singer-Sam, J., and Riggs, A. D. (1993). X chromosome inactivation and DNA methylation. Exs *64*, 358-384.

Sirenko, O. I., Lofquist, A. K., DeMaria, C. T., Morris, J. S., Brewer, G., and Haskill, J. S. (1997). Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1. Mol Cell Biol *17*, 3898-3906.

Slack, A., Bovenzi, V., Bigey, P., Ivanov, M. A., Ramchandani, S., Bhattacharya, S., tenOever, B., Lamrihi, B., Scherman, D., and Szyf, M. (2002). Antisense MBD2 gene therapy inhibits tumorigenesis. J Gene Med *4*, 381–389.

Slack, A., Cervoni, N., Pinard, M., and Szyf, M. (1999). DNA methyltransferase is a downstream effector of cellular transformation triggered by simian virus 40 large T antigen. J Biol Chem *274*, 10105-10112.

Slack, A., Pinard, M., Araujo, F. D., and Szyf, M. (2001). A novel regulatory element in the dnmt1 gene that responds to co-activation by Rb and c-Jun. Gene *268*, 87-96.

Smiraglia, D. J., and Plass, C. (2002). The study of aberrant methylation in cancer via restriction landmark genomic scanning. Oncogene *21*, 5414-5426.

Soares, J., Pinto, A. E., Cunha, C. V., Andre, S., Barao, I., Sousa, J. M., and Cravo, M. (1999). Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer *85*, 112-118.

Sommer, A., Moscatelli, D., and Rifkin, D. B. (1989). An amino-terminally extended and post-translationally modified form of a 25kD basic fibroblast growth factor. Biochem Biophys Res Commun *160*, 1267-1274.

Sonenberg, N., Morgan, M. A., Merrick, W. C., and Shatkin, A. J. (1978). A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. Proc Natl Acad Sci U S A *75*, 4843-4847.

Soultanas, P., Andrews, P. D., Burton, D. R., and Hornby, D. P. (1993). Modulation of human DNA methyltransferase activity and mRNA levels in the monoblast cell line U937 induced to differentiate with dibutyryl cyclic AMP and phorbol ester. J Mol Endocrinol *11*, 191-200.

Spicher, A., Guicherit, O. M., Duret, L., Aslanian, A., Sanjines, E. M., Denko, N. C., Giaccia, A. J., and Blau, H. M. (1998). Highly conserved RNA sequences that are sensors of environmental stress. Mol Cell Biol *18*, 7371-7382.

Spotswood, H. T., and Turner, B. M. (2002). An increasingly complex code. J Clin Invest *110*, 577-582.

Stambuk, S., and Radman, M. (1998). Mechanism and control of interspecies recombination in Escherichia coli. I. Mismatch repair, methylation, recombination and replication functions. Genetics *150*, 533-542.

Staton, J. M., Thomson, A. M., and Leedman, P. J. (2000). Hormonal regulation of mRNA stability and RNA-protein interactions in the pituitary. J Mol Endocrinol *25*, 17-34.

Stein, R., Razin, A., and Cedar, H. (1982). In vitro methylation of the hamster adenine phosphoribosyltransferase gene inhibits its expression in mouse L cells. Proc Natl Acad Sci U S A *79*, 3418-3422.

Steinmetz, K. L., Pogribny, I. P., James, S. J., and Pitot, H. C. (1998). Hypomethylation of the rat glutathione S-transferase pi (GSTP) promoter region isolated from methyl-deficient livers and GSTP-positive liver neoplasms. Carcinogenesis *19*, 1487-1494.

Stern, L. L., Mason, J. B., Selhub, J., and Choi, S. W. (2000). Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene [In Process Citation]. Cancer Epidemiol Biomarkers Prev *9*, 849-853.

Steward, N., Ito, M., Yamaguchi, Y., Koizumi, N., and Sano, H. (2002). Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. J Biol Chem *277*, 37741-37746.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Strahl, B. D., Briggs, S. D., Brame, C. J., Caldwell, J. A., Koh, S. S., Ma, H., Cook, R. G., Shabanowitz, J., Hunt, D. F., Stallcup, M. R., and Allis, C. D. (2001). Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr Biol *11*, 996-1000.

Strahl, B. D., Grant, P. A., Briggs, S. D., Sun, Z. W., Bone, J. R., Caldwell, J. A., Mollah, S., Cook, R. G., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2002). Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol Cell Biol *22*, 1298-1306.

Strahl, B. D., Ohba, R., Cook, R. G., and Allis, C. D. (1999). Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. Proc Natl Acad Sci U S A *96*, 14967-14972.

Suetake, I., Kano, Y., and Tajima, S. (1998). Effect of aphidicolin on DNA methyltransferase in the nucleus. Cell Struct Funct *23*, 137-142.

Sun, Z. W., and Allis, C. D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104-108.

Surdej, P., Riedl, A., and Jacobs-Lorena, M. (1994). Regulation of mRNA stability in development. Annu Rev Genet *28*, 263-282.

Szyf, M. (1994). DNA methylation properties: consequences for pharmacology. Trends Pharmacol Sci *15*, 233-238.

Szyf, M. (1998). Targeting DNA methyltransferase in cancer. Cancer Metastasis Rev *17*, 219-231.

Szyf, M. (2001a). The role of DNA methyltransferase 1 in growth control. Front Biosci *6*, D599-609.

Szyf, M. (2001b). Towards a pharmacology of DNA methylation. Trends Pharmacol Sci *22*, 350-354.

Szyf, M., Avraham-Haetzni, K., Reifman, A., Shlomai, J., Kaplan, F., Oppenheim, A., and Razin, A. (1984). DNA methylation pattern is determined by the intracellular level of the methylase. Proc Natl Acad Sci U S A *81*, 3278-3282.

Szyf, M., and Bhattacharya, S. K. (2002a). Extracting DNA demethylase activity from mammalian cells. Methods Mol Biol *200*, 163-176.

Szyf, M., and Bhattacharya, S. K. (2002b). Measuring DNA demethylase activity in vitro. Methods Mol Biol *200*, 155-161.

Szyf, M., Bozovic, V., and Tanigawa, G. (1991). Growth regulation of mouse DNA methyltransferase gene expression. J Biol Chem *266*, 10027-10030.

Szyf, M., Detich, N. (2001c). Regulation of the DNA Methylation Machinery and Its Role in Cellular Transformation. In Progress in Nucleic Acid Research and Molecular Biology, K. Moldave, ed. (San Diego, Academic Press).

Szyf, M., Eliasson, L., Mann, V., Klein, G., and Razin, A. (1985a). Cellular and viral DNA hypomethylation associated with induction of Epstein-Barr virus lytic cycle. Proc Natl Acad Sci U S A *82*, 8090-8094.

Szyf, M., Kaplan, F., Mann, V., Giloh, H., Kedar, E., and Razin, A. (1985b). Cell cycle-dependent regulation of eukaryotic DNA methylase level. J Biol Chem *260*, 8653-8656.

Szyf, M., Knox, D. J., Milutinovic, S., Slack, A. D., and Araujo, F. D. (2000). How does DNA methyltransferase cause oncogenic transformation? Ann N Y Acad Sci *910*, 156-174; discussion 175-157.

Szyf, M., Milstone, D. S., Schimmer, B. P., Parker, K. L., and Seidman, J. G. (1990). cis modification of the steroid 21-hydroxylase gene prevents its expression in the Y1 mouse adrenocortical tumor cell line. Mol Endocrinol *4*, 1144-1152.

Szyf, M., Rouleau, J., Theberge, J., and Bozovic, V. (1992). Induction of myogenic differentiation by an expression vector encoding the DNA methyltransferase cDNA sequence in the antisense orientation. J Biol Chem *267*, 12831-12836.

Szyf, M., Schimmer, B. P., and Seidman, J. G. (1989). Nucleotide-sequence-specific de novo methylation in a somatic murine cell line. Proc Natl Acad Sci U S A *86*, 6853-6857.

Szyf, M., Theberge, J., and Bozovic, V. (1995). Ras induces a general DNA demethylation activity in mouse embryonal P19 cells. J Biol Chem *270*, 12690-12696.

Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001). Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. J Biol Chem *276*, 25309-25317.

Takenaga, K. (1986). Modification of the metastatic potential of tumor cells by drugs. Cancer Metastasis Rev *5*, 67-75.

Tamaru, H., and Selker, E. U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

Tao, L., Nielsen, T., Friedlander, P., Zannis-Hadjopoulos, M., and Price, G. (1997). Differential DNA replication origin activities in human normal skin fibroblast and HeLa cell lines. J Mol Biol *273*, 509-518.

Teubner, B., and Schulz, W. A. (1995). Regulation of DNA methyltransferase during differentiation of F9 mouse embryonal carcinoma cells. J Cell Physiol *165*, 284-290.

Tevethia, M. J. (1984). Immortalization of primary mouse embryo fibroblasts with SV40 virions, viral DNA, and a subgenomic DNA fragment in a quantitative assay. Virology *137*, 414-421.

Tholanikunnel, B. G., Raymond, J. R., and Malbon, C. C. (1999). Analysis of the AU-rich elements in the 3'-untranslated region of beta 2-adrenergic receptor mRNA by mutagenesis and identification of the homologous AU-rich region from different species. Biochemistry *38*, 15564-15572.

Tilghman, S. M., Bartolomei, M. S., Webber, A. L., Brunkow, M. E., Saam, J., Leighton, P. A., Pfeifer, K., and Zemel, S. (1993). Parental imprinting of the H19 and Igf2 genes in the mouse. Cold Spring Harb Symp Quant Biol *58*, 287-295.

Tillmann-Bogush, M., Heaton, J. H., and Gelehrter, T. D. (1999). Cyclic nucleotide regulation of PAI-1 mRNA stability. Identification of cytosolic proteins that interact with an a-rich sequence. J Biol Chem *274*, 1172-1179.

Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. (1999). CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A *96*, 8681-8686.

Treisman, R. (1985). Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. Cell *42*, 889-902.

Tremolizzo, L., Carboni, G., Ruzicka, W. B., Mitchell, C. P., Sugaya, I., Tueting, P., Sharma, R., Grayson, D. R., Costa, E., and Guidotti, A. (2002). An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability. Proc Natl Acad Sci U S A *99*, 17095-17100.

Tsai, C. N., Tsai, C. L., Tse, K. P., Chang, H. Y., and Chang, Y. S. (2002). The Epstein-Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. Proc Natl Acad Sci U S A *99*, 10084-10089.

Tulchinsky, E., Grigorian, M., Tkatch, T., Georgiev, G., and Lukanidin, E. (1995). Transcriptional regulation of the mts1 gene in human lymphoma cells: the role of DNA-methylation. Biochim Biophys Acta *1261*, 243-248.

Vallian, S., Chin, K. V., and Chang, K. S. (1998). The promyelocytic leukemia protein interacts with Sp1 and inhibits its transactivation of the epidermal growth factor receptor promoter. Mol Cell Biol *18*, 7147-7156.

Vance, D. E., and Walkey, C. J. (1998). Roles for the methylation of phosphatidylethanolamine. Curr Opin Lipidol *9*, 125-130.

Vardimon, L., Kressmann, A., Cedar, H., Maechler, M., and Doerfler, W. (1982). Expression of a cloned adenovirus gene is inhibited by in vitro methylation. Proc Natl Acad Sci U S A *79*, 1073-1077.

Vertino, P. M., Sekowski, J. A., Coll, J. M., Applegren, N., Han, S., Hickey, R. J., and Malkas, L. H. (2002). DNMT1 is a Component of a Multiprotein DNA Replication Complex. Cell Cycle *1*, 416-423.

Vertino, P. M., Yen, R. W., Gao, J., and Baylin, S. B. (1996). De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. Mol Cell Biol *16*, 4555-4565.

Vidali, G., Boffa, L. C., Mann, R. S., and Allfrey, V. G. (1978). Reversible effects of Na-butyrate on histone acetylation. Biochem Biophys Res Commun *82*, 223-227.

Vilain, A., Vogt, N., Dutrillaux, B., and Malfoy, B. (1999). DNA methylation and chromosome instability in breast cancer cell lines. FEBS Lett *460*, 231-234.

Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet *23*, 62-66.

Wang, D., Liebowitz, D., and Kieff, E. (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell *43*, 831-840.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. (2001a). Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol Cell *8*, 1207-1217.

Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B. D., Briggs, S. D., Allis, C. D., Wong, J., Tempst, P., and Zhang, Y. (2001b). Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science *293*, 853-857.

Watson, W. H., Zhao, Y., and Chawla, R. K. (1999). S-adenosylmethionine attenuates the lipopolysaccharide-induced expression of the gene for tumour necrosis factor alpha. Biochem J *342 (Pt 1)*, 21-25.

Weidle, U. H., and Grossmann, A. (2000). Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. Anticancer Res *20*, 1471-1185.

Weinberg, R. A. (1991). Tumor suppressor genes. Science *254*, 1138-1146.

Weinshilboum, R. M., Otterness, D. M., and Szumlanski, C. L. (1999). Methylation pharmacogenetics: catechol O-methyltransferase, thiopurine methyltransferase, and histamine N-methyltransferase. Annu Rev Pharmacol Toxicol *39*, 19-52.

Weiss, A., Keshet, I., Razin, A., and Cedar, H. (1996). DNA demethylation in vitro: involvement of RNA [published erratum appears in Cell 1998 Nov 13;95(4):following 573]. Cell *86*, 709-718.

Wen, Y., and Shatkin, A. J. (2000). Cap methyltransferase selective binding and methylation of GpppG-RNA are stimulated by importin-alpha. Genes Dev *14*, 2944-2949.

Wilks, A., Seldran, M., and Jost, J. P. (1984). An estrogen-dependent demethylation at the 5' end of the chicken vitellogenin gene is independent of DNA synthesis. Nucleic Acids Res *12*, 1163-1177.

Wilson, G. M., and Brewer, G. (1999). The search for trans-acting factors controlling messenger RNA decay. Prog Nucleic Acid Res Mol Biol *62*, 257-291.

Wu, J., Herman, J. G., Wilson, G., Lee, R. Y., Yen, R. W., Mabry, M., de Bustros, A., Nelkin, B. D., and Baylin, S. B. (1996). Expression of prokaryotic HhaI DNA methyltransferase is transforming and lethal to NIH 3T3 cells. Cancer Res *56*, 616-622.

Wu, J., Issa, J. P., Herman, J., Bassett, D. E., Jr., Nelkin, B. D., and Baylin, S. B. (1993). Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. Proc Natl Acad Sci U S A *90*, 8891-8895.

Wu, J. C., and Santi, D. V. (1985). On the mechanism and inhibition of DNA cytosine methyltransferases. Prog Clin Biol Res *198*, 119-129.

Xin, Z., Tachibana, M., Guggiari, M., Heard, E., Shinkai, Y., and Wagstaff, J. (2003). Role of histone methyltransferase G9a in CpG methylation of the Prader-Willi syndrome imprinting center. J Biol Chem *278*, 14996-15000.

Yanagisawa, Y., Ito, E., Yuasa, Y., and Maruyama, K. (2002). The human DNA methyltransferases DNMT3A and DNMT3B have two types of promoters with different CpG contents. Biochim Biophys Acta *1577*, 457-465.

Yang, J., Deng, C., Hemati, N., Hanash, S. M., and Richardson, B. C. (1997). Effect of mitogenic stimulation and DNA methylation on human T cell DNA methyltransferase expression and activity. J Immunol *159*, 1303-1309.

Yang, L., Xia, L., Wu, D. Y., Wang, H., Chansky, H. A., Schubach, W. H., Hickstein, D. D., and Zhang, Y. (2002). Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. Oncogene *21*, 148-152.

Yoder, J. A., and Bestor, T. H. (1998). A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. Hum Mol Genet *7*, 279-284.

Yoder, J. A., Yen, R. W., Vertino, P. M., Bestor, T. H., and Baylin, S. B. (1996). New 5' regions of the murine and human genes for DNA (cytosine-5)-methyltransferase. J Biol Chem *271*, 31092-31097.

Yoshida, M., and Horinouchi, S. (1999). Trichostatin and leptomycin. Inhibition of histone deacetylation and signal-dependent nuclear export. Ann N Y Acad Sci *886*, 23-36.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem *265*, 17174-11719.

Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. (1995). Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A *92*, 7416-7419.

Young, J. I., and Smith, J. R. (2001). DNA methyltransferase inhibition in normal human fibroblasts induces A p21 dependent-cell cycle withdrawal. J Biol Chem *276*, 19610-19616.

Youssoufian, H., Hammer, S. M., Hirsch, M. S., and Mulder, C. (1982). Methylation of the viral genome in an in vitro model of herpes simplex virus latency. Proc Natl Acad Sci U S A *79*, 2207-2210.

Yu, F., Thiesen, J., and Stratling, W. H. (2000). Histone deacetylase-independent transcriptional repression by methyl- CpG-binding protein 2. Nucleic Acids Res *28*, 2201-2206.

Yu, F., Zingler, N., Schumann, G., and Stratling, W. H. (2001). Methyl-CpG-binding protein 2 represses LINE-1 expression and retrotransposition but not Alu transcription. Nucleic Acids Res *29*, 4493-4501.

Yuan, P. X., Huang, L. D., Jiang, Y. M., Gutkind, J. S., Manji, H. K., and Chen, G. (2001). The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. J Biol Chem *276*, 31674-31683.

Yung, R. L., and Richardson, B. C. (1994). Role of T cell DNA methylation in lupus syndromes. Lupus *3*, 487-491.

Zardo, G., Reale, A., Passananti, C., Pradhan, S., Buontempo, S., De Matteis, G., Adams, R. L., and Caiafa, P. (2002). Inhibition of poly(ADP-ribosyl)ation induces DNA hypermethylation: a possible molecular mechanism. Faseb J *16*, 1319-1321.

Zegerman, P., Canas, B., Pappin, D., and Kouzarides, T. (2002). Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. J Biol Chem *277*, 11621-11624.

Zhang, C. L., McKinsey, T. A., and Olson, E. N. (2002). Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. Mol Cell Biol *22*, 7302-7312.

Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993). Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol Cell Biol *13*, 7652-7665.

Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998). The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell *95*, 279-289.

Zhang, Y., Ng, H. H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev *13*, 1924-1935.

Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev *15*, 2343-2360.

Zhao, C. Q., Young, M. R., Diwan, B. A., Coogan, T. P., and Waalkes, M. P. (1997). Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci U S A *94*, 10907-10912.

Zhou, Q., Guo, Y., and Liu, Y. (1998). Regulation of the stability of heat-stable antigen mRNA by interplay between two novel cis elements in the 3' untranslated region. Mol Cell Biol *18*, 815-826.

Zhu, B., Zheng, Y., Angliker, H., Schwarz, S., Thiry, S., Siegmann, M., and Jost, J. P. (2000a). 5-Methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence. Nucleic Acids Res *28*, 4157-4165.

Zhu, B., Zheng, Y., Hess, D., Angliker, H., Schwarz, S., Siegmann, M., Thiry, S., and Jost, J. P. (2000b). 5-methylcytosine-DNA glycosylase activity is present in a cloned G/T mismatch DNA glycosylase associated with the chicken embryo DNA demethylation complex. Proc Natl Acad Sci U S A *97*, 5135-5139.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 28, Issue of July 12, pp. 25026–25031, 2002
Printed in U.S.A.

The Oncoprotein Set/TAF-1β, an Inhibitor of Histone Acetyltransferase, Inhibits Active Demethylation of DNA, Integrating DNA Methylation and Transcriptional Silencing*

Received for publication, March 7, 2002, and in revised form, April 25, 2002
Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M202256200

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Histone hypoacetylation and DNA hypermethylation are hallmarks of gene silencing. Although a role for DNA methylation in regulating histone acetylation has been established, it is not clear how and whether epigenetic histone markings influence DNA modifications in transcriptional silencing. We have previously shown that induction of histone acetylation by trichostatin A promotes demethylation of ectopically methylated DNA (Cervoni, N., and Szyf, M. (2001) *J. Biol. Chem.* 276, 40778–40787). The oncoprotein Set/TAF-Iβ is a subunit of the recently identified inhibitor of acetyltransferases complex that inhibits histone acetylation by binding to and masking histone acetyltransferase targets (Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) *Cell* 104, 119–130). We show here that the overexpression of Set/TAF-Iβ, whose expression is up-regulated in multiple tumor tissues, inhibits demethylation of ectopically methylated DNA resulting in gene silencing. Overexpression of a mutant Set/TAF-Iβ that does not inhibit histone acetylation is defective in inhibiting DNA demethylation. Taken together, these results are consistent with a novel regulatory role for Set/TAF-Iβ, integrating epigenetic states of histones and DNA in gene regulation and provide a new mechanism that can explain how hypermethylation of specific regions might come about by inhibition of demethylation in cancer cells.

The DNA methylation pattern is tightly correlated with chromatin structure in that transcriptionally active chromatin domains are hypomethylated, whereas inactive regions are hypermethylated (3). However, the mechanisms defining the relationship between histone hypoacetylation and DNA hypermethylation are not clear. While it is well accepted that DNA methylation can promote chromatin deacetylation and inactivation (4), a number of studies suggest that chromatin status can also alter the pattern of DNA methylation. Genome wide demethylation was shown to be induced by *n*-butyrate, an inhibitor of histone acetylation (5). Tichostatin A (TSA),[1] an inhibitor of histone deacetylase, was shown to induce selective loss of DNA methylation in *Neurospora* (6), and very recently disruption of histone methylation was shown to eliminate DNA methylation in *Neurospora* (7). We have recently shown that TSA can induce demethylation of ectopically methylated genes (1).

Hypermethylation of CpG islands has attracted considerable attention as a mechanism responsible for gene silencing during tumor suppression. It is clear that methylation of tumor suppressor genes cannot be explained by the increase in the general level of DNA methyltransferase activity observed in cancer cells. Significant effort has been therefore directed toward identification of factors that recruit DNA methyltransferases to specific promoters. In accordance with this hypothesis, a recent publication demonstrated that the oncogenic transcription factor PML-RAR fusion protein recruits DNMT1 to the retinoic acid βII promoter, resulting in methylation and silencing of this promoter (8).

An alternative potential mechanism of bringing about DNA hypermethylation is inhibition of demethylase activity. This possibility was not previously explored, since it has long been believed that the DNA methylation pattern is controlled exclusively by DNA methyltransferases and that the DNA methylation reaction is irreversible. Nevertheless, we have recently extracted DNA demethylase activity from human lung cancer cell line A549 (9) and have shown that a human embryonal kidney cell line HEK293 possesses active demethylase activity (1).

The DNA methylation state of a gene in a living cell represents a steady state, and it is hard to determine whether it is a consequence of increased DNA methylation or reduced demethylation. Moreover, it is hard to determine whether demethylation of a gene in a proliferating cell is passive, resulting from inhibition of DNA methylation during DNA synthesis or an active true removal of a methyl group. We have therefore recently developed a transient transfection system that enables studying demethylase activity in cancer cells. A reporter CMV-GFP construct is methylated *in vitro* by a CpG methyltransferase m*Sss*I and introduced into human transformed HEK293 cells. The plasmid is extracted 96 h after transfection, and its state of methylation is determined by either methylation sensitive restriction enzyme analysis or bisulfite mapping. Since we have demonstrated that this plasmid does not replicate in HEK cells (1), any change in DNA methylation must result from active demethylation. We have also shown that these constructs are not *de novo* methylated during the transient transfection period, and therefore the transient transfection assay measures exclusively active demethylation in the cell. This system could be utilized to measure the effects of specific

* This work was supported by the National Cancer Institute of Canada. Work in D. C.'s laboratory was supported by National Institutes of Health Grant RO1 DK57079. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TSA, trichostatin A; GFP, green fluorescent protein; INHAT, inhibitor of acetyltransferases; CHIP, chromatin immunoprecipitation; CMV, cytomegalovirus; HAT, histone acetyltransferase; GST, glutathione *S*-transferase.

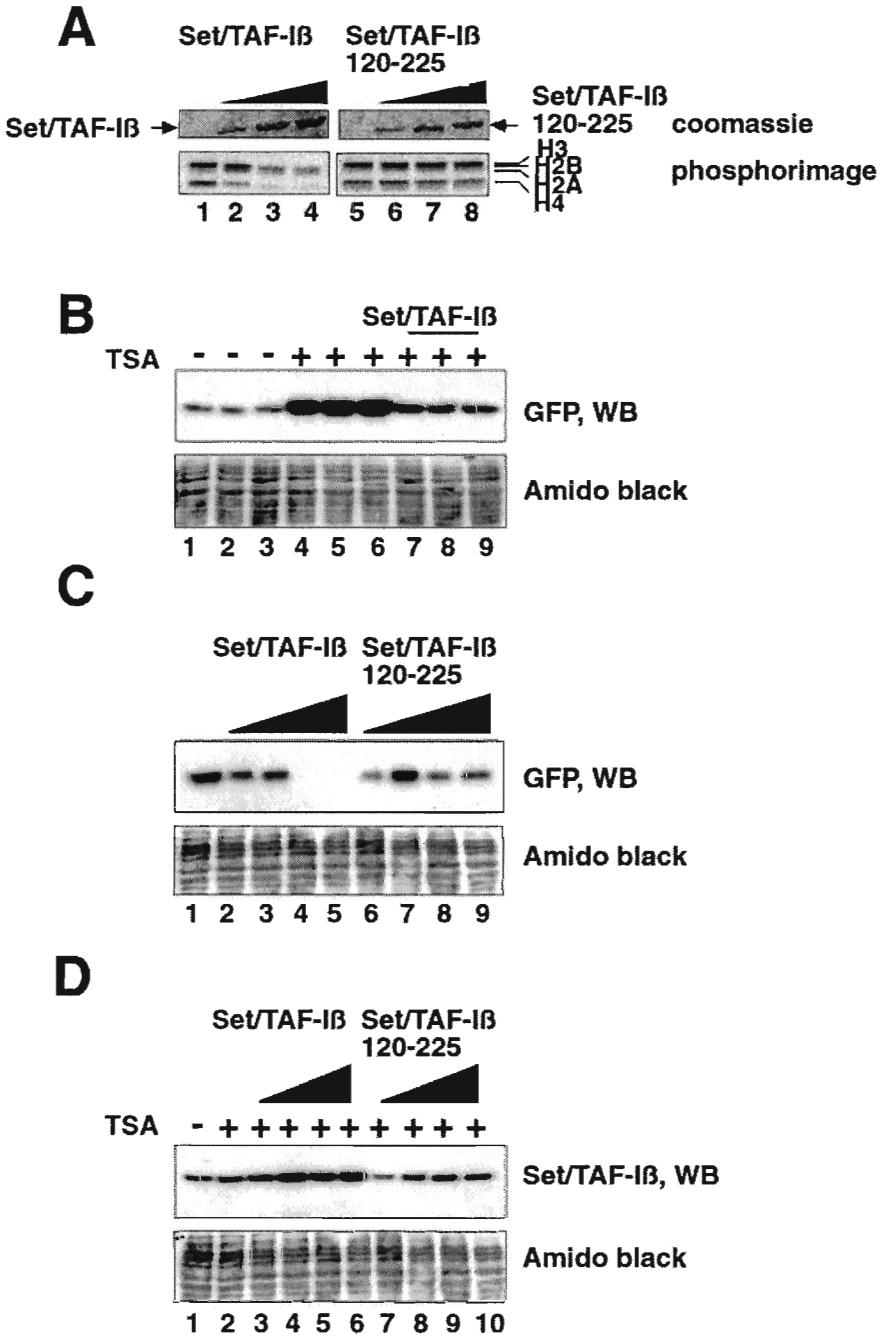

FIG. 1. *A*, Set/TAF-Iβ inhibits histone acetylation. HAT assays were performed with increasing concentrations of GST-Set/TAF-Iβ (*lanes 2–4*) and GST-Set/TAF-Iβ 120–225 (*lanes 6–8*), respectively. In *lanes 1* and *5*, p300 was incubated with histones only. The *upper panels* show Coomassie staining of both proteins used, and positions of individual histones are shown by phosphorimaging of acetylated histones (*lower panels*). *B* and *C*, Set/TAF-Iβ decreases acetylation-induced increase of GFP expression. *B*, total cell extracts from HEK293 cells transfected with either CMV-GFP plasmid alone (*lanes 1–6*) or CMV-GFP and Set/TAF-Iβ (*lanes 7–9*) in the absence (*lanes 1–3*) or presence of 0.3 μM TSA (*lanes 4–9*) were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel in triplicates. *C*, total cell extracts from HEK cells transfected with increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 2–5*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–9*), in the absence (*lane 1*) or presence of 0.3 μM TSA (*lanes 2–9*), were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. *D*, total cell extracts from HEK cells transfected with increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 3–6*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–10*), in the absence (*lane 1*) or presence of 0.3 μM TSA (*lanes 2–10*), were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. All blots were transferred to PDVF membranes. *C*, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000. *D*, Set/TAF-Iβ protein was detected using mouse monoclonal IgG 1:400 dilution, followed by peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) at 1:20,000. Signal was detected by using enhanced chemiluminescence detection kit (Amersham Biosciences). To show equal loading of the protein, membranes were stained with Amido Black (*lower panels*).

proteins on active demethylation of specific sequences in living cells. Using this system we have recently shown that histone acetylation stimulates active demethylation of ectopically methylated reporter constructs.

Although we have shown that TSA treatment could potentially stimulate demethylation, this effect is not universal, and it is clear that many methylated genes are neither demethylated nor activated following TSA treatment (10). We therefore reasoned that proteins that inhibit acetylation of histones in response to TSA might also inhibit DNA demethylation. Similar proteins might also inhibit corrective demethylation of spuriously methylated genes and lead to hypermethylation of certain sequences explaining the progressive methylation of tumor suppressor genes in cancer. Recently, experiments performed by Seo *et al.* (2) led to the discovery of a novel human cellular complex which inhibits the histone acetyltransferase (HAT) activity of transcriptional coactivators p300/CBP and PCAF. This complex called INHAT (inhibitor of acetyltransferases) comprised of the myeloid leukemia-associated oncoprotein SET/TAF-Iβ, the template-activating factor TAF-Iə, and the nuclear phosphoprotein pp32 masks the effect of histone acetyltransferases by binding to histones, thereby inhibiting transcription and serving as a novel mechanism of transcriptional regulation (2). INHAT subunits were shown to individually inhibit histone acetylation. Since INHATs influence the state of histone acetylation (2), and since histone acetylation affects chromatin structure (11, 12), we tested the hypothesis that INHATs could directly or indirectly modulate DNA methylation patterns by inhibiting DNA methylation and thus play a role in integrating the status of histone acetylation, DNA methylation, and transcriptional silencing. Since Set/TAF-Iβ's association with leukemia suggests a fundamental cellular function perhaps related to neoplastic progression, we focused primarily on the Set/TAF-Iβ oncoprotein in this study.

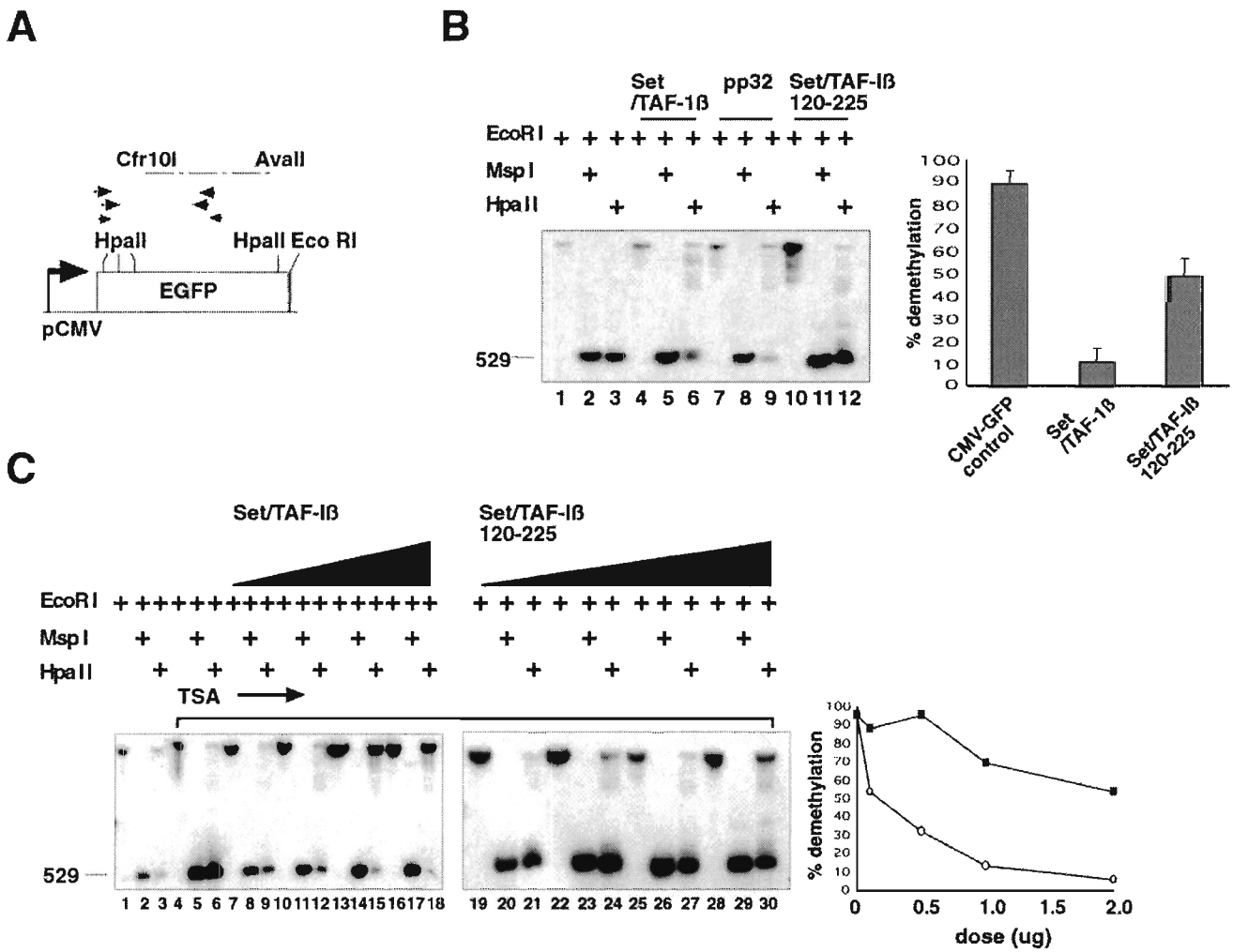

FIG. 2. **INHAT subunits inhibit demethylation of GFP.** *A*, a physical map of the CMV-GFP region analyzed is shown. *B*, *in vitro* methylated CMV-GFP plasmid was transfected alone (*lanes 1–3*) or cotransfected with either: 2 μg of Set/TAF-Iβ (*lanes 4–6*), pp32 (*lanes 7–9*), or Set/TAF-Iβ 120–225 mutant (*lanes 10–12*) plasmids and treated with a final concentration of 0.3 μM TSA. The results of three independent experiments were quantified by densitometry, and the percent demethylation for each sample was determined by calculating the ratio of *Hpa*II/*Msp*I-digested 529-bp fragment. *C*, *in vitro* methylated CMV-GFP plasmid was transfected alone (*lanes 1–6*) or with either increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–18*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 19–30*), in the absence (*lanes 1–3*) or presence of 0.3 μM TSA (*lanes 4–30*). Results were quantified as in *B* and are shown in the graph. ■, Set/TAF-Iβ 120–225 mutant; ○, Set/TAF-Iβ. For both *B* and *C*, 10 μg of isolated DNA was digested with 50 units of *Eco*RI (*B*, *lanes 1, 4, 7,* and *10*; *C*, *lanes 1, 4, 7, 10, 13, 16, 19, 22, 25,* and *28*) alone or *Eco*RI followed by either digestion with 20 units of *Msp*I (*B*, *lanes 2, 5, 8,* and *11*; *C*, *lanes 2, 5, 8, 11, 14, 17, 20, 23, 26,* and *29*) or *Hpa*II restriction enzymes (*B*, *lanes 3, 6, 9,* and *12*; *C*, *lanes 3, 6, 9, 12, 15, 18, 21, 24, 27,* and *30*), fractionated on a 1.7% agarose gel, and was then subjected to Southern blot transfer and hybridization with a ^{32}P-labeled GFP fragment (see map of probe in *A*, indicated by a *dashed line* flanked by restriction sites *Cfr*10I and *Ava*II).

MATERIALS AND METHODS

Cell Culture—HEK293 cells were plated at a density of 8×10^4/well in a six-well tissue culture dish and transiently transfected with 80 ng of methylated CMV-GFP plasmid DNA alone or cotransfected with up to a maximum of 2 μg of INHAT plasmid DNA (2) per well, using the calcium phosphate precipitation method as described previously (13). Transfections were performed in five individual wells and were repeated three times using different cultures of HEK293 cells.

In Vitro Methylation of Substrates—CMV-GFP plasmid was methylated *in vitro* by incubating 10 μg of plasmid DNA with 20 units of *Sss*I CpG DNA methyltransferase (14) (New England Biolabs Inc.) in a buffer recommended by the manufacturer containing 160 μM *S*-adenosylmethionine, at 37 °C for 2 h. After repeating this procedure three times, full protection from *Hpa*II digestion was observed.

Histone Acetylation Assays—The assay was performed essentially as described previously (2) utilizing increasing concentrations of wild type Set/TAF-Iβ and mutant Set/TAF-Iβ proteins. In short, ~1 pmol of baculovirus-expressed and purified p300 was incubated with (Fig. 1*A*, *lanes 2–4* and *6–8*) or without (Fig. 1, *lanes 1* and *5*) increasing concentrations of purified wild type or mutant GST-Set/TAF-Iβ as indicated in the legend to Fig. 1 for 5 min at 40 °C before the addition of ~1 μg of purified core histones and [^{14}C]acetyl-CoA (1000 pmol/μl, Sigma) and incubation continued for an additional 30 min. HAT assays were performed in the presence of 10 mM sodium butyrate. Reaction products were separated by SDS-PAGE and analyzed by a phosphorimager (2). GST alone had no effect in the assay system.

Bisulfite Mapping—Bisulfite mapping was performed as described previously with minor modifications (15). 5 μg of sodium bisulfite-treated DNA samples were subjected to PCR amplification using the first set of primers described below. PCR products were used as templates for subsequent PCR reactions utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA cloning kit (we followed the manufacturer's protocol), and the clones were sequenced using the T7 sequencing kit (Amersham Biosciences; we followed the manufacturer's protocol, procedure C). The primers used for the enhanced green fluorescent protein (pEGFP-1) (CLONTECH) (GenBank™ accession number U55761) were: GFP5′, 1,5′-gttattatggtgagtaaggg-3′; GFP5′ (nested), 5′-ggggtggtgtttattttgg-3′; GFP3′, 1,5′-tataactattataatatactcca-3′; GFP3′ (nested), 5′-cttataccccaaaatattacc-3′.

Chromatin Immunoprecipitation (CHIP) Assay—CHIP assays (16) were performed by following the Upstate Biotechnology CHIP assay kit protocol (catalog number 17-295). HEK293 cells were transfected with 80 ng of *in vitro* CMV-GFP plasmid, using the calcium phosphate method (see above). A final concentration of 0.3 μM TSA was added or

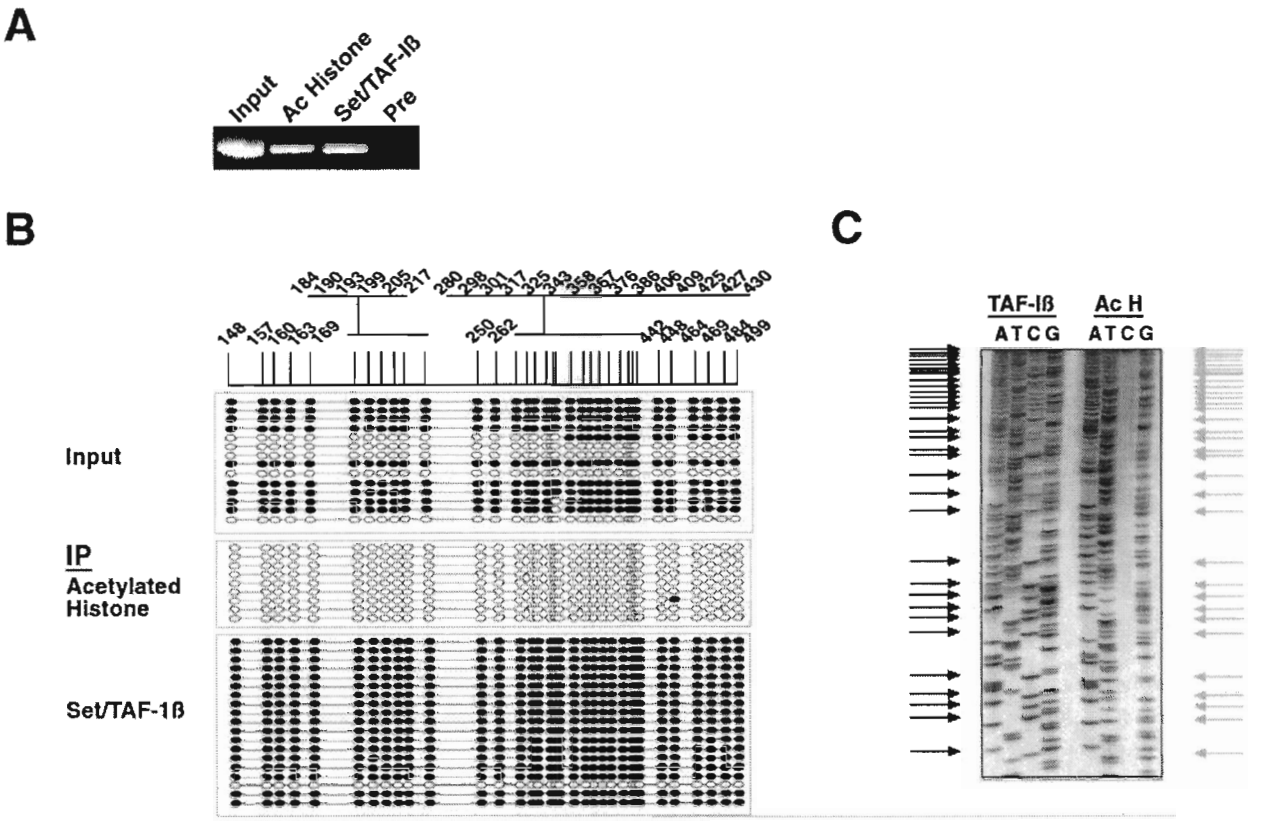

FIG. 3. **Bisulfite mapping and CHIP analysis of the association of transfected plasmids with Set/TAF-Iβ.** *A*, HEK293 cells were cotransfected with *in vitro* methylated GFP plasmid and 1 μg of Set/TAF-Iβ plasmid, treated with a final concentration of 0.3 μM TSA, formaldehyde cross-linked after 96 h, and subjected to a chromatin immunoprecipitation assay using antibodies against acetylated histone H3 (*Ac Histone*), Set/TAF-Iβ, or rabbit preimmune serum (*Pre*). *Input* denotes 10% of total DNA prior to immunoprecipitation. The GFP sequence was amplified from purified DNA by PCR. Primers (indicated in Fig. 2*A* as *solid arrows*) were diluted to 50 μM and are described under "Materials and Methods." A representative PCR is shown. *B*, immunoprecipitated DNA was subjected to bisulfite mapping analysis. *Hatched arrows* (displayed in Fig. 2*A*) indicate the location of both the outside and nested primers used to amplify bisulfited DNA. Each *line* within the *boxes* represents an independent clone. A *filled circle* represents a methylated CG dinucleotide, and an *empty circle* represents a demethylated CG dinucleotide. Clones from TSA-treated DNA not immunoprecipitated (*Input*), or immunoprecipitated with anti-acetylated H3 antibody or anti-Set/TAF-Iβ, are presented. *C*, representative sequencing gels of immunoprecipitated clones using either anti-acetylated H3 (*Ac H*) or anti-Set/TAF-Iβ (*TAF-Iβ*) antibodies. *Black arrows* point to methylated CGs from clones pulled down with anti-Set/TAF-Iβ antibody, and *gray arrows* point to demethylated CGs (converted to Ts) from clones pulled down with anti-acetylated histone H3 antibody

not added to fresh medium 24 h after transfection. Formaldehyde was added to the culture media at a final concentration of 1%, 96 h post-transfection and incubated at 37 °C for 10 min. Chromatin was immunoprecipitated using either an anti-acetylated histone H3 antibody (Upstate Biotechnology) as recommended by the manufacturer or the anti-Set/TAF-Iβ antibody (2) or rabbit preimmune serum, as a control. One-tenth of the lysate was kept to quantitate the amount of DNA present in different samples before immunoprecipitation (*input*). PCR reactions on DNA purified from non-immunoprecipitated samples (*input*) and immunoprecipitated samples (*Ac H*, *Set/TAF-Iβ*, and *Pre*) were repeated exhaustively using varying amounts of template to ensure that results were within the linear range of the PCR. The following primers were used to amplify the GFP gene: GFP 5′, 5′-caagggcgaggagctgtt-3′; GFP 3′, 5′-cggccatgatatagacgttg3′.

Western Blot Analysis—Total cell extracts were prepared using standard protocols and resolved on SDS-polyacrylamide gel electrophoresis (12.5%). After transferring to polyvinylidene difluoride membrane (Amersham Biosciences) and blocking the non-specific binding with 5% skim milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000, and an enhanced chemiluminescence detection kit (Amersham Biosciences). Set/TAF-Iβ protein was detected as described previously (17).

Multiple Tumor Blot Analysis—The matched tumor/normal expression assay membrane was hybridized using a ^{32}P-labeled Set/TAF-Iβ cDNA, according to the CLONTECH Laboratories Inc. Matched Tumor/Normal Expression Array User Manual (catalog number 7840-1). To obtain final expression values, each hybridization signal was quantified by densitometry and normalized against signal obtained by hybridization with a cDNA probe for ubiquitin.

RESULTS

INHAT Set/TAF-1β Inhibits Expression of a Cotransfected Methylated CMV-GFP Reporter Gene in a Dose-dependent Manner—We utilized a previously described transient transfection-based assay system to study active demethylation of ectopically methylated DNA and the different parameters that might affect it. In prior studies we have shown that *in vitro* methylated CMV-GFP reporter plasmid is actively demethylated 72–96 h following transfection into human embryonal kidney HEK293 cells and that this demethylation is dramatically enhanced when histone hyperacetylation is induced pharmacologically with TSA (1). If Set/TAF-Iβ functions by inhibiting histone acetylation, and also functionally interacts with methylated CMV-GFP, then overexpression of Set/TAF-Iβ should inhibit expression of CMV-GFP. To test this, Set/TAF-Iβ plasmid was cotranfected into human embryonic kidney (HEK293) cells, with *in vitro* methylated CMV-GFP plasmid, in the presence or absence of TSA (72 h), and harvested 96 h post-transfection. Cell extracts were prepared and subjected to Western blot analysis using antibodies directed against GFP protein (Fig. 1, *B* and *C*) or against the amino-terminal region of the Set/TAF-Iβ oncoprotein (which only recognizes the endogenous and wild type Set/TAF1β construct) (Fig. 1*D*). GFP expression is significantly increased in the presence of TSA compared with no TSA treatment (Fig. 1*B*) (1); and in agreement with previous

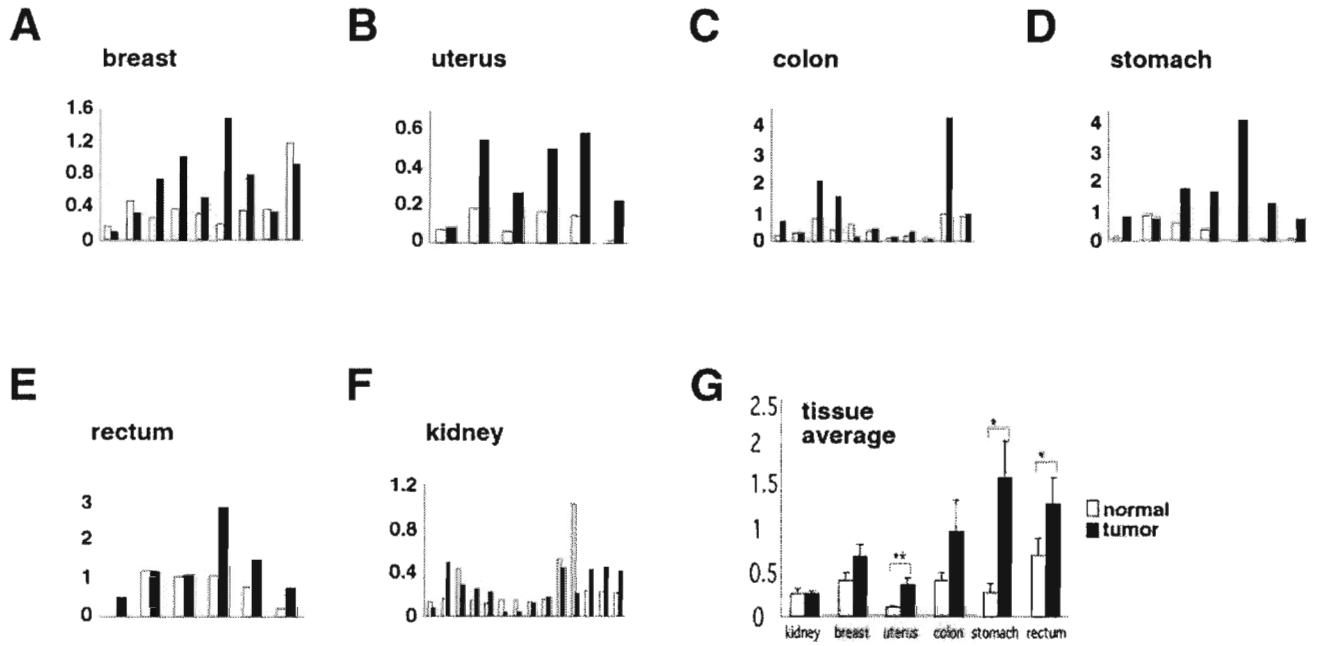

FIG. 4. **Set/TAF-Iβ oncogene is up-regulated in tumor tissues.** The matched tumor/normal expression assay membrane was hybridized using a ^{32}P-labeled Set/TAF-Iβ cDNA. Histograms represent values analyzed by densitometry from Set/TAF-Iβ signal normalized against ubiquitin signal. *Black bars* represent values obtained from tumor tissues, and *white bars* represent values obtained from normal tissues. The following tissues were analyzed: breast (*A*), uterus (*B*), colon (*C*), stomach (*D*), rectum (*E*), kidney (*F*). *G*, the tissue average of Set/TAF-Iβ message was calculated and found to be significantly greater in uterus, stomach, and rectum (**, $p < 0.005$; *, $p < 0.05$) when compared with Set/TAF-Iβ signal from normal tissues. Colon ($p = 0.053$) and breast ($p = 0.063$) were nearly significant.

results (2), Fig. 1*A* shows that while the wild type Set/TAF-Iβ inhibits p300-mediated histone acetylation in a dose-dependent manner, the mutant Set/TAF-Iβ 120–225, which lacks the previously determined HAT inhibitory domain (INHAT domain) and histone binding domain, fails to inhibit histone acetylation (Fig. 1*A* and Ref. 2). Cotransfection with Set/TAF-Iβ (Fig. 1, *B* and *C*), but not the mutant Set/TAF-Iβ 120–225 plasmid (Fig. 1*C*), attenuated GFP expression to a level that is only slightly above the basal value obtained in the absence of TSA (Fig. 1*B*), in a dose-dependent manner (Fig. 1, *C* and *D*), establishing a role for the INHAT activity of Set/TAF-Iβ in regulating GFP expression.

INHAT Subunits Inhibit Demethylation of Ectopically Methylated CMV-GFP Transiently Transfected into HEK293 Cells in a Dose-dependent Manner—Since different subunits of the INHAT complex inhibited expression of CMV-GFP, we determined their effect on DNA demethylation induced by TSA (1). DNA was isolated from HEK293 cells cotransfected with methylated GFP DNA and Set/TAF-Iβ, pp32, or Set/TAF-Iβ 120–225 mutant and was treated with 0.3 μM TSA for 72 h. A map of the analyzed GFP gene is presented in Fig. 2*A*. DNA was first linearized with the *Eco*RI restriction enzyme, followed by digestion with *Msp*I (which cleaves the sequence CCGG) or *Hpa*II (which cleaves the sequence CCGG only when it is not methylated). The results of this experiment demonstrate that histone deacetylase inhibitor (TSA) treatment alone results in nearly complete demethylation (90%) as indicated by the detection of a fully demethylated *Hpa*II-digested fragment (529 kb) as shown previously (Fig. 2*B*, *lane 3* and *graph bar 1*) (1). Remarkably, addition of individual INHAT subunits Set/TAF-Iβ and pp32, which block histone acetylation, inhibit demethylation of the reporter gene, as indicated by the 9-fold reduction in the relative abundance of the *Hpa*II-digested fragment with Set/TAF1β (Fig. 2*B*, compare *lane 3* with *lanes 6* and *9*, and *graph bar 2 versus bar 1*). The Set/TAF-Iβ 120–225 mutant, which does not inhibit histone acetylation, had a minimal effect (2-fold) on blocking DNA demethylation (Fig. 2*B*, compare *lane 12* with *lane 6*, and *graph bar 3*). There is a dose-response relationship between demethylation and inhibition of histone acetylation. The relative abundance of the fully demethylated 0.529-kb *Hpa*II fragment was reduced from 95% (with TSA alone) to nearly undetectable levels (6%) with increasing doses of Set/TAF-Iβ (Fig. 2*C*, *lanes 6*, *9*, *12*, *15*, *18*, and *graph open circles*), but not with the Set/TAF-Iβ 120–225 mutant (Fig. 2*C*, *lanes 21*, *24*, *27*, *30*, and *graph closed squares*). In summary, these experiments demonstrate that INHAT subunits inhibit demethylation of ectopically methylated DNA in a dose-dependent manner.

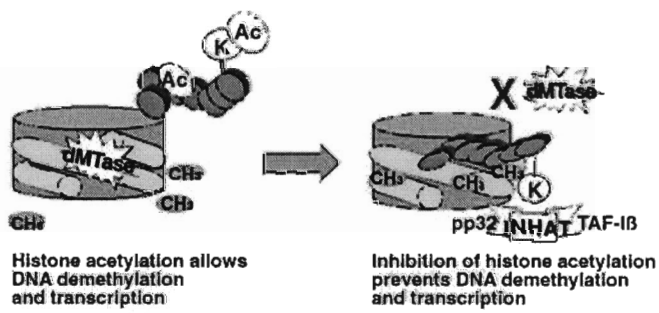

FIG. 5. **Model illustrating how endogenous proteins such as demethylase can access and demethylate DNA within the nucleosome when histone tails are acetylated.** INHATs' ability to mask histones and therefore prevent histone acetylation inhibit the association between demethylase and the nucleosome-wrapped DNA. The resulting phenotype is hypermethylated DNA.

INHAT Subunit Set/TAF-Iβ Inhibits Demethylation by Directly Interacting with the Chromatin Associated with It—Set/TAF-Iβ might inhibit demethylation of CMV-GFP DNA indirectly by inducing other activities required for demethylation or they might act directly on CMV-GFP by binding to its chromatin and preventing its acetylation and, as a consequence, its demethylation. If Set/TAF-Iβ acts on histones associated with the target DNA, then CMV-GFP DNA associated with Set/TAF-Iβ

should remain hypermethylated, whereas DNAs that escaped Set/TAF-Iβ binding should be associated with acetylated histones and hypomethylated (1). To address this issue we used the CHIP assay with anti-acetyl-histone H3 and anti-Set/TAF-Iβ antibodies on cells cotransfected with methylated GFP and 1 μg of Set/TAF-Iβ (a concentration of Set/TAF-Iβ that results in partial inhibition of demethylation as shown in Fig. 2*C*) and treated with TSA (Fig. 3). The results presented in Fig. 3*A* show that the CMV-GFP gene sequence was immunoprecipitated with both the anti-acetylhistone H3 and anti-Set/TAF-Iβ antibodies but not with preimmune rabbit serum. GFP plasmid DNAs isolated from both acetylhistone H3 and Set/TAF-Iβ-immunoprecipitated samples were then subjected to bisulfite mapping to determine their respective methylation patterns (Fig. 3*B*). GFP DNA isolated before immunoprecipitation (*Input*) was partially demethylated as anticipated (Fig. 2*C*). All DNAs immunoprecipitated with acetylhistone H3 antibody displayed almost complete demethylation (Fig. 3, *B* and *C*). Remarkably, however, DNAs immunoprecipitated with anti-Set/TAF-Iβ antibodies appeared completely hypermethylated except for one demethylated clone. This result shows that Set/TAF-Iβ associates with methylated transfected CMV-GFP chromatin and facilitates the maintenance of the methylated status.

Set/TAF-1β Is Overexpressed in Multiple Tumors—To test the hypothesis that the level of expression of INHATs might vary under pathological conditions where aberrations in chromatin acetylation and DNA methylation are commonly observed, we used a cDNA expression array (Atlas™) containing mRNA from various tumors and their respective non-tumor tissues (Fig. 4). The membrane was hybridized with full-length ^{32}P-labeled Set/TAF-Iβ oncoprotein cDNA and quantified by densitometry. From the multiple samples assayed for each tissue type, an increased expression of Set/TAF-Iβ in certain tumor tissues relative to normal tissues was observed (Fig. 4, *A–F*). The tissue averages from uterus, colon, stomach, and rectum displayed a 2-fold or greater increase in Set/TAF-Iβ expression in tumor tissues compared with normal tissues (Fig. 4*G*).

DISCUSSION

This paper demonstrates that a chromatin-modifying protein can determine the state of methylation of certain ectopically methylated sequences by inhibiting DNA demethylation. There are several possibilities by which INHAT subunits may regulate DNA methylation. First Set/TAF-Iβ, by virtue of its ability to inhibit histone acetylation, maintains target DNAs in hypoacetylated form. Since histone hyperacetylation is necessary for DNA demethylation of ectopically methylated DNA (1), Set/TAF-Iβ-targeted DNAs remain hypermethylated. Second, the histone binding and HAT inhibitory functions of Set/TAF-Iβ are not necessary for its role in the maintenance of DNA hypermethylation. However, the greatly reduced ability of the Set/TAF-Iβ mutant (defective in histone binding and INHAT functions) to protect methylated DNA as compared with the wild type Set/TAF-Iβ eliminates this possibility. Third, INHAT functions by directly recognizing methylated DNAs and protecting them from being targeted by DNA demethylases, which is supported by the fact that the Set/TAF-Iβ mutant still retains a minor ability to inhibit demethylation. Finally INHAT may function by both binding to methylated DNAs and preventing histone acetylation. While the last two remain formal possibilities and are testable, based on our results we suggest a model depicted in Fig. 5 directly linking HAT-inhibitory property, INHAT, in the establishment of an inverse relationship between histone acetylation and DNA methylation in gene silencing. Based on our previous results and the results shown here, we propose that the balance of demethylase and INHATs in cells may therefore determine the final DNA methylation pattern. Histone hypoacetylation due to an elevation in the cellular concentration of factors such as INHATs (Fig. 4*G*) may prevent access of demethylases to chromatin, therefore preserving the existing ectopic hypermethylation.

This paper identifies for the first time a potential mechanism of inhibition of active demethylation and further supports our previous hypothesis that cancer cells bear a level of demethylase activity that demethylates ectopically methylated sequences. Further extensive experiments are obviously required to test whether such a mechanism participates in precipitating the hypermethylated state of specific tumor suppressor genes. An obvious question that has to be addressed is how could inhibitors of demethylation cause hypermethylation of normally unmethylated genes such as tumor suppressors. We would like to speculate that spurious methylation events do occur during normal replication and that they are normally removed from transcribed genes that are associated with hyperacetylated histones by demethylase(s). Interaction of proteins such as Set/TAF-Iβ with histones associated with a gene lead to inhibition of acetylation, and as a consequence corrective demethylation is inhibited. This can lead to progressive hypermethylation of a gene.

In summary, this paper demonstrates that a protein that inhibits histone acetylation also blocks demethylation as well as expression of ectopically methylated DNA. This is to our knowledge the first example of a class of proteins shown to affect a methylation pattern by inhibiting demethylation. These data redirect our understanding of how DNA methylation patterns are generated, maintained, and pathologically altered in cancer and provide novel potential targets for regulation of aberrant methylation patterns, such as those found in tumors or transformed cells.

While our experiments suggest that Set/TAF-Iβ could potentially affect DNA methylation states by inhibiting demethylation, further experiments will be necessary to demonstrate that hypermethylation of specific tumor suppressor genes involves interaction with proteins like Set/TAF-Iβ. We are currently searching for endogenous genes targeted by INHATs, to test our model and confirm the results of our transient system. Other important questions that have to be resolved are what are the mechanisms responsible for up-regulation of Set/TAF-Iβ during tumorigenesis and what may target Set/TAF-Iβ to certain genes and not others? Nevertheless, our data illustrates some of the first principles integrating the process of demethylation, histone hypoacetylation, and DNA hypermethylation in the establishment of silent chromatin loci.

REFERENCES

1. Cervoni, N., and Szyf, M. (2001) *J. Biol. Chem.* **276,** 40778–40787
2. Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) *Cell* **104,** 119–130
3. Razin, A., and Cedar, H. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2725–2728
4. Razin, A. (1998) *EMBO J.* **17,** 4905–4908
5. Szyf, M., Kaplan, F., Mann, V., Giloh, H., Kedar, E., and Razin, A. (1985) *J. Biol. Chem.* **260,** 8653–8656
6. Selker, E. U. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9430–9435
7. Tamaru, H., and Selker, E. U. (2001) *Nature* **414,** 277–283
8. Di Croce, L., Raker, V. A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S., and Pelicci, P. G. (2002) *Science* **295,** 1079–1082
9. Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6107–6112
10. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999) *Nat. Genet.* **21,** 103–107
11. Chen, H., Tini, M., and Evans, R. M. (2001) *Curr. Opin. Cell Biol.* **13,** 218–224
12. Jenuwein, T., and Allis, C. D. (2001) *Science* **293,** 1074–1080
13. Rouleau, J., Tanigawa, G., and Szyf, M. (1992) *J. Biol. Chem.* **267,** 7368–7377
14. Nur, I., Szyf, M., Razin, A., Glaser, G., Rottem, S., and Razin, S. (1985) *J. Bacteriol.* **164,** 19–24
15. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) *Nucleic Acids Res.* **22,** 2990–2997
16. Crane-Robinson, C., Myers, F. A., Hebbes, T. R., Clayton, A. L., and Thorne, A. W. (1999) *Methods Enzymol.* **304,** 533–547
17. Nagata, K., Saito, S., Okuwaki, M., Kawase, H., Furuya, A., Kusano, A., Hanai, N., Okuda, A., and Kikuchi, A. (1998) *Exp. Cell Res.* **240,** 274–281