NOTE TO USERS

Regulation of the epigenome and its implications in cancer therapy

By

Snezana Milutinovic

Department of Pharmacology and Therapeutics

McGill University, Montreal

August 2004

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

Your file Votre référence
ISBN: 0-494-12911-5
Our file Notre référence
ISBN: 0-494-12911-5

Dedicated with love to my son Matia, my husband Ljubomir,
and my parents, Mirjana and Milivoje

Abstract

The regulation of the genome by the epigenetic modifications of DNA methylation and histone modification is increasingly recognized as a vital factor in the development, physiology and pathology of vertebrates. There is mounting evidence suggesting that both aberrant DNA methylation and histone modifications are common events in cancer. This has lead to the establishment of both DNMTs and HDACs as important targets in cancer therapy. There are currently several clinical trials that are testing inhibitors of both DNMTs and HDACs as anticancer agents. This thesis attempts to understand the roles that DNMT1 and HDAC1 play in the regulation of gene expression and epigenomic inheritance. In the first chapter, we examined the effects of DNMT1 inhibitors on gene expression and found that DNMT1 can regulate gene expression independent of its DNA methyltransferase activity. This novel role of DNMT1 has challenged a widely accepted theory that the mechanism of DNMT1 inhibitors involves inhibition of the catalytic activity of DNMT1, thus leading to demethylation and reexpression of tumor suppressors previously silenced by methylation. In chapter 2, we further examined different roles of DNMT1. We showed that different DNMT1 inhibitors inhibit different DNMT1 functions and produce different effects on gene expression. Our data suggests that inhibition of DNMT1 enzymatic activity can produce serious long-term effects as a result of massive non-selective demethylation of the genome. In contrast, reduction of DNMT1 levels was shown to result in a rapid arrest of cell growth, limited demethylation and induction of stress-response genes. We hypothesize that these effects are a result of the activation of an epigenetic check point that has evolved to protect the cell from undergoing replication in the absence of DNMT1. In chapter 3, we further explore the roles of DNMT1 in methylation independent regulation of gene expression, which has been suggested to involve recruitment of histone modifying proteins. However, we show that DNMT1 can regulate gene expression in both DNA methylation and histone modification independent manner. In chapter 4, we examine the role of PCNA in the coordination of genome and epigenome replication. We show that in addition to

ensuring concurrent DNA synthesis, DNA methylation and chromatin assembly, PCNA also recruits HDAC1 activity. Our results suggest a mechanism for the inheritance of histone modification. Taken together, our results have uncovered novel roles of the epigenetic proteins DNMT1 and HDAC1 and have raised important issues regarding the targeting of different functions of these proteins in cancer therapy.

Résumé

La régulation du génome par le biais de modifications épigénétiques telles que la méthylation de l'ADN et la modification des histones est reconnue de plus en plus comme un facteur essentiel au développement, à la physiologie et aux pathologies des Vertébrés. De nombreux travaux décrivent une méthylation aberrante de l'ADN et/ou une modification des histones déficiente comme des événements fréquents dans les cas de cancer. Ceci a conduit légitimement à considérer les DNMTs et les HDACs comme des cibles de choix en vue de thérapies anticancéreuses. D'ailleurs, plusieurs essais cliniques mesurant les effets anticancéreux d'inhibiteurs des DNMTs et HDACs, sont actuellement en cours. Cette thèse tente d'évaluer la part prise par DNMT1 et HDAC1 dans la régulation de l'expression génique et de l'hérédité épigénomique. Au sein du premier chapitre, nous nous sommes penchés sur les effets des inhibiteurs de DNMT1 sur l'expression génique et nous avons découvert, par ce biais, que DNMT1 régule celle-ci mais curieusement indépendemment de son activité ADN méthyltransférase. Toutefois, ce nouveau rôle joué par DNMT1 va à l'encontre d'une théorie largement acceptée selon laquelle le mécanisme d'action des inhibiteurs de DNMT1 implique l'inhibition de son activité catalytique, laquelle conduit ainsi à une déméthylation et à une réexpression de gènes suppresseurs de tumeurs préalablement mis sous silence par méthylation. Dans le chapitre 2, nous nous sommes plus profondément intéressés aux différents rôles joués par DNMT1. Nous montrons ainsi que chaque type d'inhibiteurs de DNMT1 agit au niveau de ses différentes fonctions et engendre de nombreux effets sur l'expression génique. De plus, nos données suggèrent que l'inhibition de l'activité enzymatique de DNMT1 produit des effets à long terme tels qu'une déméthylation massive et non sélective du génome. Au contraire, la réduction du niveau de DNMT1 dédié aux fonctions cellulaires entraine un arrêt rapide de la croissance cellulaire, une déméthylation modérée et une activation de gènes de réponse au stress. Nous émettons l'hypothèse que ces effets soient en réalité le résultat de l'activation d'un point de contrôle épigénétique ayant évolué dans le but de protéger la cellule d'une réplication en absence de DNMT1. Au sein du chapitre 3, nous explorons plus en avant les rôles de DNMT1 dans l'expression génique méthylation-indépendante, laquelle implique le

recrutement de protéines modificatrices d'histones. Nous montrons que DNMT1 module l'expression génique de façon indépendante à la fois de la méthylation de l'ADN mais aussi de la modification des histones. Dans le chapitre 4, nous examinons enfin le rôle de PCNA dans la coordination de la réplication de l'ADN et celle de l'épigénome. Nous montrons qu'en plus d'assurer la synchronisation de la synthèse de l'ADN, de sa méthylation et de l'assemblage de la chromatine, PCNA recrute également l'activité HDAC1. Nos résultats laissent entrevoir un nouveau rôle pour HDAC1 dans la transmission fidèle des modifications des histones au travers de la division cellulaire. L'ensemble de nos travaux dévoile de nouveaux rôles pour les protéines DNMT1 et HDAC1 et soulève d'importantes questions quant au ciblage de leurs différentes fonctions en vue de thérapies anti-cancéreuses.

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Acknowledgements

My most sincere gratitude goes to my supervisor Dr. Moshe Szyf for being an extraordinary teacher, a powerful inspirational force, a source of constant energy and brilliant ideas, and above all a caring supervisor ensuring the wellbeing of his students. I am especially grateful for his understanding and tolerance during the difficult times of my pregnancy and motherhood. I feel truly lucky for having the opportunity to be educated in this exceptional environment where fun and learning always go together.

I would like to extend my gratitude to my advisor Dr. Brian Collier and my former supervisor Dr. Paul Clarke for their advice and support during difficult times, their endless efforts with writing reference letters, and last but not the least their daily wit and humor.

I would also like to thank Dr. Alfredo Ribeiro-Da-Silva for his advices and help with the confocal microscopy.

My very special thanks goes to Johanne Theberge who is the foundation of this lab that stands still as the new students come in chaos and take years until they come to appreciate all her work and dedication. Thank you for your superior organizational and experimental help, and most of all your patience and tolerance during all these years.

I would also like to thank all former and present lab members for creating such inspiring and amusing environment that made the long hours and weekend work often seem like a privilege rather then a requirement. The mix of such diverse cultures, personalities, interests and discussion topics is what made this experience particularly rewarding. I am thankful for the friendship and collaborations of Nadia Cervoni, Rebecca Grant, Magalie Rocheville, David Knox, Felipe Araujo, Nancy Detich, Ian Weaver, Gula Sadvakassova, Sergiy Dymov, Alexander Unterberger, Steve Andrews, Jerome Torrisani, Stefan Hamm, Ana Catalina D'Allesio, Drew Slack, Paul Campbell,

Jing-Ni Ou, Shelley Brown, Sachin Tendulkar, Anagha Tendulkar, Veronica Bovenzi, Qiangli Zhuang, Huiping Ren, Sanjoy Bhattacharya, Shyam Ramchandani.

A special thanks goes to Jerome Torrisani for the time and effort that he put into translating my abstract into French.

I would also like to thank Pamalla Moore, Helene Duplessis, Mona Azar and Tina Tremblay for their kindness and excellent administrative assistance.

I would also like to thank McGill University for McGill Majors/Philip Baily Award (2001-2002), Alex Dworkin Scholarship in Oncology (2000-2001) and Faculty of Medicine Internal Studentship (1999-2000) for the support during my Ph.D. training.

I am most thankful to my parents, Mirjana and Milivoje, who made such a great sacrifice of leaving their lives in Serbia to come to a new continent and give my brother and me an opportunity to live in a peaceful and prosperous country. Their courage, love and support is what enabled me to complete this degree. Without my mother's unconditional help with taking care of my son, earning this degree may not have been possible.

My final thanks goes to my son Matia for enduring nine months in this laboratory and for providing me with the reason and inspiration to focus and finish this degree. My husband, Ljubomir, deserves an equal thank you for being a source of unwavering love, friendship and encouragement.

Statement of Contributions

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

Chapter 1: DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21(WAF1/CIP1/sdi1).

Snezana Milutinovic, J. David Knox and Moshe Szyf.

Figure 1. All the experiments were done and quantified by Snezana Milutinovic.

Figure 2. The p21 western blots were done by Snezana Milutinovic; DNMT1 western blot was done by J. David Knox.

Figure 3. All the experiments were done and quantified by Snezana Milutinovic.

Figure 4. All the experiments and mapping was done by Snezana Milutinovic.

Figure 5. All the experiments were done by Snezana Milutinovic.

Figure 6. All the experiments were done by Snezana Milutinovic.

Figure 7. All the experiments were done by Snezana Milutinovic.

The text of the manuscript was prepared by Moshe Szyf and Snezana Milutinovic.

Chapter 2: Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes.

Snezana Milutinovic*, Qianli Zhuang*, Alain Niveleau and Moshe Szyf.

* Authors made equal contribution to this work.

Figure 1A, E: The sequence alignment and the DNMT1 western was done by Snezana Milutinovic.

Figure 1B, C, D: The DNMT activity assay, DNMT1 RT and its quantification was done by Qianli Zhuang.

Figure 2A, C: The thymidine incorporation and FACS analysis was performed by Qianli Zhuang.

Figure 2B: The thymidine incorporation was done by Snezana Milutinovic.

Figure 3: All the experiments were done by Qianli Zhuang.

Figure 4: All the experiments were done by Qianli Zhuang.

Figure 5: All the experiments were done by Qianli Zhuang.

Figure 6. All the experiments were done by Qianli Zhuang.

Table 1: The data presented in this table was obtained by Snezana Milutinovic.

Figure 7: All the experiments were done by Snezana Milutinovic.

Table 2: The data presented in this table was obtained by Qianli Zhuang.

Figure 8: A couple of PCRs were done by Gula Sadvakassova. The rest of the experiments and graphing was done by Snezana Milutinovic and Qianli Zhuang.

The text of the manuscript was prepared by Moshe Szyf and Snezana Milutinovic.

Chapter 3: DNA Methyltransferase 1 Knock Down Induces Gene Expression by a Mechanism Independent of DNA Methylation and Histone Deacetylation

Snezana Milutinovic, Shelley E. Brown, Qianli Zhuang, and Moshe Szyf

Figure 1: All the experiments were done by Snezana Milutinovic.

Figure 2: All the experiments were done by Snezana Milutinovic.

Figure 3A: The mapping of the BIK promoter was done by Snezana Milutinovic.

Figure 3B: The bisulfite mapping experiments were done by Shelley E. Brown.

Figure 4: All the experiments were done by Snezana Milutinovic.

Figure 5: All the experiments were done by Snezana Milutinovic.

Figure 6: All the experiments were done by Snezana Milutinovic.

Figure 7: All the experiments were done by Snezana Milutinovic.

Figure 8: All the experiments were done by Snezana Milutinovic.

Figure 9: All the experiments were done by Snezana Milutinovic.

Figure 10: All the experiments were done by Snezana Milutinovic.

The text of the manuscript was prepared by Moshe Szyf and Snezana Milutinovic

Chapter 4: Proliferating Cell Nuclear Antigen Associates with Histone Deacetylase Activity, Integrating DNA Replication and Chromatin Modification.

Snezana Milutinovic, Qianli Zhuang, and Moshe Szyf.

Figure 1: All the experiments were done by Snezana Milutinovic.

Figure 2: All the experiments were done by Snezana Milutinovic.

Figure 3: All the experiments were done by Snezana Milutinovic.

Figure 4: All the experiments were done by Snezana Milutinovic.

Figure 5: The samples for FACS analysis were prepared by Snezana Milutinovic, and the FACS analysis itself was performed by Qianli Zhuang.

The text of the manuscript was prepared by Moshe Szyf and Snezana Milutinovic

Contributions to original knowledge

In this thesis, I presented the following original results:

1. Inhibition of DNMT1 by antisense oligonucleotides and hairpin inhibitor induce mRNA and protein levels of tumor suppressor *p21*.
2. *p21* promoter is completely unmethylated in A549 cells.
3. Activation of *p21* by DNMT1 antisense and hairpin inhibitor is independent of p53 activation.
4. Inhibition of DNMT1 by antisense results in the induction of transcriptional activity of the *p21* promoter.
5. Inhibition of DNMT1 by antisense oligonucleotide in A549 cells results in the quick and dramatic reduction of the fraction of cells that synthesized DNA.
6. Inhibition of DNMT1 activity by 5-aza-CdR results in a slower and weaker reduction of the fraction of cells that synthesized DNA.
7. Knock down of DNMT1 by antisense results in the intra-S-phase arrest which is overcome after longer treatments.
8. Knock down of DNMT1 by antisense results in limited global DNA demethylation, whereas inhibition of DNMT1 activity by 5-aza-CdR results in extensive global DNA demethylation in both A549 and T24 cells.
9. The demethylation of *p16* promoter is much quicker and stronger following inhibition of DNMT1 activity by 5-aza-CdR then by knock down of DNMT1 protein by antisense oligonucleotides.
10. Gene array analysis of A549 cells following inhibition of DNMT1 activity by 5-aza-CdR reveals the induction of genes known to be silenced by methylation.
11. Gene array analysis of A549 cells following DNMT1 knock down with antisense reveals the induction of stress responsive genes.
12. Knock down of DNMT1 by antisense induces stress response genes *p21*, *BIK*, and *HSPA2* in a methylation and histone modification independent manner.
13. Knock down of DNMT1 induces Sp1 responsive promoters in a methylation independent manner.

14. Sp1 and Sp3 proteins occupy *p21*, *BIK* and 4xSp1 promoters in A549 cells and their occupancy does not change following knock down of DNMT1 by antisense oligonucleotides.
15. An Sp1 site within the *p21* promoter is identified that is essential for induction of this promoter by DNMT1 knock down
16. HDAC1 and PCNA interact in A549, HEK and MRHF cells.
17. The PCNA-interacting domain is identified within HDAC1, and is found to be different from previously identified DNMT1-interacting domain.
18. PCNA and HDAC1 colocalize in the nuclei of A549 as determined by immunostaining.
19. PCNA associates with HDAC activity in HEK293 cells, and this HDAC activity is sensitive to inhibition by Trichostatin A.
20. Trichostatin A arrests A549 cells at the G2/M phase of the cell cycle.

List of abbreviations:

DNMT - DNA methyltransferase

HDAC - histone deacetylase

HAT - histone acetyltransferase

PCNA - proliferating cell nuclear antigen

HP1 - heterochromatin protein 1

CAF-1 - chromatin assembly factor-1

TSA - Trichostatin A

AdoMet - S-adenosylmethionine

AdoHcy - S-adenosylhomocysteine

COMT - catechol-O-methyltransferase

Rb - retinoblastoma protein

CG/CpG - cytosine and guanine

5-MCDG - 5-methylcytosine DNA glycosylase

MBD - methyl-CpG binding domain

5-aza – 5-azacytidine

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

MeCP - methyl-CpG-binding protein

NuRD - nucleosome remodeling and deacetylation

ATR-X - alpha-thalassemia mental retardation

RLGS - Restriction landmark genomic scanning

APC - adenomatous polyposis coli

EBV - Epstein Barr Virus

SV40 - simian virus 40

UTR - untranslated region

dMTase – demethylase

GST - glutathione *S*-transferase

FACS - fluorescence-activated cell-sorting

BrdUrd – thymidine analog bromodeoxyuridine

GENERAL INTRODUCTION

I. Goal of the thesis and literature review

Epigenetics is emerging as one of the key areas of biological research investigating the plasticity of the eukaryotic genome. One of the most intriguing processes in biology is the way by which complex eukaryotic organisms store the vast amount of DNA into their nucleus, which enables launching of multiple fine tuned gene expression programs and creating a diversity of cell types. While the genome is fixed in all cells of a multicellular organism, its expression is programmed by the epigenome, which consists of chromatin structure and DNA methylation. The genome is mainly compartmentalized into transcriptionally competent euchromatin and transcriptionally incompetent heterochromatin. This is achieved by packaging DNA into nucleosomes, which associate with and are modified by a wide variety of protein complexes. In addition, the DNA itself is covalently modified by DNA methylation through the activity of a family of DNA methyltransferase enzymes (DNMTs). Cell specific DNA methylation patterns are established during development and it was long believed that they remain fixed throughout life. However, recent data suggests that both chromatin and DNA methylation are dynamic and work hand in hand to modulate gene expression in response to physiological, environmental and pathological cues. Indeed, it is well established that chromatin and DNA methylation are tightly correlated, such that active chromatin associates with unmethylated DNA accessible to the transcriptional machinery, while inactive chromatin associates with hypermethylated DNA. Although it is unclear which of the two components of the epigenome, chromatin modification and DNA methylation, is the primary cause of switches in gene expression, they have been shown to bind and complement each other. For example, histone deacetylase inhibitors were shown to induce DNA demethylation while chromatin repressor complexes were shown to recruit DNA methyltransferases. Similarly, DNA methylation silences gene expression and causes histone deacetylation. Although the epigenome is dynamic there has to be a balance between the stability and plasticity to maintain identity within cell lineages. How is the

stability of the epigenome maintained in distinct cell types? This is achieved by replicating the genome and the epigenome concurrently in the S-phase of the cell cycle thus ensuring that the DNA is properly methylated and packaged into chromatin in the daughter cells. Any loss of either genetic or epigenetic information would be disastrous to a cell resulting in death or abnormal function, leading ultimately to cancer or other diseases. One of the main topics of this thesis will deal with the replication of the epigenome and the protective mechanisms that sense problems associated with it.

Three changes in the DNA methylation and its machinery are a hallmark of cancer: 1. Deregulated expression of DNA methyltransferases (DNMTs). 2. Regional hypermethylation of tumor suppressor genes. 3. Global hypomethylation. Regional hypermethylation has attracted most of the attention in the last few years. It has been assumed that the increased DNMT1 in cancer causes hypermethylation and silencing of tumor suppressor genes. Currently, several programs in a number of pharmaceutical companies are developing anticancer drugs which target DNA methylation. Since demethylation induced by such drugs might induce metastasis it is important to determine whether inhibition of DNA methylation is an appropriate goal. Although it has been demonstrated that increased DNA methyltransferase is prevalent in cancer and that forced expression of DNA methyltransferase can transform cells, it is still possible that DNA methyltransferase causes transformation by DNA methylation independent mechanism. If DNMT1 causes cancer by a DNA methylation independent mechanism it would be proper to inhibit these mechanisms rather than DNA methylation and thus avoid the potential adverse effects of demethylation. It is therefore of great importance to understand how DNMT1 causes transformation. The majority of this thesis will deal with the methylation independent regulation of gene expression by DNMT1 and its implications on the design of the inhibitors of DNMTs as anticancer agents.

The literature review will focus in detail on the topics that will provide a better understanding of the rationale behind the studies of the thesis and will aid in the interpretation of the obtained results.

II. Chromatin and its roles

(i) Chromatin structure and function

The genetic information is organized within the eukaryotic cell nucleus in a highly conserved structural polymer, termed chromatin. The basic repeating unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped approximately two turns around an octamer of two of each core histone proteins, H2A, H2B, H3, and H4. This packaging creates 10 nm fibers, which resemble beads on a string when viewed under the electron microscope in denaturing conditions. The linker histones of the H1 class associate with DNA between single nucleosomes and further organize chromatin into 30 nm helical fibers. The more complex packaging of chromatin into higher order structures is less clear, but it is believed that its folding and unfolding have a major impact on genomic function and gene activity (Fischle et al., 2003). Based on the level of its compactness, chromatin is broadly divided into two categories: open and accessible 'euchromatin', associated with active genes, and condensed and inactive 'heterochromatin', associated with inactive genes. The term 'heterochromatin' was originally used by Emil Heitz in 1928 to describe the type of chromatin that stained densely with carmine acetic acid throughout the cell cycle in several different species of moss (Heitz, 1928). Since then, the definition of heterochromatin has been expanded to include a broader set of characteristics. The type of chromatin that is visibly condensed during the interphase is typically gene-poor, consisting mainly of repetitive DNA, such as satellite sequences, transposable elements and retroviruses. This chromatin is termed constitutive heterochromatin and is associated with highly condensed telomeres and pericentromeric regions. On the other hand, facultative heterochromatin is defined as developmentally regulated chromatin capable of undergoing transition between the heterochromatic and euchromatic structure. This type of chromatin is associated with genes that are silenced at certain times, but need to be activated at other times during development or in response to different cellular signals (Dillon and Festenstein, 2002).

The degree of chromatin condensation is mainly determined by the strength of the histone-DNA and histone-histone association. Histones are highly conserved proteins that consist of globular domain and a more flexible, amino terminus called 'histone tail'. Histone tails protrude from the nucleosomal core and are rich in positively charged side chains that interact with negatively charged DNA and with other histones. These interactions are regulated through neutralization or reversal of histone tail charges by a number of post-translational modifications. These include acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues. Combination of these covalent marks on single histones, single nucleosomes and nucleosomal domains establish local and global patterns of chromatin modification that are critical for a number of cellular processes such as nuclear organization, centromere function, recombination, repair and gene expression.

(ii) Histone modifications and gene expression

The first association between functional state of chromatin and its histone tail modifications came from the demonstration that transcriptionally active chromatin is particularly enriched in acetylated histones, while the silenced chromatin is hypoacetylated (Jeppesen and Turner, 1993; Pogo et al., 1966). This is consistent with the idea that acetylation neutralizes positive charges of histone tails and weakens their association with negatively charged DNA, rendering the chromatin more open, and the DNA more accessible to the transcriptional machinery. All core histones can be acetylated at several lysine residues, and the level of acetylation of any particular lysine is likely to result from a balance of the activities of two families of enzymes: histone acetyltransferases and deacetylases.

(iii) Histone acetyltransferases (HATs)

Histone acetyltransferases (HATs) can be grouped into at least two classes: type A HATs and type B HATs. While type A HATs are localized in the nuclei and most

likely acetylate nucleosomal histones as well as non-histone proteins, type B HATs can be found in cytoplasmic fractions and are responsible for acetylating newly synthesized histones before their translocation into the nucleus for chromatin assembly (Hasan and Hottiger, 2002). Type A HATs can be further classified into five groups including Gcn5-related GNATs, MYSTs, p300/CBP HATs, general transcription factor HATs such as TAFII250, and nuclear hormone related HATs such as SRC1 and ACTR (Carrozza et al., 2003). Although all HATs catalyze the transfer of the acetyl moiety from acetyl coenzyme A to positively charged amino groups of lysine residues, they exhibit substrate preference for specific lysines of specific histone proteins, which makes them non-redundant in many cellular processes.

(iv) Histone deacetylases (HDACs)

In the opposing deacetylation reaction, histone deacetylases remove the acetyl groups and thereby reestablish the positive charge in the histones. There are two structurally unrelated families of deacetylases that have been evolutionary conserved from prokaryotes to humans and include histone deacetylases (HDACs) and Sir2-like deacetylases, or sirtuins. Although these families catalyze deacetylase reaction by completely different mechanisms, they are both involved in silencing transcription by histone deacetylation, and are also involved in other cellular processes through their activity on non-histone proteins. Since 1996, when the first human deacetylase HDAC1 was cloned (Taunton et al., 1996), eleven human HDACs and their multiple isoforms that share homology in their deacetylase domains have been characterized (Petrie et al., 2003). They are broadly divided into Class I and Class II on the basis of homology to the yeast HDACs Rpd3 and Hda1, respectively. Class I HDACs (HDAC1,2,3 and 8) are nuclear proteins that are generally small in size and ubiquitously expressed. In contrast, Class II HDACs (HDAC4,5,6,7 and 9) are larger and their expression patterns tend to be tissue-specific and regulated by their transport into the nucleus. HDAC11 shares homology with both HDAC classes (Petrie et al., 2003).

(v) HDACs and HATs targeting to promoters

In general, HATs and HDACs do not act on chromatinized histones, unless they are recruited to specific chromatin sites through their interaction with transcription factors or other chromatin associated complexes. This is of crucial importance for achieving differential regulation of different chromatin regions found within the same cellular milieu and exposed to the same global levels of HATs and HDACs. In accordance with this, there are numerous examples of co-activator and co-repressor complexes recruiting HATs and HDACs, respectively. For instance, c-myc and E2F family of transcription factors recruit Gcn-5 containing HAT complexes; Sp1 and Sp3 transcription factors recruit p300 HAT activity; nuclear hormone receptors recruit Tip 60 HAT activity etc. (Carrozza et al., 2003). On the other hand, HDAC1 has been shown to form a repressor complex with YY1 and Rb transcription factors; HDAC4,5,7 and 9 repress MEF family of transcription factors etc. In addition, HDACs are found in large multiunit complexes such as Sin3, NuRD, and CoREST (Grozinger and Schreiber, 2002), while HATs are found in complexes such as SAGA, PCAF, and ADA (Roth et al., 2001). It is interesting to note that both HATs and HDACs can be present in the same multiunit complexes (Yamagoe et al., 2003), or that they can act as switches by competing for the same binding site, as in the case of the transcription factor MEF2 (Grozinger and Schreiber, 2002). Therefore, the transcriptional activity of a particular gene will depend on the openness of its chromatin structure, which will in turn depend on the availability of particular HATs and HDACs, their recruitment by repressors and activators, and specific modifications that influence these interactions.

(vi) Histone codes

Although this model of regulation of gene expression by open and closed chromatin may generally hold true, there is mounting evidence suggesting that this regulation is more complex and involves interplay of many different histone tail modifications. For example, there are around 50 differentially acetylated isoforms of the four core

histones, and with the combinatorial effect of other modifications such as histone methylation, phosphorylation and ubiquitination, they may run into thousands for a single nucleosome. This led to the hypothesis of the existence of 'histone code' that can be 'read and interpreted' by non-histone effector proteins, leading to sequential histone modifications or recruitment of other cellular factors (Strahl and Allis, 2000).

(vii) Histone methylation

Another histone modification that has been implicated as an integral part of the 'histone code,' is histone methylation. Recent intense research efforts have identified that methylation can occur either on lysine (mono-,di-, or trimethylation) or arginine residues (mono-, or dimethylation) and is catalyzed by two groups of enzymes: lysine methyltransferases SUV39, G9A, SET2, and ESET, and arginine methyltransferases PRMT1 and CARM1 (Bannister et al., 2002). It is interesting that lysine methylation can be associated with either active or silenced genes, depending on which particular lysine residue is methylated and to what extent. For instance, methylation of H3 on K9 is associated with repression, whereas K4 of H3 is found in both active and inactive genes when dimethylated, but it is exclusively found in active genes when trimethylated. The finding that the same histone modification can mediate separate, and sometimes opposing effects is in accordance with the model where 'histone code' plays a major role in the regulation of chromatin function (Figure 1.).

(viii) Proteins recognizing specific histone modifications

In agreement with this hypothesis, different protein structures were identified that are capable of recognizing specific histone modifications. For example, protein structure known as bromodomain that binds to acetylated lysine residues of the histone tails is present in many complexes containing HAT or chromatin remodeling activity, such as p300, Gcn-5, P/CAF, and SWI/SNF (Dyson et al., 2001). Similarly, chromodomains recognize methylated lysine residues of histone tails and are found in proteins such as heterochromatin protein 1(HP1), silencing protein Polycomb (Pc), and histone

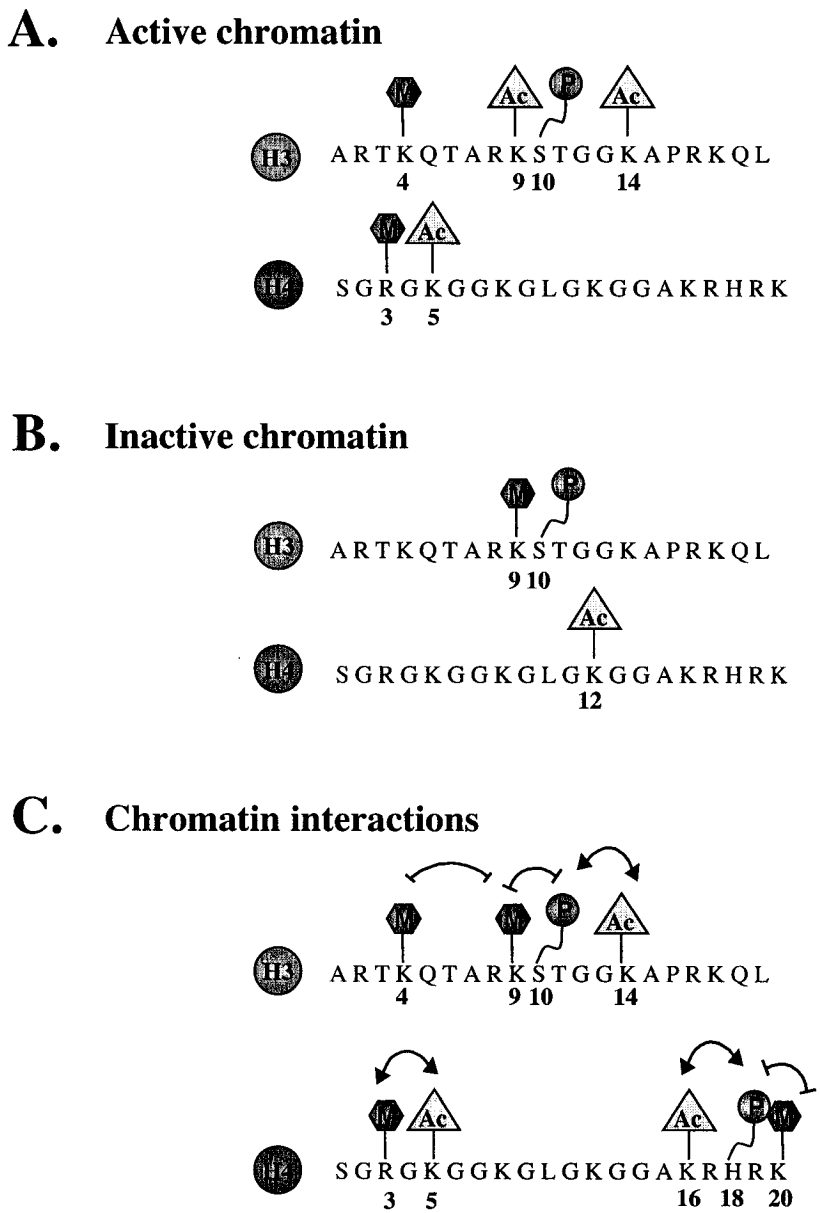


Figure 1. Histone code and gene expression. An example of acetylation (Ac), methylation (M) and phospohorylation (P) modifications on histone H3 and H4 which are associated with transcriptionally active (A.) and inactive (B.) chromatin. C. Examples of synergistic (arrowhead line) and antagonistic (blocked line) interactions that add additional layer of complexity to the histone code.

methyltransferase SUV39H1 (Jones et al., 2000). The presence of such domains is believed to mediate at least some of the epigenetic cross talk responsible for the enhancement of a particular chromatin state in a given gene. The following are some examples of numerous studies showing that different modifications within or between histone tails can mutually affect each other: Methylation of H3-K4 and H3-K9 are mutually exclusive, and they facilitate and inhibit subsequent H3 acetylation, respectively (Wang et al., 2001a); methylation of H4-R3 can promote acetylation of some, but inhibit acetylation of other residues in H4 (Wang et al., 2001b); methylation of H3-K9 interferes with the phosphorylation of H3-S10 (Rea et al., 2000) (Figure 1.). In addition to the local cross talk responsible for the fine-tuning of the expression of specific genes, it is proposed that a similar cross talk may play a role in the control of larger genomic regions, such as euchromatin and heterochromatin, or even in the control of a whole chromosome as in the case of X-inactivation (Fischle et al., 2003).

(ix) The inheritance of epigenetic states

Every time a cell undergoes DNA replication, in addition to faithfully duplicating its genome it also needs to retain proper genomic regulation by faithfully duplicating its chromatin configuration. This entails proper inheritance of the DNA methylation pattern, reassembly and positioning of nucleosomes, and the reestablishment of the histone modification status (Kass and Wolffe, 1998; Krude, 1999). The proliferating cell nuclear antigen (PCNA) serves as a processivity factor for DNA polymerases during DNA replication, and it is also becoming increasingly recognized as an important player in the inheritance of chromatin states. PCNA was shown to linger on the DNA for a short time following DNA replication, and it is believed to act as a loading platform for proteins responsible for the epigenetic inheritance (Shibahara and Stillman, 1999). In accordance, it was shown that the maintenance DNA methyltransferase DNMT1 is recruited to the replication foci through its interaction with PCNA (Chuang et al., 1997), resulting in the concurrent replication and methylation of DNA (Araujo et al., 1998a). The other protein complex that binds to PCNA is chromatin assembly factor 1 (CAF-1), which assembles newly synthesized

histones H3 and H4 onto the replicated DNA (Shibahara and Stillman, 1999). The newly deposited histones are acetylated at the lysine residues, and their deacetylation is known as an essential step in the maturation of chromatin (Annunziato and Seale, 1983; Sobel et al., 1995). In general, transcriptionally active euchromatin is rich in acetylated histones and it is replicated early in the S-phase, while the inactive heterochromatin is hypoacetylated and it tends to be replicated later in S-phase (Sadoni et al., 1999). It was recently proposed that this deacetylation is carried out by HDAC2, which is recruited to the replication foci through its interaction with DNMT1. Since this recruitment was limited to the replication foci of the late S-phase, it was proposed that HDAC2 is the enzyme responsible for the maturation of chromatin through histone deacetylation (Rountree et al., 2000). However, this model is incomplete since it cannot account for the early S-phase deacetylation events, which are probably necessary for the fine-tuning of different euchromatic regions. The data presented in chapter 4 suggests an additional mechanism for the inheritance of the state of histone modifications through the direct interaction of PCNA and HDAC1 (Figure 2.). In addition, this data proposes an additional mechanism for the antitumorigenic effects of the histone deacetylase inhibitor trichostatin A (TSA). TSA has been shown to rapidly activate a number of genes by increasing the acetylation of the histones, resulting in opening of the chromatin structure surrounding these genes (Strahl and Allis, 2000). This mechanism was shown to be responsible for the induction of tumor suppressor genes, such as *p21*, *p14*, *p16* and *gelsolin*, and it was proposed that these genes mediate the anticancer activity of TSA and related HDAC inhibitors. A number of HDAC inhibitors are currently used in clinical trials as anticancer agents in solid tumors and different haematological malignancies (Richon et al., 2001). The data presented in chapter 1 shows that treatment of cancer cells with TSA results in a G2/M arrest of the cell cycle, suggesting the existence of a check point that monitors the state of histone acetylation and ensures that only cells having proper chromatin divide. It is interesting to note that HDAC inhibitors and DNMT inhibitors show synergistic effects on growth arrest in cancer cells. There are a number of examples where this synergism results in activation of tumor suppressors (Zhu and Otterson, 2003). However, data presented in this thesis raises the possibility

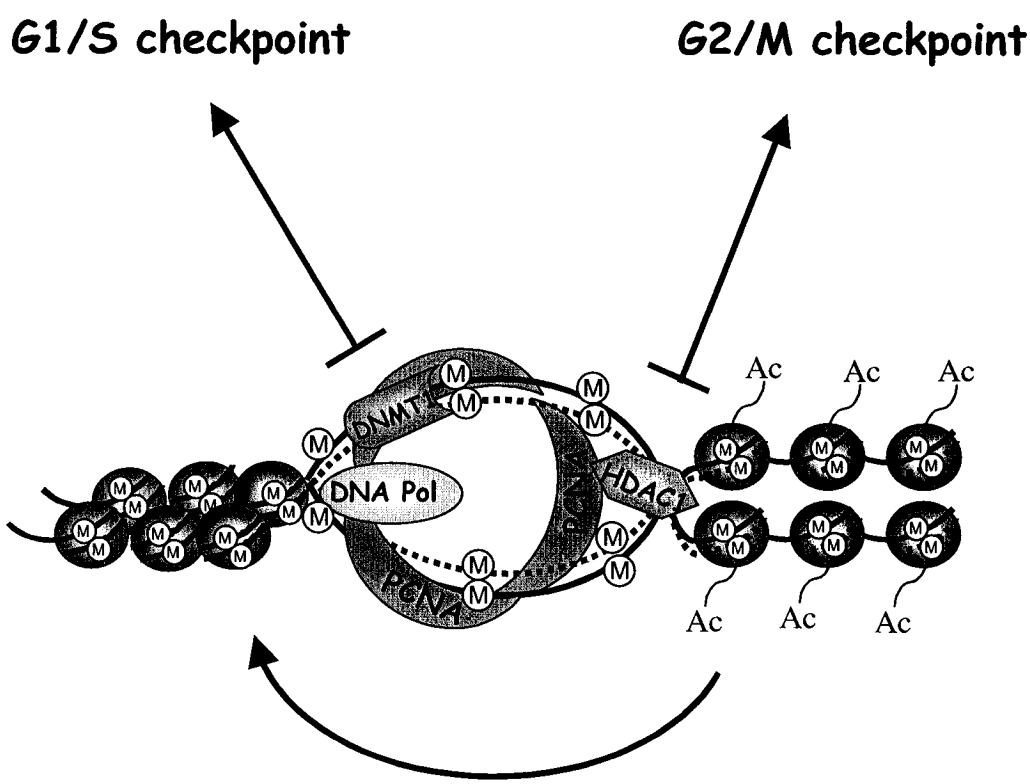


Figure 2. Epigenetic inheritance during replication. PCNA recruits DNMT1 to the replication fork thus ensuring that the methylation pattern is faithfully replicated on the nascent strand. Following replication, DNA is repackaged into chromatin. The newly deposited histones are acetylated and they undergo deacetylation as an essential step in chromatin maturation. We propose that PCNA serves as a loading platform for HDAC1 thus enabling deacetylation of the nascent histones. The depletion of DNMT1 activates the G1/S checkpoint resulting in cell cycle arrest at G1/S. Inhibition of HDAC1 with TSA results in the cell cycle arrest at the G2/M, suggesting that the putative G2/M checkpoint might have been activated.

that this synergism is also due to the activation of two independent check points, one inhibiting cells at the G1 and the other at the G2/M phase of the cell cycle (Figure 2.).

III. General methylation and demethylation reactions

Methylation reactions play a role in a wide variety of metabolic processes ranging from metabolism of xenobiotics and synthesis of neurotransmitters (Vance and Walkey, 1998; Weinshilboum et al., 1999; Zhu, 2002), to the complex functions such as regulation of DNA and chromatin structure (Robertson, 2002). These reactions are catalyzed by different families of methyltransferase enzymes (MTases), which don't share much of the sequence homology except for the functionally conserved structure termed "AdoMet dependent fold". As suggested by the name, S-adenosylmethionine (AdoMet) is used as a donor for the methyl group that is transferred to carbon, nitrogen or oxygen atom of a substrate molecule. Twenty MTases have been structurally characterized so far, and include seven DNA MeTases (DNMTs), four protein MeTases, five RNA MeTases, and four small molecule MeTases. The diversity of methylation substrates is in accordance with its roles in a variety of normal physiological functions as well as different pathological states. This section will concentrate on DNA methylation and will provide only a brief overview of other methylation reactions.

(i) Small and macromolecule methylation

The first methylation reaction was described more than a century ago when Wilhelm His discovered that dogs excreted N-methylpyridine following administration of pyridine (His, 1887). Since then, a wide variety of endogenous substrates, such as small molecules, proteins, RNA and DNA, have been shown to be modified by methylation (Weinshilboum et al., 1999). In addition, exogenous compounds including many drugs have been shown to undergo methylation or demethylation before they are cleared from the body (Weinshilboum et al., 1999). Small molecule methylation is of prime pharmacological interest since it is involved in the

interconversion of many neurotransmitters. For instance, catechol-O-methyltransferase (COMT) methylates the hydroxyl group at the C3 position of the catechol ring (Kopin, 1985). This process is common to a number of catecholamine neurotransmitters, such as dopamine and (nor)epinerphrine, and is responsible for their inactivation at the synapse (Kopin, 1985). Since in Parkinson's disease there is a progressive depletion of dopamine release, its analogue levodopa (L-DOPA) is used as a therapy. Like dopamine, L-DOPA is inactivated by COMT. Two inhibitors of COMT have been developed, entacapone and tolcapone, and are used as adjunct therapy to extend the duration of L-DOPA activity (Inzelberg et al., 2000). S-methylation is involved in the metabolism of many sulphydryl drugs. Thiopurine methyltransferase (TPMT) methylates antineoplastic drug thiopurine (6-mercaptopurine), and the major toxicity factor of this drug is a consequence of a widespread polymorphism of TPMT enzyme (Weinshilboum et al., 1999). Thiol methyltransferase (TMT) methylates anti-rheumatic drug penicillamine and is also a determining step in the depletion of the antihypertensive agent captopril (Weinshilboum et al., 1999). N-methyation is also a contributing factor in drug toxicity. Nicotinamide N-methyltransfererase (NNMT) and histamine N-methyltransferase polymorphisms have been characterized. In addition, demethylation of a number of drugs such as morphine, caffeine and codein is carried out in a relatively non-specific manner by the various p450 enzymes (Kirkwood et al., 1997; Rasmussen et al., 1998).

(ii) RNA methylation

Methylation at 2'OH group of the sugar moiety is one of the major posttranscriptional modifications involved in the maturation of almost all classes of RNAs, and is important for their transport, transcription and stability (Maden, 1990). Although it was shown that tRNA can be methylated by a methyltransferase protein alone, rRNA, snRNA and mRNA require ribonucleoproteins for this activity (Galardi et al., 2002). These are small nucleotide RNAs (snRNA) compelexed with methyltransferase protein and are termed 'snorps'. The snRNA component is responsible for sequence

specificity by base-pairing with the target RNA, while the protein catalyzes the reaction (Decatur and Fournier, 2003). Another important modification of RNA is methylation of 5' ends of the newly synthesized mRNA transcripts. RNA (guanine-7-) methyltransferase is responsible for addition of 7-methylguanosine cap soon after the initiation of transcription. This modification facilitates transcript processing, transport, and turnover (Furuichi and Shatkin, 1989). In addition, this cap structure is recognized by the initiation factor eIF-4E, and is a rate limiting step in the recruitment of ribosome to the translation start site (Marcotrigiano et al., 1997).

(iii) Protein methylation

Recently, methylation has emerged as an extensively studied modification of proteins which occurs on carboxyl groups or side chains of the amino acids lysine, arginine, and histidine (Aletta et al., 1998). These modifications are involved in multitude of cellular processes such as aging and repair of proteins, cellular signaling, stress response and chromatin remodeling. Several fundamental observations indicated that methylation plays an important role in signal transduction, and that this process may be reversible and dynamic, much like regulation of proteins by phosphorylation (Aletta et al., 1998). Methylation of Ras oncogene protein by prenylcysteine carboxyl methyltransferase (pcCMT) is responsible for its targeting to the membrane, and is essential step in epidermal growth factor signaling (Chiu et al., 2004). Ras methylation was proposed to be reversible (Philips et al., 1993). Another protein shown to undergo reversible methylation of the carboxyl group is one of the main cellular phosphatases, PP2A, which is involved in processes such as kinase regulation, DNA replication, growth and transformation. It is interesting to note that, similarly to DNA demethylation reaction (Ramchandani et al., 1999), the removal of methyl group from the PP2A protein results in the release of methanol as a byproduct (Lee et al., 1996). Side chain methylation is also involved in a variety of cellular processes including RNA processing and transport (Lin et al., 2000), transcription and growth factor regulation (Gary and Clarke, 1998; Sommer et al., 1989), and chromatin structure modification (Kouzarides, 2002). Histones, which are the building blocks of

chromatin, are methylated on the side chains of amino acids arginine and lysine (Kouzarides, 2002). Similarly to DNA methylation pattern, methylation and other histone modifications form tissue and gene specific pattern, which is termed 'histone code'. These complex histone modifications provide encoded information necessary for orchestrating numerous DNA-based processes, such as transcription, replication and chromatin organization.

IV. DNA methylation

DNA methyalation occurs in bacteria, fungi, plants and animals, however its role varies widely among different organisms. In bacteria, two bases, adenine and cytosine, can be methylated into N6-methyladenine, and N4-methylcytosine or 5-methylcytosine, respectively. These modifications reside in a specific sequence context of the bacterial genome and are used in combination with the methylation specific restriction endonucleases to distinguish and defend itself from invading bacteriophages. In addition to its function as a primitive immune system, DNA methylation is also involved in a number of other cellular processes in bacteria 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). With the increasing complexity of the higher order organisms, the roles and patterns of DNA methylation become more complex as well. Although in higher eukaryotes the only modified base is 5-methylcytosin its levels and sequence location differ greatly between different organisms. While the unicellular fungus *S. cervisiae* doesn't have DNA methylation, other multicellular fungi such as *Neurospora crassa* and *Aspergillus flavus* contain less than 2% methylation, restricted mainly to transposons and other repeats. Plants however, display up to 40% of methylation in either CNG or CG sequence context, which may be in accordance with their higher need for adaptation to the environment that they cannot escape (Jeltsch, 2002). In mammals, up to 70-90% of all CGs are methylated. Here, methylation is almost exclusively present in the CG dinucleotide context, except for low levels of CA, CT and CC methylation in early embryonic development (Ramsahoye et al., 2000). There are

different theories about the evolution and importance of DNA methylation. Some suggest that the main role of such widespread methylation is to silence selfish genetic elements that have accumulated in the genome over the course of the evolution (Yoder and Bestor, 1998). Other hypothesis suggest that DNA methylation evolved as a mechanism to reduce background transcriptional noise in the more complex organisms containing increasing number of genes (Bird, 1993). More importantly, DNA methylation has been proposed to regulate gene expression, as will be discussed in detail below (section V).

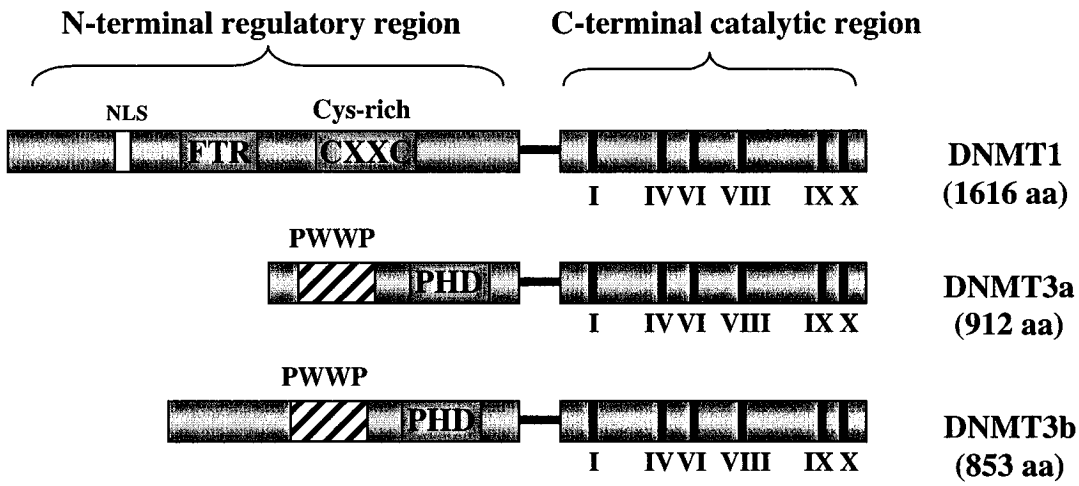
In general, CGs are underrepresented in the genome occurring at ~20% of their expected frequency, and are usually heavily methylated. However, there are regions of the genome where the CG content is 5-10 fold enriched, and these are referred to as CG islands. Genomic analysis has revealed that ~60% of all genes contain CG islands within their regulatory regions, including most housekeeping genes and around half of tissue specific genes (Antequera, 2003). This immediately suggests the involvement of CGs in the gene regulation, such that the promoters of housekeeping genes and genes expressed in a given cell type remain unmethylated and active, while other tissue specific genes become methylated and silenced (Razin and Szyf, 1984). The particular methylation pattern is established during the germ cell and embryo reprogramming through the sequential waves of *de novo* methylation and demethylation in the early development (Reik et al., 2001). Most of these patterns become fixed for life, especially when only one of the two alleles is expressed, as in the case of parentally imprinted genes and X chromosome inactivation. These patterns are faithfully maintained during the cell division by maintenance methylation, but it is becoming evident that they are subject to modification during differentiation, aging and disease. Cancer is the most studied pathological state tightly linked to abnormal changes of the methylation pattern and the machinery responsible for its establishment, maintenance and interpretation. There is a long line of evidence showing involvement of DNMTs in cellular transformation and tumor progression, but in depth discussion of this issue will follow in later sections.

(i) DNA methyltransferase enzymes and their roles

Although the existence of 5-methylcytosine was discovered in calf thymus more than half a century ago (Hotchkiss, 1948), the first murine DNA (5-methylcytosine) methyltransferase (DNMT1) was cloned much later (Bestor, 1988), and it was followed by the cloning of human DNMT1 (Yen et al., 1992). Since then only two other active DNMTs have been identified, DNMT3a and DNMT3b (Figure 3.), and two other homologous proteins whose function is still unclear, DNMT2 and DNMT3L. DNMTs are generally composed of two parts, a diversified amino terminal region and a relatively conserved carboxy terminal region. C-terminal houses AdoMet binding and the catalytic motifs similar to those of the prokaryotic 5-mC methyltransferases. Initial steps of cytosine methylation involve flipping of the base out of the double helix and a nucleophilic attack by the enzyme's catalytic cystein residue on the 6^{th} position of the cytosine carbon ring. This results in an intermediate complex between the enzyme and the base, with an activated 5^{th} position of the cytosine, which further attacks the methyl group of AdoMet. This is followed by the release of the AdoHcy and the dissociation of the enzyme and the base (Jeltsch, 2002) (Figure 4.).

DNMT1 is the most abundant of all DNMTs, and it contains the largest N-terminal regulatory domain responsible for such functions as the substrate specificity (Araujo et al., 2001), allosteric activation by methylated DNA (Bacolla et al., 2001), protein targeting to the replication fork (Leonhardt et al., 1992), and interaction with various proteins such as PCNA (Chuang et al., 1997), HDAC1 and 2 (Fuks et al., 2000; Rountree et al., 2000), Rb and E2F1 (Robertson et al., 2000a). Since DNMT1 has a significant preference for hemimethylated DNA relative to unmethylated DNA, it was assigned the role of maintenance methyltransferase. It is proposed that during DNA replication the newly synthesized non-methylated strand and the methylated parental strand create a preferred substrate for DNMT1, which copies the parental methylation pattern and ensures its proper inheritance during the cell division (Bestor, 1988). The replication of the epigenome along with the genome is of crucial importance for

A.



B.

	DNMT1	DNMT3a	DNMT3b
PCNA	+		
HDAC1	+	+	+
HDAC2	+	+	+
SUV39H1	+	+	+
HP1	+	+	+
Rb	+		
DMAP1	+		
DNMT3a	+		+
DNMT3b	+	+	

	DNMT1	DNMT3a	DNMT3b
Sumoylation enzymes		+	
MeCP2	+		
MBD2	+		
MBD3	+		
PML-RAR	+	+	
hSNF2H			+
RP58		+	

Figure 3. Schematic of the structure of catalytically active DNMTs and the summary of their interacting proteins. A. C-terminal catalytic region and its conserved motifs (black bars) are shown. N-terminal regulatory region containing NLS (nuclear localization signal), FTR (fork targeting region), Cys-rich region (containing CXXC Zn finger domain), PWWP domain (involved in cell growth and differentiation) and PHD domain (Zn finger) are shown for each DNMT. B. The proteins known to interact with each of the DNMTs are shown with crosses.

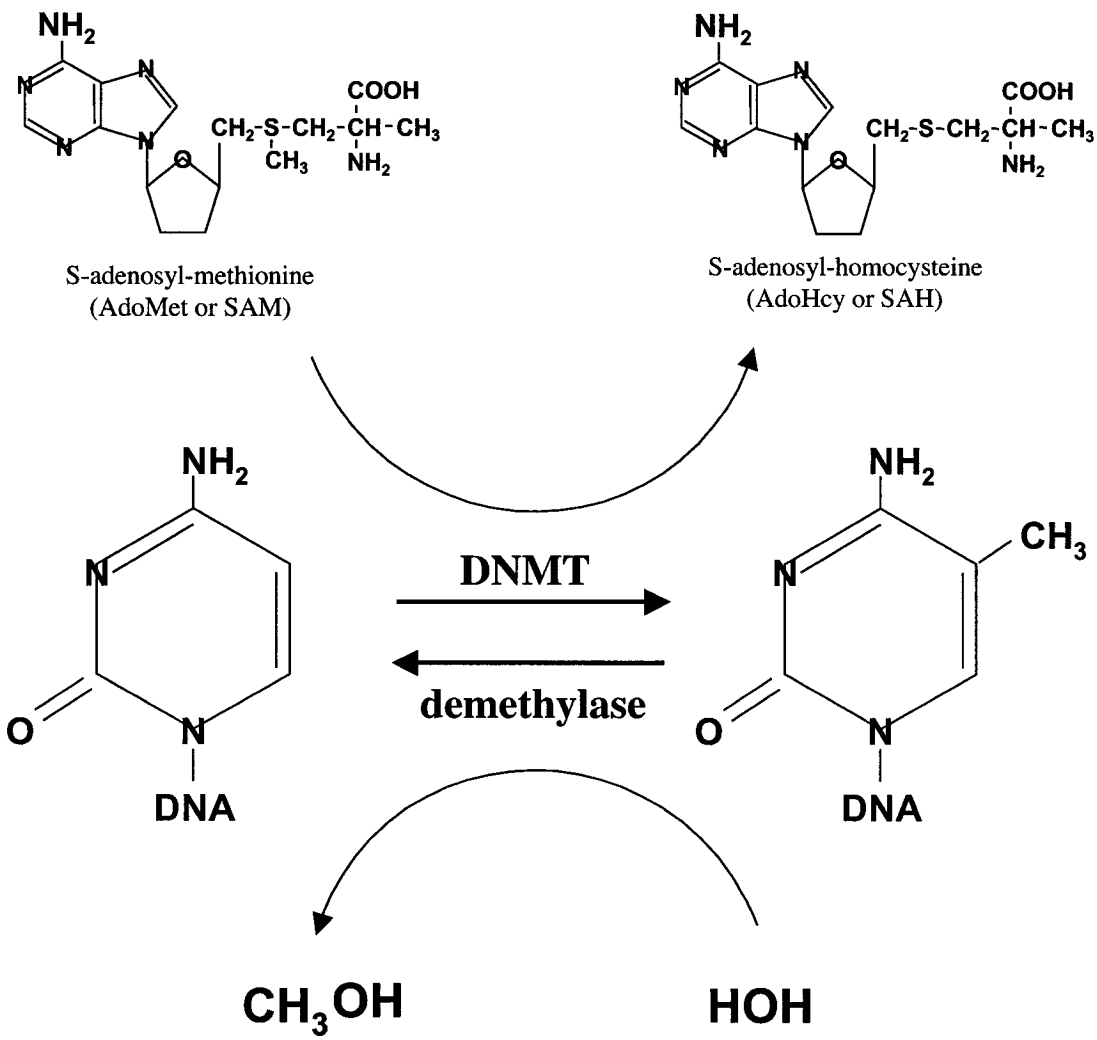


Figure 4. The DNA methylation and demethylation reactions. DNMT catalyzes the transfer of a methyl group from S-adenosyl-methionine (AdoMet or SAM) onto the 5th position of the cytosine ring in the DNA. DNA demethylase catalyzes the hydrolytic cleavage of the bond between the methyl group and the cytosine ring releasing the methyl group in the form of methanol.

cellular integrity, and this issue will be further discussed later in this section. The importance of DNMT1 for methylation and proper development was confirmed by the knockout experiments. It was shown that mouse embryos bearing homozygous mutation of DNMT1 are delayed in development, and do not survive past mid-gestation. At the same time it was shown that embryonic stem cells bearing the same mutation are viable, but similar to mutated embryos, their total levels of 5mC were reduced to around 30% of that of wild type levels (Li et al., 1992). Although these experiments confirmed the importance of DNMT1 in development and maintenance of methylation, they suggested that in addition to DNMT1 there must be other methyltransferase enzymes responsible for the residual methylation observed in the knockout cells.

Further studies using DNMT1 knockout cells suggested that they contain a de novo methyltransferase activity since the integration of the proviral DNA into these cells resulted in its de novo methylation (Lei et al., 1996). These observations led to the continued search for de novo methyltransferases and the discovery of two new mammalian enzymes, DNMT3a and DNMT3b (Okano et al., 1998). Similarly to DNMT1, these two enzymes are composed of catalytic C-terminal region, and a smaller regulatory N-terminal region which was found to mediate various protein-protein interactions (Pradhan and Esteve, 2003). In contrast to DNMT1, these two enzymes lack a domain responsible for substrate preference and show equal affinity for hemi-methylated and unmethylated DNA. In addition, their expression seems to be much higher in ES cells and early embryos than in differentiated somatic cells (Okano et al., 1998). Based on these characteristics DNMT3a and DNMT3b were assigned the role of *de novo* methyltransferases. There are two well-documented instances where such a *de novo* activity is necessary for the reprogramming of the epigenome. The first occurs in the early germ cells development, when there is a genome-wide wave of demethylation, and is followed by *de novo* methylation a few days later (Reik et al., 2001). The second occurs shortly after fertilization, when paternal genome in the egg is almost completely demethylated. This is followed by cleavage divisions where passive demethylation takes place because Dnmt1 is

excluded from the nucleus, and around the time of implantation there is a genome wide reprogramming by *de novo* methylation. Futher support for this hypothesis comes from knockout experiments. It was shown that mice with homozygous mutation of DNMT3a appear normal at birth but die by 4 weeks of age, while there were no viable mice with DNMT3b mutations. The study of the methylation levels revealed that DNMT3a and DNMT3b seem dispensable for maintaining previously established methylation patterns, but are essential for de novo methylation of specific sequences. For instance, DNMT3b was required for methylation of the satellite repeats in the centromeric region. This is in accordance with the observation that people with mutations in DNMT3b gene develop ICF syndrome (immunodeficiency, centromeric instability, and facial anomalies) characterized by the hypomethylation of certain satellite sequences (Hansen et al., 1999).

Although it is widely accepted that DNMT3a and DNMT3b are the enzymes responsible for establishment of the *de novo* methylation pattern, and that the inheritance of this pattern during the multiple cell divisions is achieved by DNMT1 maintenance methyltransferase, there are instances in which this simplified classification of roles may not be true. For example, the overexpression of DNMT1 in cancer cell lines leads to de novo methylation of the CpG islands (Vertino et al., 1996) and recent studies suggest that DNMT3a and DNMT3b are also required for maintenance methylation of certain sequences (Chen et al., 2003; Liang et al., 2002). The overlapping functions may be a consequence of the recently reported physical interactions of these three enzymes (Kim et al., 2002). It is suggested that maintenance and de novo methyltransferases cooperate in silencing both single copy genes and repetitive sequences (Liang et al., 2002; Rhee et al., 2002).

In addition to the active DNMTs, there are also two other homologous proteins that don't seem to be capable of methylating DNA. DNMT2 is a small protein lacking the regulatory N-terminal domain, but having all of the conserved methyltransferase motifs. Although this protein exhibits no methyltransferase activity *in vitro*, and mice with the targeted mutation of its putative catalytic site showed no defect in the

methylation patterns (Okano et al., 1998), recent reports suggest that it may have some catalytic activity. Drosophila DNMT2 was shown to specifically methylate cytosines in CpA and CpT dinucleotide context (Kunert et al., 2003), and mouse DNMT2 expressed in Drosophila was shown capable of methylating cytosines in non-CpG dinucleotide context (Mund et al., 2004). On the other hand, DNMT3L almost certainly lacks methyltransferase activity since it harbors a mutation of the catalytic cysteine residue. Targeted disruption of this gene suggested that it plays a role in silencing of maternally imprinted genes, and this activity is probably mediated through its association with other proteins (Bourc'his et al., 2001). For instance, DNMT3L was shown to interact with DNMT3a and DNMT3b, and was proposed to regulate their methyltansferase activity (Chedin et al., 2002). In addition, its association with HDAC1 suggests that it participates directly in the transcriptional repression and the establishment of genomic imprints (Aapola et al., 2002).

(ii) DNA demethylation

Until recently it was believed that methylation is a unidirectional reaction, and that the only determinant of the methylation pattern is the presence and the activity of DNMTs. Global and gene-specific demethylation that was observed during development was explained by the replication in the absence of maintenance DNA methyltransferase (Razin and Riggs, 1980). A reduction of global levels of DNMT activity would result in global demethylation, while masking of specific sequences by transcription factors would lead to gene specific demethylation (Monk et al., 1991). Although this passive demethylation model may hold true, it cannot explain those demethylation events that are occurring in the absence of replication. For instance, the mouse paternal genome undergoes global demethylation within hours following fertilization before the first round of DNA replication commences (Oswald et al., 2000). During the differentiation of the mouse myoblasts into myotubules, the first exon of *myogenin* becomes demethylated within minutes (Lucarelli et al., 2001). In addition to its roles in development, active demethylation seems to modulate gene expression in somatic tissues as well. The activation of the fully differentiated T

lymphocytes results in a rapid promoter demethylation of a tissue-specific gene *interleukin-2*, and is not blocked by the inhibitors of DNA replication (Bruniquel and Schwartz, 2003). Perhaps the most interesting is the recent report that neural plasticity may be mediated through active demethylation of the brain-derived neurotrophic factor promoter (Martinowich et al., 2003). Neurons are an excellent example of a tissue that would benefit greatly from the reversibility of methylation. Since they are postmitotic, non-dividing, they need replication independent mechanisms for alteration of gene expression programs in order to achieve the long-lasting functional and structural changes necessary for their plasticity. Indeed, many neurological disorders have been shown to be a consequence of inappropriate function of the methylation machinery (Mattson, 2003).

Cancer is another example where a classic concept of fixed DNA methylation patterns and their inheritance cannot explain the paradoxical observation that both regional hypermethylation and global hypomethylation occur simultaneously in cancer cells (Baylin et al., 1991). In order to explain the global hypomethylation in the cells where high levels of DNMTs exist, the existence of an active demethylase was predicted (Szyf et al., 1985a). A decade later, it was shown that mouse embryonal P19 cells stably transfected with Ras oncogene possess high levels of such demethylase activity (Szyf et al., 1995).

Three mechanisms of active demethylation have been proposed to date. The first comes from the studies of chicken embryo (Jost et al., 1995) and mouse myoblast differentiation (Jost et al., 2001), and is based on the nucleotide excision repair. It is proposed that the enzyme responsible for this activity is 5-methylcytosine DNA glycosylase (5-MCDG), which initially removes the methylated base resulting in the cleavage of the abasic sugar and repair by replacement with cytosine. However, the affinity of 5-MCDG for hemimethylated DNA substrate is much higher than for substrates methylated on both strands (Jost et al., 1995), which implies that DNA first needs to undergo one round of passive demethylation in order to become a good substrate for active demethylation. Since this model involves at least one round of

replication it cannot explain the aforementioned demethylation events. The second proposed mechanism involved the excision of the whole methylated nucleotide and replacement by an unmethylated one. However, it is difficult to conceive that the global demethylation would involve genome wide excision and repair, since this would compromise genome integrity at the critical point in development (Weiss et al., 1996). Therefore, the search for a true demethylase continued and a few years later a *bona fide* demethylase activity was purified from human lung cancer cell line A549 (Ramchandani et al., 1999). The study of the demethylation reaction revealed the mechanism involving hydrolytic cleavage of the C-C bond between the methyl group and the cytosine ring releasing the methyl group in the form of methanol (Ramchandani et al., 1999) (Figure 4.). Cloning of the enzyme possessing a demethylase activity was done virtually by searching an EST database for DNA sequences homologous to the previously identified methyl-CpG-binding domain (MBD) (Bhattacharya et al., 1999). This domain is common to proteins able to recognize methylated DNA (Cross et al., 1997) and it was assumed that any enzyme that catalyzes demethylation should be able to recognize methylated DNA. The identified protein was termed MBD2b/dMTase and was shown to actively demethylate DNA both *in vitro* (Bhattacharya et al., 1999) and *in vivo* and was suggested to play a role in transcriptional activation (Cervoni et al., 2002; Cervoni and Szyf, 2001; Detich et al., 2003; Detich et al., 2002). The demethylase activity was found to be processive (Cervoni et al., 1999), which is probably critical for the rapid demethylation observed in the early development.

MBD2 was also independently cloned and identified as a methylated DNA binding repressor (Hendrich and Bird, 1998; Tweedie et al., 1999), which created a controversy in the field, with some groups disputing the demethylase activity of MBD2b/dMTase (Hendrich et al., 2001; Tweedie et al., 1999). The opposing functions of MBD2 and MBD2b/dMTase are further discussed in section V-ii.

This discovery demonstrated that DNA methylation is a reversible biological modification, and that the observed patterns of methylation result from an equilibrium

of methylation and demethylation activities. These findings have a profound effect on our understanding of all aspects of DNA methylation, from its establishment during development, to its maintenance in the somatic tissues and its role in cancer and other pathological states.

V. DNA methylation and gene expression

The idea that DNA methylation is involved in the regulation of gene expression has been around long before any of the DNA methyltransferase proteins were discovered. Early observations showed that DNA that is rich in 5mC is harder to digest with micrococcal nucleases because it is associated with more densely packaged nucleosomes (Razin and Cedar, 1977). Further studies indicating that regulatory regions of inactive genes are often methylated, led to the hypothesis that DNA methylation is involved in gene silencing (Razin and Riggs, 1980). However, the question of whether methylation precedes and is an active player in gene silencing, or whether it is just a reflection of the gene activity status remained unresolved. Multiple studies that followed have demonstrated that in vitro methylation of genes ectopically introduced into vertebrate cells become suppressed, suggesting that DNA methylation is a trigger for gene silencing (Stein et al., 1982; Vardimon et al., 1982). Other early studies used a different approach to analyze the importance of methylation in gene silencing, by examining the effects of 5-azacytindine (5-aza) and its deoxy analog 5-aza-2'-deoxycytidine (5-aza-CdR), a potent DNA methylation inhibitor, on the expression of methylated genes. It was shown that human HPRT gene residing in the heavily methylated inactive X-chromosome could be reactivated by 5-aza (Jones et al., 1982). However, treatment of adult chicken with 5-aza resulted in demethylation of the embryonic beta globin gene, but this demethylation did not result in gene expression (Ginder et al., 1983). Similar phenomenon was observed in case of *c-mos* and *beta globin* genes in the mouse embryonal fibroblasts (Hsiao et al., 1984) suggesting that additional factors, possibly related to the specific cell type, may be required for the expression of these loci. These and other similar observations suggested that although DNA methylation may be important for gene expression its

effects are dependent on other cellular factors such as the presence of the right transcriptional machinery in a given cell type or the chromatin structure of a given gene. In addition, DNA methylation itself was proposed to affect gene expression through different mechanism.

(i) DNA methylation and direct repression

The most direct mechanism by which DNA methylation can interfere with transcription is to prevent binding of transcription factors or basal transcriptional machinery to the DNA. Most mammalian transcription factors have CG-rich binding sites and many have CGs in their DNA recognition elements. There are a number of examples where binding of transcription factors was shown to be inhibited by methylation of their cognate sequences, as in the case of c-AMP response element (CRE) (Iguchi-Ariga and Schaffner, 1989), AP-2 (Comb and Goodman, 1990), c-Myc (Prendergast and Ziff, 1991), ATF-like factor and retinoblastoma binding factor 1 (Ohtani-Fujita et al., 1997). In all of these studies *in vitro* binding assays suggested that methylation of DNA can physically interfere with the transcription factor binding, and the corresponding *in vivo* experiments showed that this interference resulted in the transcriptional repression. However, this simple mechanism can not account for all the repressive effects of methylation since not all the transcription factors contain a CG dinucleotide in their recognition sequence, and since some transcription factors do not differentiate between methylated and unmethylated sites. One such example is the ubiquitous transcription factor Sp1, which was shown to bind equally well to methylated and unmethylated DNA (Harrington et al., 1988; Holler et al., 1988). However, more recent data suggested that binding of Sp1 to its consensus sequence could be prevented by methylation (Mancini et al., 1999; Zhu et al., 2003). The explanation for these conflicting reports may lay in the fact that methylation can indirectly interfere with Sp1 binding through the recruitment of other factors which can in turn preclude Sp1 binding (Kudo, 1998). In fact, there is accumulating evidence that a more universal mechanism by which DNA methylation regulates gene

expression involves indirect repression through the recruitment of complexes acting on chromatin structure.

(ii) Indirect repression of methylated genes through methyl-CpG-binding proteins

A breakthrough in understanding the tight relationship between DNA methylation, inactive chromatin and gene silencing came with the discovery of the protein family capable of 'reading and interpreting' DNA methylation signals. In 1992, the first protein called MeCP2 (methyl-CpG-binding protein 2) was purified and cloned, and although capable of recognizing single CpG sites, it was shown to localize mainly with the inactive, densely methylated pericentromeric heterochromatin (Lewis et al., 1992). The region of MeCP2 protein responsible for recognizing methylated DNA was identified and termed methyl-CpG binding domain (MBD) (Nan et al., 1993). The search for proteins containing homologous domains resulted in the discovery of four other proteins, MBD1 (Cross et al., 1997), MBD2, MBD3 and MBD4 (Hendrich and Bird, 1998) (Figure 5.). Although all these proteins are considered members of the same MBD family, they do not share sequence similarity outside of the MBD domain, except in the case of MBD2 and MBD3, which are 70% identical and are believed to have originated by gene duplication from a common ancestral protein (Hendrich and Tweedie, 2003). Transcriptional repressor activity of MBDs is mediated through their association with multiprotein repressor complexes that modify chromatin by both ATP-dependent nucleosome remodeling and histone deacetylase activities. For example, MeCP2 was shown to recruit transcriptional repressor complex Sin3A/HDAC through its transcriptional repressor domain (TRD) (Nan et al., 1998) and to direct H3-K9 methylation through its association with histone methyltransferase activity (Fuks et al., 2003b). MBD1 was also shown to recruit histone deacetylase (Ng et al., 2000) and histone methyltransferase activity (Fujita et al., 2003b). Transcriptional repression by these complexes in vivo was relieved by the deacetylase inhibitor trichostatin A, indicating that deacetylation of histones (and/or of other proteins) is a major component of this repression mechanism. The role of other

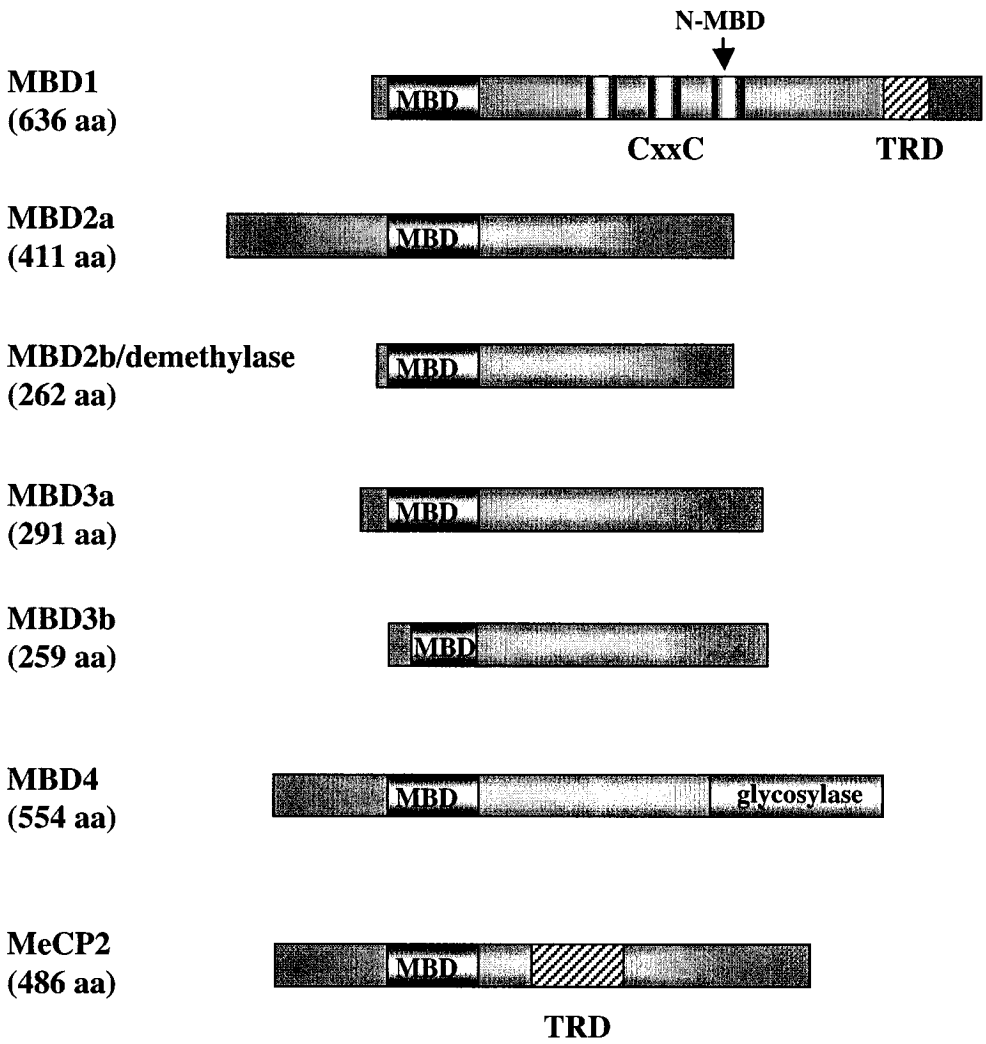


Figure 5. Schematic of the structure of MBD family of proteins. The only region homologous among all proteins is methyl-CpG-binding domain (MBD). MBD2 and MBD3 proteins also exhibit homology outside of MBD domain, and are 70% identical. Some MBDs have different splice variants (note variable aa length). MBD1-3 and MeCP2 can act as transcriptional repressors by binding to methylated DNA. MBD1 has three CxxC Zn finger domains, one of which can also bind to non-methylated DNA through its non-mCpG binding domain (N-MBD). MBD4 has a glycosylase domain that exhibits mismatch repair activity. Deletion analysis of some proteins identified transcriptional repression domains (TRDs).

MBD family members in transcriptional repression remains unclear. MBD4 does not seem to posses any repressor activity; rather it is reported to be a thymine DNA glycosylase responsible for repairing T-G mismatches resulting from deamination of 5mC-G base pair (Hendrich et al., 1999). Methylated CpG dinucleotides are considered 'mutational hotspots' due to their high frequency of spontaneous deamination, and MBD4 was proposed to have evolved to protect the genome from this kind of mutagenesis, and has recently been implicated in the cellular response to DNA damage induced by cytotoxic agents (Sansom et al., 2003b).

The two homologues, MBD2 and MBD3, both exist as two isoforms responsible for different cellular roles. Both MBD3a and MBD3b were found to be integral components of NuRD (nucleosome remodeling and deacetylation) complex, but they could not be shown to specifically bind methylated DNA (Hendrich and Bird, 1998; Zhang et al., 1999). Instead, it was proposed that transcriptional silencing by NuRD complex requires other factors for its recruitment to methylated sites. One such factor was suggested to be MBD2, which was originally chromatographically purified in a complex with NuRD, and these were collectively named MeCP1 (Meehan et al., 1989). MeCP1 was shown to preferentially bind, remodel and deacetylate nucleosomes associated with methylated DNA (Feng and Zhang, 2001). As mentioned in the previous section, MBD2b isoform of this protein was also shown to posses demethylase activity (Bhattacharya et al., 1999), and was suggested to play a role in transcriptional activation rather than transcriptional repression (Cervoni et al., 2002; Cervoni and Szyf, 2001; Detich et al., 2003). The opposing functions of these two isoforms may be mediated through their association with different cellular factors. MBD2b lacks the N-terminal domain of MBD2, and it is possible that this domain targets the complexes responsible for gene repression. Alternatively, the same isoforms may be responsible for different activities depending on the cellular context, as in the case with many transcription factors that act as either activators or repressors, depending on their partners (Attisano and Wrana, 2000; Xu et al., 1999). In addition, particular sequence and/or chromatin context may itself determine the modification, by being more or less accessible for a particular enzymatic activity. This is illustrated

in the study where MBD2b exhibited different levels of demethylase activity in the same cellular context depending on the type of methylated promoter that was examined (Detich et al., 2002).

Further insight into the function and importance of MBD family of proteins was gained through the knockout studies. MBD3 deletion was the most severe, and MBD3-/- mice died during early embryogenesis (Hendrich et al., 2001). Mutations in the human MeCP2 were found in patents with Rett syndrome, a severe neurodevelopmental disorder occurring in females at a frequency of 1:15,000 (Amir et al., 1999). MeCP2 deletion in mice produced a Rett-like phenotype and confirmed the importance of MeCP2 in the normal brain function (Guy et al., 2001). Recently reported MBD1-/- mice developed normally but showed some deficits in adult neurogenesis and hippocampal function (Zhao et al., 2003). MBD2-/- mice developed normally but were found to have defects in maternal behavior (Hendrich et al., 2001). The analysis of methylation patterns of MBD2-/- mice did not offer support for MBD2 role as either a transcriptional repressor or a demethylase. All of the imprinted and X-linked genes that were examined showed normal expression, and the only example that showed slightly increased expression was Il4 gene in differentiating T-cells (Hutchins et al., 2002). In addition, demethylation during embryogenesis also appeared normal. Taken together, these mutational analysis suggested that different members of the MBD family play distinct roles in the development and the adult life. However, viability and gross normality of some MBD deletions suggest that there are probably multiple layers of control of gene expression which can sometimes compensate for each other and ensure that loss of one component does not immediately result in a potentially disastrous misinterpretation of the methylation signal.

(iii) The crosstalk between DNA methylation and chromatin structure

The tight connection between DNA methylation and inactive chromatin can be interpreted in different ways depending on the examination angle. The existence of

MBD proteins, which recognize and bind methylated DNA, and are capable of recruiting chromatin remodeling complexes, suggests that DNA methylation is the first event in gene silencing and that it is responsible for marking sites for chromatin inactivation and silencing. In support of this model, a number of studies showed that when methylated DNA is exogenously introduced into the cells, it directs the formation of the inactive chromatin, which becomes deacetylated and methylated at H3-K9 (Hashimshony et al., 2003; Keshet et al., 1986). Furthermore, detailed examination of the effects of inhibitors of DNA methylation and histone deacetylation on the expression of some methylated genes, suggested that although these two modifications act synergistically, DNA methylation is a dominant factor in gene silencing. This conclusion was based on the fact that inhibition of DNA methylation by 5-azaCdR lead to promoter demethylation and induction of gene expression, whereas inhibition of HDAC activity by TSA alone was not enough to induce expression of the same genes (Cameron et al., 1999; Fahrner et al., 2002). However, the interpretation of these results is limited by the fact that TSA affects only histone deacetylation and not the other histone modifications involved in the formation of inactive chromatin. It is possible that certain modifications, such as methylation of H3-K9, are enough to keep a given gene in a silenced state, regardless of its state of acetylation. Alternatively, inhibition of HDAC activity may not necessarily result in increased histone acetylation (Fahrner et al., 2002), possibly due to the presence of other factors such as INHATs, which mask histones and prevent their acetylation (Cervoni et al., 2002; Seo et al., 2001). Therefore, although these results are in agreement with the causal role of DNA methylation in gene silencing, it is possible that an alternative hypothesis may be true as well.

The model proposing that inactive chromatin structure precipitates DNA methylation, whereas active chromatin leads to demethylation has emerged from interpretation of recent data. Genetic analyses in a variety of species from fungus to humans, have shown that mutations of genes involved in chromatin remodeling and histone modification also affect DNA methylation to a different degree. For instance, histone methyltransferases DIM-5, from the fungus *Neurospora Crassa*, and Kryptonite, from

the plant *Arabidopsis Thaliana*, were shown to be important for normal DNA methylation (Jackson et al., 2002; Tamaru and Selker, 2001). In mice, deletion of the chromatin remodeling protein Lsh lead to global loss of DNA methylation (Dennis et al., 2001), while ES cells lacking histone MTase G9a showed defects in the establishment and maintenance of DNA methylation at the imprinted loci (Xin et al., 2003). Similar observations were made in people with an X-linked genetic disease ATR-X (alpha-thalassemia mental retardation). This disease is characterized by developmental abnormalities, severe mental retardation, facial dysmorphism, and alpha-thalassemia, and was found to result from mutations in ATR-X gene. This gene encodes a SWI/SNF-like protein. SWI/SNF proteins are involved in chromatin remodeling, and ATR-X was therefore proposed to act as a transcriptional regulator within a heterochromatin environment (Berube et al., 2000). ATR-X patients demonstrate DNA methylation defects in select regions of their genomes, suggesting that disturbances in chromatin remodeling result in altered DNA methylation (Gibbons et al., 2000).

In accordance with the hypothesis that DNA methylation reflects the dynamic changes in chromatin, it was shown that activation of chromatin leads to DNA demethylation. For example, HDAC inhibitor sodium butyrate was shown to trigger global demethylation in Epstein Barr Virus (EBV) transformed cells in a replication-independent manner (Szyf et al., 1985a). Similarly, inhibition of HDAC activity by TSA resulted in selective DNA demethylation in *Neurospora Crassa* (Selker, 1998). In human cells, treatment with two different HDAC inhibitors, TSA and valproate, resulted in active DNA demethylation directed by histone acetylation (Cervoni and Szyf, 2001; Detich et al., 2003). Based on these data, it was proposed that the pattern of DNA methylation results from the dynamic methylation and demethylation reactions, and that it is the chromatin structure that directs the equilibrium of these reactions (Szyf, 2003).

The mechanism by which chromatin structure directs DNA methylation pattern could work either through determining the accessibility of DNA to methyltransferases and

demethylases, or through the recruitment of these enzymes by the chromatin associated factors. For instance, it was shown in *Arabidopsis* that DNA methyltransferase CHROMOMETHYLASE 3 (CMT 3) associates with heterochromatin protein HP1 homologue, which in turn binds methylated H3-K9. It is proposed that DNA methylation is targeted to inactive chromatin through these interactions (Jackson et al., 2002). Similarly, it was shown in mammalian cells that histone methyltransferase SUV39H1 and heterochromatin protein HP1beta recruit both DNMT1 and DNMT3a (Fuks et al., 2003a), raising the possibility that chromatin structure may be responsible for directing both *de novo* and maintenance methylation.

An alternative mechanism is also possible where DNA methyltransferases and demethylases would be recruited to specific sites of action through their association with transcriptional activators and repressors. One such example is the recruitment of DNMT1 and DNMT3a to the RARbeta2 promoter by the leukemia-promoting PML-RAR fusion protein. It was suggested that PML-RAR could act as both an activator and a repressor, depending on the cellular environment. In the absence of retinoic acid it recruits both DNMT and HDAC activities, causing gene hypermethylation and silencing, while in the presence of retinoic acid it turns into an activator and induces demethylation and gene reexpression (Di Croce et al., 2002). Similarly, DNA demethylation and expression of the kappa immunoglobulin gene during development was shown to require transcription factor NF-kB (Kirillov et al., 1996; Kistler et al., 1997).

In conclusion, the data presented above suggest that the hypothesis that DNA methylation leads to chromatin inactivation, may not necessarily contradict the hypothesis that chromatin structure directs the methylation state. Rather, the cross-talk between DNA methylation and chromatin appears to be bidirectional, serving to reinforce the two modes of epigenetic control of gene expression, and to ensure a rapid and orchestrated response to a multitude of different cellular signals.

(iv) Methylation-independent regulation of gene expression by DNMTs

In recent years it has become evident that DNMTs are multifunctional proteins that have functions independent of their methyltransferase activities. As previously discussed, silencing of gene expression by DNA methylation is a well-established role of DNMTs that requires their enzymatic activity. However, recent data suggest that DNMTs may also be involved in control of gene expression through their interaction with other proteins. All three active mammalian methyltransferases, DNMT1, DNMT3a, and DNMT3b, have been shown to repress transcription by recruiting HDAC activity, and the deletion analysis of DNMTs indicated that their catalytic domains are dispensable for this silencing activity (Bachman et al., 2001; Fuks et al., 2000). In addition, DNMT3L homologue, which is not a functional methyltransferase due to the mutations in the critical residue of its catalytic domain, was shown to be required for the establishment of the maternal imprints (Bourc'his et al., 2001). The discovery that DNMT3L also represses transcription through the recruitment of HDAC1 activity offered an explanation for its roles in gene silencing and further strengthened the notion that DNMTs have functions independent of their methyltransferase activities (Aapola et al., 2002; Deplus et al., 2002). The recruitment of DNMT repressor activity to specific sites was suggested to occur through the interaction of specific transcription factors with DNMTs, which are in turn associated with HDAC activity. For example, DNMT3a is recruited to specific sequences through its interaction with a DNA binding repressor protein RP58. The co-repressor function of DNMT3a was shown to be methylation-independent and probably mediated through the recruitment of HDAC activities (Fuks et al., 2001). Likewise, in human Hela cells, DNMT1 and HDAC1 were found in a complex with transcription factors Rb and E2F1 (Robertson et al., 2000a). Rb is the most studied tumor suppressor and the master regulator of the cell cycle, and at least one of its functions involves binding E2F transcription factors and recruiting repressor activities to promoters containing E2F responsive elements. As the cells progress from G1 to S phase, Rb releases E2F, which then becomes free to activate genes involved in DNA synthesis or those required for S phase entry (Ferreira et al., 2001). The finding that DNMT1 is part of the Rb/E2F repressor complex raises the possibility that DNMT1

could also participate in the regulation of the genes involved in the cell cycle. In fact, co-transfection of DNMT1, Rb and E2F1 resulted in suppression of unmethylated co-transfected promoter of the tumor suppressor *p14 (ARF)* (Robertson et al., 2000a). However, this model was only partially supported by the study of the regulation of the endogenous *p14* promoter (Nguyen et al., 2002). In this study it was found that although *p14* promoter is unmethylated, it becomes acetylated upon treatment with DNA methylation inhibitor 5-azacytidine. It was suggested that 5-azacytidine reduces the free pool of DNMTs in the cell, leading to the reduced recruitment of DNMT/HDAC repressor complex, resulting in an increase in acetylation of the targeted *p14* promoter. Surprisingly, *p14* gene expression remained unchanged despite an increase in its acetylation status, presumably because of the existence of other dominant regulators of its transcription. One interesting point raised in these studies is that the recruitment of DNMT1 through these complexes does not result in methylation of the promoter targeted for repression. An obvious question is why DNMT1 does not methylate a promoter to which it is bound. One possible explanation comes from a recent study showing that Rb binds to DNMT1 and inhibits its catalytic activity by disrupting interaction between DNMT1 and DNA (Pradhan and Kim, 2002). It is tempting to speculate that DNMT1 plays a role in the orchestrated progression from G1 to S phase of the cell cycle, and that Rb controls this acitivity by recruiting DNMT1 to the sites destined for repression. By inhibiting DNMT1 catalytic activity, Rb could also ensure that this repression is transient and that it does not involve DNA methylation, which is believed to be a more permanent repression mark. If this hypothesis is true, frequently observed inactivation of Rb during cancer progression could also contribute to the aberrant methylation of tumor suppressors, which is also a common event in tumorigenesis. In addition, Rb has recently been shown to regulate DNMT1 gene at the transctiptional level through the recruitment of E2F/Rb/HDAC repressor complex (Kimura et al., 2003), and at the posttranscriptional level through decreasing DNMT1 mRNA stability (Slack et al., 1999). It remains to be seen if DNMT1 itself participates in this repression activity as part of a feedback regulatory mechanism.

The data presented in chapters 1-3 of this thesis is in agreement with the methylation-independent roles of DNMT1, and show some of the first examples of unmethylated endogenous genes regulated by DNMT1 levels. The finding that knock down of DNMT1 results in induction of a tumor suppressor *p21* and growth arrest is consistent with its role in the regulation of the cell cycle. Also, the induction of stress response genes following knock down of DNMT1 suggests that cells have developed a mechanism responsible for sensing DNMT1 levels and interpreting its loss as a potential epigenomic stress. The examination of the mechanism of induction of these genes by knock down of DNMT1 has revealed that it does not include changes in either acetylation nor methylation of histones associated with their promoters. This is contrary to previously discussed reports of the HDAC mediated regulation of gene expression by DNMT1, and suggests a novel role of DNMT1 which is independent of both DNA methylation and histone modification. This novel role of DNMT1 was also observed in a recent report where an integrated microarray system was used for simultaneous assessment of DNA methylation, histone acetylation and gene expression of regulatory regions of ~1500 genes. It was found that genes induced by 5-azacytidine through a methylation-independent mechanism can be divided into two groups. A smaller group contained genes whose activation was accompanied by histone acetylation, and a larger group contained those genes that were activated in a histone acetylation independent way (Shi et al., 2002). The possible mechanism and a putative role of Sp1 transcription factor in recruiting this novel function of DNMT1 will be discussed in chapter 3.

Taken together, DNMT1 and the other DNMTs seem to be multifunctional proteins that, in addition to their prime role as DNA methyltransferase enzymes, also have other functions in gene silencing (Figure 6.). Many of these functions appear to be involved in the control of cell growth, and alterations of different components of methylation machinery may directly contribute to the transformation and cancer progression (Szyf, 2003). The following section will provide a detailed discussion of the involvement of DNA methylation in cancer and its targeting in anticancer therapy.

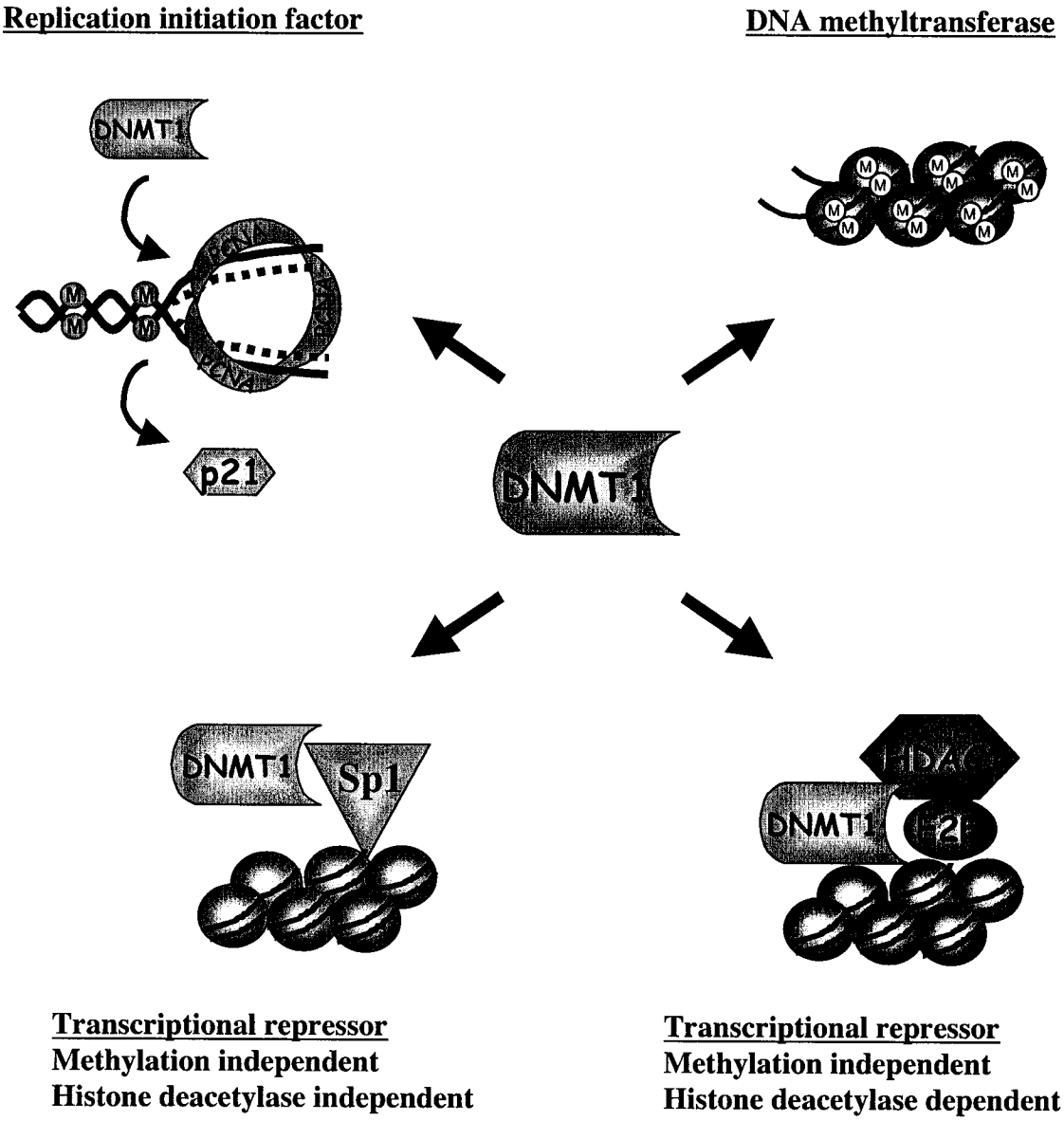


Figure 6. Multiple functions of DNMT1. In addition to its enzymatic activity as a DNA methyltransferase, DNMT1 also acts as a replication initiation factor by competing with replication inhibitor p21 for the same binding site on PCNA. DNMT1 can also act as a transcriptional repressor independent of its methyltransferase activity. This repressor activity can be either HDAC dependent, or, as our data suggests, HDAC independent.

VI. DNA methylation machinery and cancer therapy

(i) Regional hypermethylation versus global hypomethylation

The observations that DNA methylation patterns are altered in cancer cells relative to normal cells have been around for two decades, and are believed to result in abnormal expression of a wide variety of genes (Jones, 1996). The first attempts to correlate aberrant DNA methylation and cancer focused on determining the total levels of 5mC, and observed lower levels of DNA methylation in tumor versus normal tissues (Lu et al., 1983). Initially, the hypomethylation was observed in oncogenes and was believed to be responsible for their activation and tumor progression (Feinberg and Vogelstein, 1983; Nambu et al., 1987). However, later studies discovered that hypomethylation observed in cancers is not restricted to oncogenes, but occurs throughout the genome, mostly in the normally methylated parasitic and repetitive sequences such as satellite DNA, LINE-1 elements, and retroviruses (Ehrlich, 2002). It is believed that hypomethylation of such transposable elements may render them competent for transcription and recombination, thus contributing to the genomic instability observed in cancers (Eden et al., 2003; Florl et al., 1999; Wong et al., 2001). In addition, some genes involved in tumor invasion and metastasis were shown to become hypomethylated in cancer (Guo et al., 2002; Rosty et al., 2002; Shteper et al., 2003). In some tumor types DNA hypomethylation was found to be a biological marker of tumor progression with the potential prognostic use (de Capoa et al., 2003; Soares et al., 1999).

The paradoxical observation that global hypomethylation is accompanied by regional hypermethylation in the same cancer cell has caused difficulties in the interpretation of the events leading to aberrant methylation and its roles in tumorigenesis. Early studies discovered that many tumor suppressor genes are inactivated by methylation in cancer cells, providing a direct link between DNA methylation and abnormal cell growth (Esteller et al., 2002a). For example, the hypermethylation of promoters of tumor suppressor genes Rb and Von Hippel Lindau (VHL) has been detected in familial

cases of retinoblastoma and renal cancer, as well as other cancers, and is believed to be a primary inactivation event (Herman et al., 1994; Stirzaker et al., 1997). Methylation of other tumor suppressors, such as p14 (ARF), and cyclin-dependent kinase inhibitors p15 and p16, has also been detected in a wide variety of tumors (Herman et al., 1996b; Herman et al., 1995; Lowe and Sherr, 2003). In addition to cell cycle genes that are directly involved in the control of tumor growth, inactivation by hypermethylation has been observed in genes involved in other cellular pathways targeted in cancer. Some of these include genes involved in the response to DNA damage such as repair enzymes hMLH1 and MGMT, and a multifunctional protein BRCA1 (Esteller et al., 2002b). Inactivation of these genes results in higher mutation rates and genomic instability leading to increase in cancer predisposition and progression (Villemure et al., 2003). In addition, members of the cadherin family of cell adhesion molecules are often methylated in cancer. Since these proteins are responsible for keeping the tissue integrity by controlling cell attachments and motility, their inactivation has been correlated with increased invasion and metastasis (Hirohashi and Kanai, 2003). With the increased awareness of the existence of the tumor suppressor hypermethylation in cancer, novel genomic approaches have been developed for a more global assessment of the methylation patterns. Restriction landmark genomic scanning (RLGS) and the microarray-based techniques, that can simultaneously assess the methylation status of thousands of CpG islands, have found that hypermethylation occurs in most tumors and varies between different tumor types (Adorjan et al., 2002; Dai et al., 2001). Particular sets of hypermethylated genes were found to be characteristic of particular tumor classes, and were used to predict the tumor class of unknown samples, suggesting that they could have important diagnostic significance (Adorjan et al., 2002).

One question that still remains unresolved is how is the methylation pattern aberrantly modified in the same cancer cell in two opposite directions, causing both hypomethylation and hypermethylation. Since hypomethylation occurs mainly in sparsely distributed CpGs, while hypermethylation occurs mainly in CpG islands within the regulatory regions of genes, it seems that different factors are responsible

for each. Since dynamic nature of DNA methylation requires activities of both DNA methyltransferase and demethylase enzymes, alteration of their expression or their targeting could offer an explanation for the co-existence of hypo and hypermethylation.

(ii) Role of DNMTs in cancer

In parallel with the existence of aberrant methylation patterns in cancer cells, it has been observed that DNMT1 expression is increased in many tumors (Issa et al., 1993). Several cellular pathways that are involved in tumorigenesis have been shown to induce DNMT1 levels. For example, DNMT1 transcription was induced by different components of the protooncogenic Ras signaling pathway and it was shown that DNMT1 is essential for their transformation capacity since inhibition of DNMT1 by antisense led to reversal of transformation (Bakin and Curran, 1999; Bigey et al., 2000; MacLeod et al., 1995; MacLeod and Szyf, 1995). Aberrant Ras signaling is a common event in cancer (Ayllon and Rebollo, 2000), and is probably responsible for some of the induced DNMT1 levels observed in cancers. Another pathway that is tightly linked to cancer, and can contribute to elevated DNMT1 levels, is the APC/beta catenin/TCF pathway. Mutations of the adenomatous polyposis coli (APC) tumor suppressor gene initiates the majority of colorectal cancers by failing to inactivate beta-catenin/Tcf transcription factor complex, resulting in its constitutive activation (Barker et al., 2000). It was shown in mice that knockout of APC gene leads to intestinal neoplasia, and that reduction of DNMT1 activity in these mice reduced tumorigenesis, suggesting that DNMT1 is an important downstream effector in the APC pathway (Eads et al., 1999; Laird et al., 1995). Similar conclusions were drawn from APC-/- colon cancer cells, where the expression of wild type APC lead to downregulation of DNMT1 mRNA and concomitant inhibition of growth of these cancer cells (Campbell and Szyf, 2003).

In addition, some cancer causing viruses, such as simian virus 40 (SV40) and Epstein Barr Virus (EBV), have been shown to induce DNMT1 levels. Large T antigen is one

of the protein products of the SV40 virus whose transforming activity is exerted mainly through the inactivation of Rb and p53 pathways (Gazdar et al., 2002). Following the observation that cells transformed with SV40 virus show increased expression of DNMT1 (Chuang et al., 1997), it was discovered that this upregulation of DNMT1 occurs mainly at the posttranscriptional level and that it depends on Rb inactivation (Slack et al., 1999). Transformation by large T antigen was dependent on DNMT1 since its knockdown by antisense resulted in the inhibition of transformation (Slack et al., 1999). Another oncogenic viral protein, latent membrane protein 1 (LMP1) produced by EBV, has been shown to upregulate DNMT1, DNMT3a and DNMT3b in a few cancer cell lines (Tsai et al., 2002). In parallel, transfection of LMP1 into these cells resulted in the methylation and inactivation of E-cadherin gene, resulting in increase of the invasive ability of these cells. Inhibition of DNA methylation by 5-azacytidine led to E-cadherin demethylation and expression, which in turn blocked cell migration ability (Tsai et al., 2002). Taken together, the observations that DNMT1 lays downstream of different oncogenic pathways may offer a mechanism to explain its elevated levels commonly observed in cancer.

A separate line of evidence implicating DNMT1 in tumorigenesis comes from observations that overexpression of DNMT1 in fibroblasts results in transformation (Bakin and Curran, 1999; Wu et al., 1993). Similarly, another study in fibroblasts demonstrated that DNMT1 overexpression results in the time-dependent increase in methylation of several CpG islands (Vertino et al., 1996). On the other hand, reduction of DNMT1 levels by antisense causes demethylation of tumor suppressors and reverses tumorigenesis both in vivo and in vitro (Fournel et al., 1999; MacLeod and Szyf, 1995; Ramchandani et al., 1997). Although most of the research on the connection between DNA methylation and cancer concentrated on DNMT1, there is emerging evidence that DNMT3a and DNMT3b may also be overexpressed in cancer (Mizuno et al., 2001; Robertson et al., 1999; Yakushiji et al., 2003). Similar to DNMT1, DNMT3b was shown to contribute to the transforming activities of SV40 T antigen and activated Ras (Soejima et al., 2003). In addition, DNMT3b was shown to be specifically required for cancer cell survival since its depletion resulted in cancer

cell apoptosis whereas it did not affect normal cells. Also, depletion of DNMT3b resulted in demethylation and reactivation of the normally silenced tumor suppressor gene RASSF1A (Beaulieu et al., 2002).

Taken together, these data support a generally accepted paradigm that overexpression of DNMTs results in hypermethylation and inactivation of tumor suppressors, allowing uncontrolled cell growth and tumor formation. On the other hand, inhibition of DNMTs leads to tumor suppressor demethylation and reactivation, resulting in the inhibition of tumor growth. Although it cannot be disputed that upregulation of DNMTs and hypermethylation of tumor suppressors are common events in cancer that can directly contribute to tumorigenesis, there is emerging evidence suggesting that these are two independent events. Correlation studies of increased expression of DNMT1, 3a and 3b, and hypermethylation of various tumor suppressors failed to establish any cause-effect relationship in a number of different cancer types (Eads et al., 1999; Ehrlich, 2002; Oue et al., 2001; Sato et al., 2002; Yakushiji et al., 2003). For example, comparison of oral carcinoma samples matched with normal mucosa samples found that there is a high incidence of both methylation of the tumor suppressor p16 and upregulation of all three active DNMTs in cancer samples. However, there was no correlation found between these events suggesting that different mechanisms are responsible for each (Yakushiji et al., 2003). If the increase in DNMT levels is not essential for the tumor suppressor methylation, then the obvious question is what is the mechanism responsible for the hypermethylation commonly observed in cancer. One possible explanation comes from a recent study demonstrating that p16 silencing can be achieved through histone H3-K9 methylation independent of DNA methylation. In this case inactivation of p16 was followed by methylation many cell passages later, suggesting that it was targeted by the inactive chromatin structure rather then being a result of the increased DNMT levels (Bachman et al., 2003). Another possible mechanism explaining hypermethylation independent of DNMT overexpression is the aberrant recruitment of DNMTs to tumor suppressor genes. This is illustrated in the report that leukemia-promoting PML-RAR fusion protein induces gene hypermethylation and silencing by recruiting DNMT1 and

DNMT3a to its target promoters, and that hypermethylation contributes to its leukemogenic potential (Di Croce et al., 2002).

In conclusion, although overexpression of DNMTs can lead to hypermethylation of some tumor suppressors, correlation analysis in different tumor types suggest that other mechanisms must exist that are responsible for inappropriate targeting of DNMT activity to tumor suppressor genes. This also leads to an alternative hypothesis on the possible mechanisms by which elevated DNMTs might promote tumorigenesis.

(iii) Methylation independent roles of DNMTs in cancer

Since DNMTs are multifunctional proteins involved in different cellular activities, their elevated levels may promote tumorigenesis independently of their methyltransferase activities. As discussed in section IV-ii, DNMTs were shown to recruit HDAC activities and repress transcription independent of their catalytic domain (Bachman et al., 2001; Fuks et al., 2000). The finding that DNMT1 associates with Rb/E2F complex, which is critical for the regulation of cell growth, raises the possibility that DNMT1 is involved in the control of the orchestrated progression from G1 to S phase of the cell cycle (Robertson et al., 2000a). It is possible that either elevated levels of DNMTs or their mistargeting results in their increased recruitment to genes responsible for the inhibition of cell growth, resulting in their inactivation. In accordance, inhibition of DNMTs in cancer cells either by antisense or by 5-azacytidine treatment was shown to induce a number of unmethylated genes involved in the regulation of cell growth (chapters 1-3). One possible mechanism responsible for mistargeting of DNMTs could be the formation of aberrant complexes due to their improper expression during the cell cycle. Analysis of the expression levels of active DNMTs showed that DNMT1 and DNMT3b mRNA levels were almost undetectable in arrested cells and that they increased dramatically upon entrance into S, while DNMT3a levels were less sensitive to the alterations in the cell cycle (Robertson et al., 2000b; Szyf et al., 1991). Comparison of the methyltransferase activity between cancer and normal cells revealed that cancer cells retain higher activity even during

the cell arrest suggesting that DNMT regulation may be disrupted in cancer cells (Robertson et al., 2000b). Regulation of DNMT1 levels during the cell cycle was found to involve a posttranscriptional mechanism, and a 3'UTR element within DNMT1 mRNA was identified and it was shown to be sufficient to mediate this regulation. In addition, overexpression of DNMT1 lacking the 3'UTR was shown to be able to transform NIH-3T3 cells, whereas inclusion of the 3'UTR prevented transformation (Detich et al., 2001).

One important reason for the regulation of DNMTs during the cell cycle would be to ensure the coordinate replication of the genetic and epigenetic material. Since DNA replication is semi conservative, newly synthesized DNA molecule is hemimethylated and the methylation pattern needs to be copied from the parental strand onto the nascent strand. This maintenance methylation occurs concurrently with DNA replication (Araujo et al., 1998a), and it is carried out by DNMT1, which is associated with the replication foci during S phase (Leonhardt et al., 1992). Targeting of DNMT1 to the replication fork is achieved through it binding to the proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997). A recent study demonstrated that DNMT1 has a higher affinity for DNA which is associated with PCNA, and that this DNA is more efficiently methylated than free DNA (Iida et al., 2002). This may be one of the mechanisms responsible for the specificity of DNMT1 for the newly synthesized hemimethylated DNA. Another level of regulation of DNMT1 activity is based on the fact that the tumor suppressor p21 competes with DNMT1 for binding to PCNA (Chuang et al., 1997). When p21 levels are high during Go/G1 phase, p21 competes out DNMT1 and forms an inhibitory complex with PCNA, therefore preventing inappropriate DNA methylation during this phase. As the cells enter into S phase, DNMT1 levels rise and it can now compete out p21 and enable PCNA to assemble at the replication fork. Additional control of DNMT1 binding to PCNA could also be achieved through DNMT1-Rb interaction, which was proposed to preclude DNMT1 binding to PCNA. Therefore, DNMT1 seems to play a regulatory function in the initiation of replication by promoting assembly of the replication fork. In turn, this function of DNMT1 seems to be regulated with the cell cycle through its

interaction with cell cycle regulatory proteins. In accordance, it was shown that knock down of DNMT1 by antisense oligonucleotides results in the inhibition of DNA replication, suggesting that the presence of DNMT1 in the fork is critical not only for methylation but also for replication itself (Knox et al., 2000). The data presented in chapters 2 and 3 of this thesis demonstrate that DNMT1 knock down by antisense triggers an intra-S-phase arrest accompanied by the induction of stress response genes. This is probably a consequence of an activated check point that senses lack of DNMT1 in the replication fork and arrests its progression in order to prevent a potentially disastrous loss of the methylation pattern. The nature of this checkpoint is currently under investigation in our laboratory.

In conclusion, DNMTs have emerged as multifunctional proteins involved in different cellular processes that can affect cell growth either directly, through protein-protein interactions, or indirectly, through silencing of tumor suppressor genes. In addition, DNMTs seem to be capable of silencing genes through both methylation dependent and methylation independent mechanisms (Figure 6.). Understanding which of these functions of DNA methylation machinery are essential for tumorigenesis, and what are the consequences of their inhibition, is of crucial importance for the design and use of DNMT inhibitors as anticancer agents.

(iv) Targeting DNMTs in anticancer therapy

A few different agents that target DNA methylation machinery are currently in use either as anticancer drugs in clinical trials, or as research tools for the study of DNA methylation. The mechanism of action of DNMT1 inhibitors used in this thesis is depicted in Figure 7. Based on their mechanisms of action they could be broadly divided into two groups, those that act as nucleoside analogues and get incorporated into DNA, and those that function independent of DNA incorporation. The first DNA methylation inhibitors that were discovered were 5-azacytidine (5-aza) and 5-aza-2'-deoxycytidine (5-aza-CdR), which are nucleoside analogues of cytidine and 2'-deoxycytidine. Once inside the cells, these pro-drugs first undergo phosphorylation

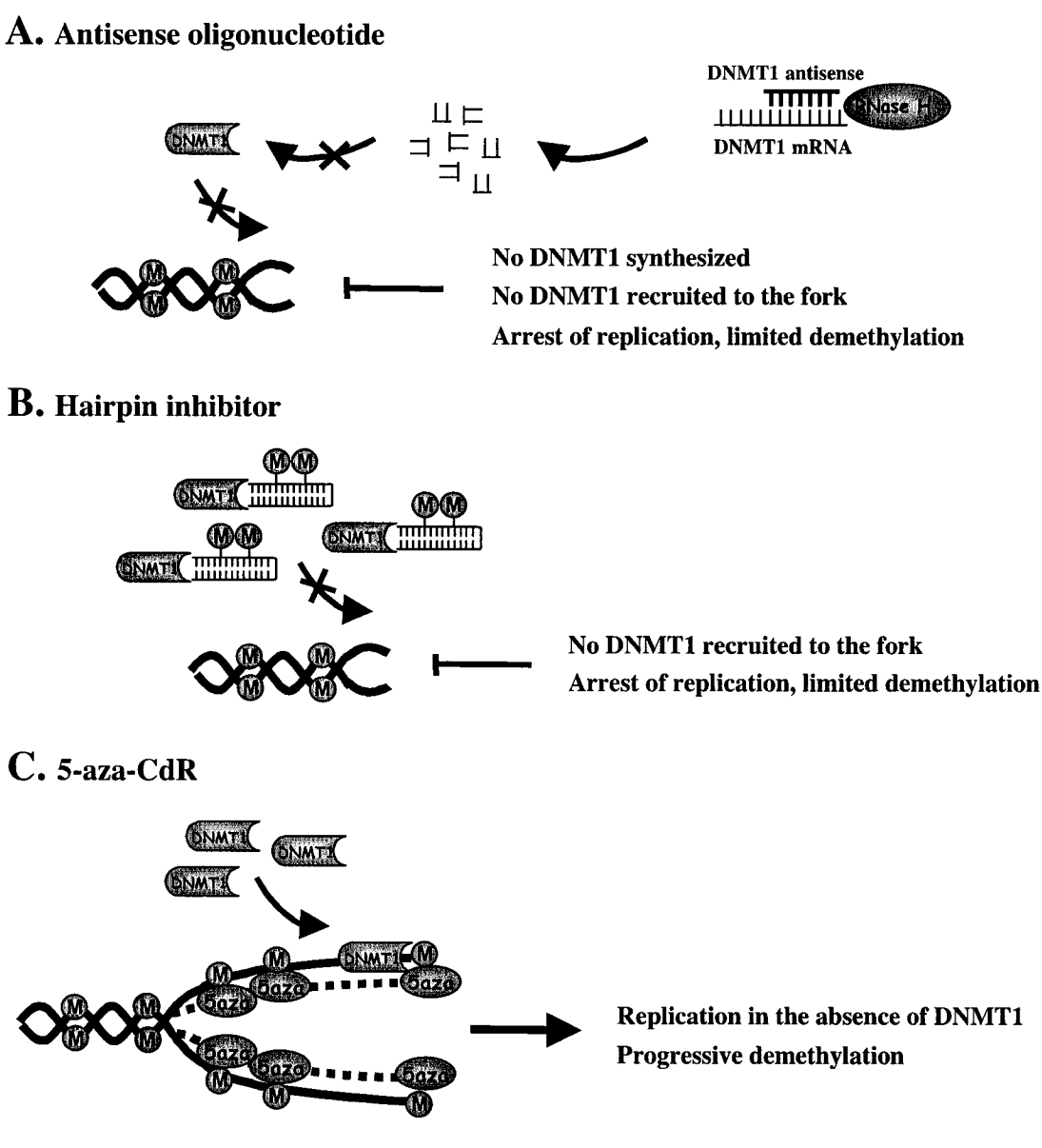


Figure 7. Mechanism of action of DNMT1 inhibitors. A. Antisense oligonucleotides directed against DNMT1 mRNA cause its degradation leading to DNMT1 depletion. In the absence of DNMT1 protein replication is arrested resulting in limited demethylation. B. Hairpin inhibitors bind DNMT1 irreversibly resulting in its limited availability at the replication fork. This also leads to replication arrest and limited demethylation. C. 5-aza-CdR molecules are incorporated into newly synthesized DNA strands and trap DNMT1 molecules during replication. This results in progressive DNA demethylation.

and they are then incorporated into the newly synthesized DNA. However, 5-aza is much less potent since it is a ribonucleoside, and is therefore predominantly incorporated into the newly synthesized RNA (Lubbert, 2000). Since the initial discovery of these drugs in 1964 (Sorm et al., 1964), they were used in clinical trials as anticancer agents in patients with different cancers, mostly leukemias (Lubbert, 2000). However, it took almost two decades until it was discovered that they act as DNA demethylating agents (Jones and Taylor, 1980; Jones and Taylor, 1981). When DNMT1 tries to methylate newly synthesized DNA, it encounters these analogues and forms covalent complex with them (Bouchard and Momparler, 1983). Trapping of DNMT1 onto the DNA results in the replication in its absence, leading to passive DNA demethylation that increases with each round of replication. The mechanism responsible for the antitumorigenic activities of these drugs is believed to work primarily through demethylation and activation of tumor suppressor genes, resulting in the inhibition of cell growth. There are numerous examples of hypermethylated tumor suppressor genes that are reactivated by treatment with 5-aza-CdR (Karpf and Jones, 2002). In addition, since trapping of DNMT1 onto DNA dilutes its free concentration in the cell (Jones, 1985b), it is possible that methylation independent functions of DNMT1 may also be inhibited by these drugs. Although the effect of these drugs on DNMT3a and DNMT3b levels has not been reported, it is likely that their activities are inhibited in a similar way.

Despite the fact that a number of clinical trials assessed the anticancer potential of 5-azaC and 5-aza-CdR, these drugs never gained much popularity. All trials reported toxicities commonly observed with the nucleoside analogues such as leucopenia (Lomen et al., 1975), myelosuppression and GI toxicity (Shnider et al., 1976), granulocytopenia (Velez-Garcia et al., 1977), diarrhea and phlebitis (Gaynon and Baum, 1983). In addition, these drugs cause extensive DNA demethylation, and, as discussed in section VI-i, demethylation could lead to genomic instability and increased tumor invasion. In fact, there are multiple studies showing that treatment of cancer cells with 5-aza-2'-deoxycytidine increases their metastatic potential through the activation of pro-metastatic genes (Frost et al., 1987; Guo et al., 2002; Habets et

al., 1990; Sato et al., 2003). Similarly, mice bearing hypomorphic *dnmt1* allele exhibited wide genomic hypomethylation and developed T cell lymphomas, cautioning against the use of demethylating agents in cancer therapy due to serious secondary effects. This issue will be further discussed in chapter 2.

It has been recently reported that another cytidine analogue, zebularine, inhibits DNA methylation, induces tumor suppressor genes and inhibits tumorigenesis in mice. Unlike 5-aza and 5-aza-CdR, which are highly unstable and toxic, zebularine, can be given orally due to its higher stability, and it seems to cause less toxicity (Cheng et al., 2003). However, similar to the other nucleoside analogues, the basic mechanism of action of zebularine involves trapping DNMT1 onto the DNA, suggesting that it would precipitate similar unwanted effects resulting form the extensive DNA demethylation.

Among the non-nucleoside analogue inhibitors of DNA methylation, antisense oligonucleotides targeted to DNMT1 mRNA are the agents most widely used in research, and are currently being tested in clinical trials in patients with different tumors such as head and neck, kidney, lung, and some leukemias. These antisense oligonucleotides bind to DNMT1 mRNA and target it for degradation, with the net result of knock down of DNMT1 mRNA and protein. Therefore, this mechanism of DNMT1 inhibition affects all of its functions, including both methylation dependent and independent ones. The data presented in chapter 2 illustrates some of the fundamental differences between the effects of DNMT1 antisense and 5-aza-CdR on cell growth and DNA demethylation. DNMT1 antisense causes a much faster and stronger inhibition of cell growth, therefore limiting reduction of global methylation levels due to passive DNA demethylation. On the contrary, 5-aza-CdR inhibits growth much slower, resulting in a marked reduction of the overall methylation. The rapid arrest of cell growth by DNMT1 antisense oligonucleotides is probably a consequence of the inhibition of the methylation independent activities of DNMT1, resulting in both induction of unmethylated cell cycle inhibitors (chapter 2 and 3), and inhibition of DNA replication (Knox et al., 2000). It is therefore possible that this

treatment would avoid some of the serious secondary effects resulting from extensive DNA demethylation. The side effects reported in patients included transient fatigue, anorexia, fever and chills and elevated liver enzymes (Stewart et al., 2003). However, further studies are necessary to assess the long-term effects of this drug in patients.

Another non-nucleoside analogue inhibitor of DNA methylation is an oligonucleotide hairpin inhibitor (Bigey et al., 1999). This oligonucleotide contains complementary sequences of which only one is methylated, so that upon its hybridization a hemimethylated hairpin is formed. This hairpin resembles a preferred, hemimethylated substrate for DNMT1, but since it contains modified backbone, DNMT1 binds to it and becomes trapped. This leads to the reduction of DNMT1 molecules available for different cellular roles, and this reduction may, in some respects, resemble the one resulting from DNMT1 inhibition with antisense oligonucleotides. In fact, hairpin inhibitors have also been shown to cause a rapid growth arrest (Knox et al., 2000), and to induce p21 tumor suppressor through a methylation independent mechanism (chapter 1). One possible mechanism by which hairpin inhibitors arrest DNA replication is by disrupting PCNA-DNMT1 complex (Araujo et al., 2001), which seems to be important for the initiation of replication, as previously discussed.

Two other agents that have been shown to possess DNA demethylation potential are structurally related drugs, procaine and procainamide, which are already in clinical use as anti-arrhythmic and a local anesthetic, respectively. Although these drugs do not act as nucleoside analogues, they were both shown to cause extensive DNA demethylation, probably through binding of the CpG rich sequences of DNA. This demethylation was accompanied by activation of hypermethylated tumor suppressors and growth inhibition (Cornacchia et al., 1988; Lin et al., 2001; Scheinbart et al., 1991; Villar-Garea et al., 2003). Although these drugs do not exhibit serious side effects common to nucleoside analogues, procainamide has been reported to cause a lupus-like autoimmune disease probably resulting from demethylation of DNA in T cells (Cornacchia et al., 1988).

With the exception of DNMT1 antisense, all of the inhibitors mentioned above could potentially inhibit action of all DNMTs. However, since DNMT3a and DNMT3b have been shown to prefer unmethylated DNA substrates, a hemimethylated hairpin inhibitor would probably be less effective in inhibiting these two enzymes. In addition, specific antisense oligonucleotides targeting DNMT3a and 3b were recently developed, and it was shown that only DNMT3b antisense inhibits cell growth and induces apoptosis of cancer cells (Beaulieu et al., 2002). Further studies are needed to assess the potential of these inhibitors in arresting cell growth and cancer therapy.

In summary, although it is clear that hypermethylation of tumor suppressors is a common event in cancer, it is not clear whether their demethylation resulting from inhibition of DNA methyltransferase activity is the primary antitumorigenic effect of DNMT inhibitors. This thesis supports an alternative hypothesis that the methylation-independent effects of DNMT inhibitors are mainly responsible for the rapid cell growth arrest. Understanding the exact mechanisms of DNMT involvement in tumorigenesis is of crucial importance for a design of better drugs that could target specific functions of DNMTs and limit the side effects. Also, depending on tumor characteristics, different tumor types may respond differentially to inhibition of specific DNMT functions, so a better structure-function understanding could aid in designing the right therapy for a particular tumor.

(v) Role of DNA demethylase in cancer

As previously discussed, global DNA hypomethylation and regional hypermethylation are common events that occur simultaneously in cancer, suggesting the existance of multiple defects in DNA methylation machinery. Since DNMT levels are generally elevated in cancer cells, it would be hard to imagine that the observed hypomethylation results from a decreased DNA methyltransferase capacity of these cells. However, a recent discovery of MBD2b/demethylase, together with the reports of its deregulated expression in cancer, could offer a potential explanation for the global hypomethylation. For example, MBD2b/demethylase was found to be more

highly expressed in breast and ovarian cancer tissues when compared with the corresponding normal tissues (Billard et al., 2002; Hattori et al., 2001; Vilain et al., 1999). In addition, MBD2 was identified as one of the tumor antigens that react only with serum of colorectal cancer patients and not with serum of normal patients (Scanlan et al., 2002). One potential mechanism that could explain elevated MBD2 levels in cancer could be its induction by oncogenes. It was shown that expression of Ras oncogene in mouse embryonal P19 cells could induce MBD2 mRNA levels (unpublished data), as well as global DNA demethylation (Szyf et al., 1995). As previously discussed, DNMT1 is also activated by Ras oncogene (Bakin and Curran, 1999; MacLeod et al., 1995; MacLeod and Szyf, 1995), suggesting that the same transformation event could contribute to both hypo- and hypermethylation. However, it is important to note that further studies are needed to establish a direct connection between MBD2 levels and hypomethylation observed in cancer.

Since hypomethylation has been suggested to play an active role in cancer progression, either by causing genomic instability or inducing pro-cancer genes, MBD2 inhibitors were tested as anticancer agents. Antisense knock down of MBD2 using either adenoviral and plasmid vectors, or antisense oligonucleotides inhibited anchorage independent growth of a number of different cancer cell lines as well as xenograft tumors implanted in mice. However, knock down of MBD2 did not affect normal growth of either cancer cells, or normal fibroblasts (Campbell et al., 2004; Slack et al., 2002). This observation is consistent with the finding that MBD2-/- mice are viable and fertile (Hendrich et al., 2001), and suggests that use of MBD2 antisense in anticancer therapy would have limited side effect on normal cell growth. Similarly, antisense oligonucleotides targeting MBD2 exhibited antitumorigenic activity both in mice and in cancer cell lines grown in anchorage independent conditions. Cells grown in normal conditions were not affected, and there was minimal toxicity observed in mice (Campbell et al., 2004). An additional line of evidence supporting the involvement of MBD2 in tumorigenesis comes from a study showing that MBD2 knockout in min-/- mice reduces their susceptibility to developing colorectal cancers (Sansom et al., 2003a).

There are also data that suggests that methyl enriched diets can protect the genome against global hypomethylation (Ross, 2003; van der Westhuyzen, 1985). A potential mediator of this protection is believed to be methyl group donor S-adenosylmethionine (AdoMet), which was shown to protect rats from the development of liver cancer. This protection was inhibited by 5-aza-CdR, and it was proposed that the mechanism of AdoMet action involves stimulation of DNMT activity resulting in increased DNA methylation (Pascale et al., 2002). However, a new mechanism that was recently proposed suggests that AdoMet increases DNA methylation by inhibiting demethylase activity. In accordance, AdoMet inhibited MBD2 activity in vitro, and was shown to inhibit endogenous demethylase activity and anchorage independent growth of cancer cells (Detich et al., 2003).

In conclusion, this data suggests that DNA methylation pattern is a result of a dynamic interplay of two opposing enzymatic activities, DNA methyltransferase and demethylase. The involvement of both of these enzymes in tumorigenesis can explain the paradoxical observations that both hypo and hypermethylation changes occur in cancer cells (Figure 8.). Similarly, although inhibition of DNA methyltransferase and demethylase activities in anticancer therapy seems contradictory, both of these show great potential in inhibiting cancer growth. The key to these phenomena probably lies in the fact that these enzymes are multifunctional proteins whose exact function in space and time is determined by complex interactions with other epigenetic and cellular players.

Summary

The preceeding literature review provides overwhelming evidence that the correct epigenetic regulation of gene expression is essential for the proper function of a cell and an organism as a whole. The regulation of the proteins involved in establishment and maintenance of the epigenome is therefore essential, and any aberrant expression, activity or mistargeting of these proteins could lead to aberrant gene expression and

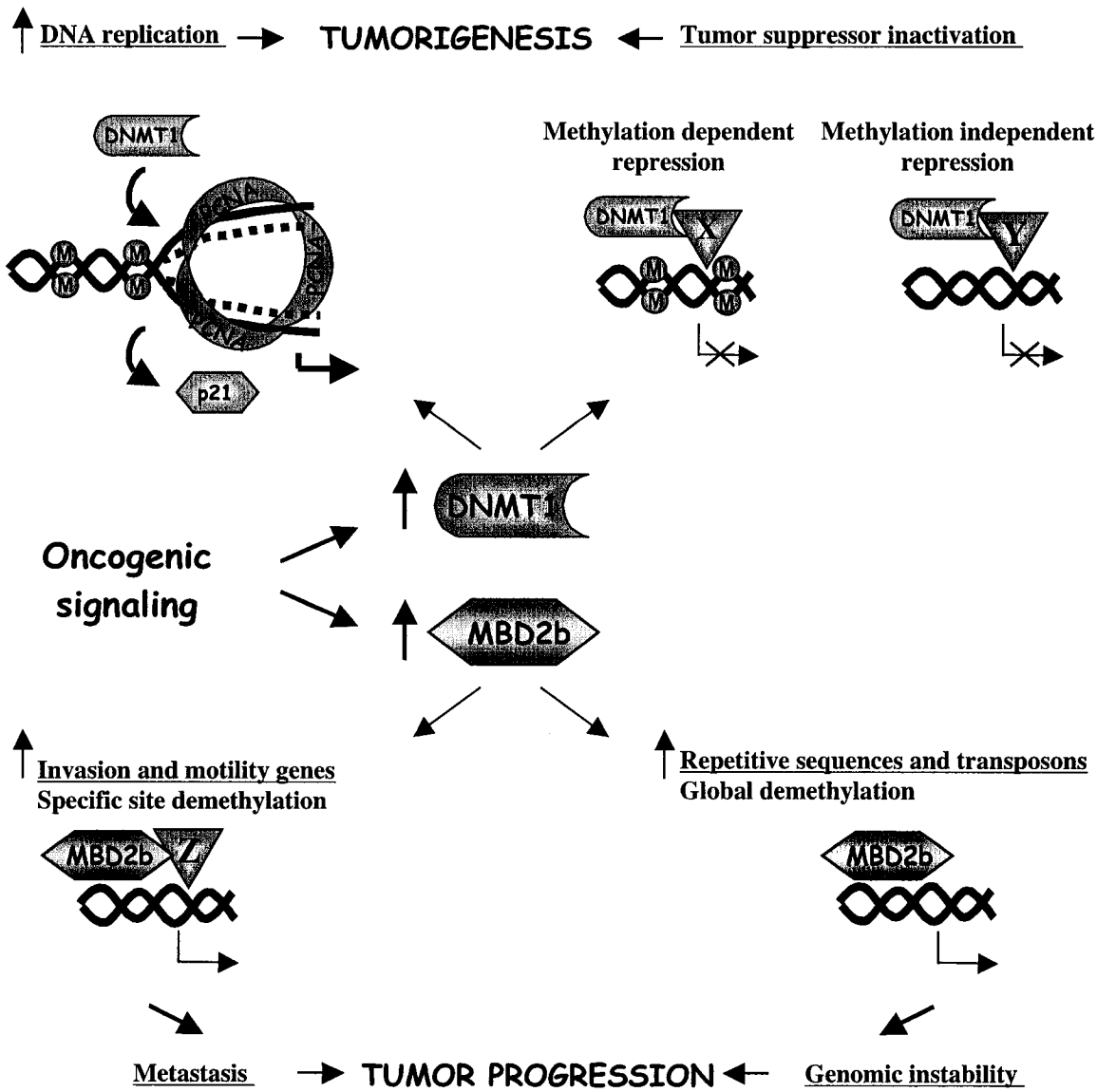


Figure 8. The role of DNMT1 and MBD2b/demethylase in tumorigenesis. Oncogenic signals can lead to induction of both DNMT1 and MBD2b. Higher levels of DNMT1 promote entry into S phase through promoting initiation of DNA replication. In addition, higher levels of DNMT1 or its mistargeting through specific factors (X and Y) repress tumor suppressor genes by both methylation dependent and independent mechanisms. These events are important steps in tumorigenesis. On the other hand, increased MBD2b levels cause global hypomethylation, resulting in genomic instability as a result of the induction of normally silenced repetitive sequences and transposons. In addition, either increased MBD2b levels or its mistargeting through specific factors (Z) result in induction of genes involved in metastasis. These events are important steps in tumor progression.

disease. In cancer, frequently observed regional hypermethylation of tumor suppressors as well as elevated levels of DNMT1 have established this enzyme as a valid target in anticancer therapy. Inhibitors of DNMT1 cause arrest of cancer cell growth, and the generally accepted mechanism of their action is believed to be demethylation and reexpression of the silenced tumor suppressors such as p16. However, the findings that DNMT1 overexpression does not always correlate with tumor suppressor hypermethylation, prompted us to investigate whether DNMT1 plays a role in tumorigenesis independent of its methyltransferase activity. In chapter 1, we studied the effects of DNMT1 inhibition on gene expression in a cell line that has a deletion of the tumor suppressor p16 gene. We specifically looked at the expression of other known tumor suppressors and cell cycle regulators and found that unmethylated tumor suppressor p21 is induced rapidly following inhibition of DNMT1. This induction was shown to be at the transcriptional level and was the first example showing methylation-independent effects of DNMT1 on gene expression. In this study two different inhibitors of DNMT1 were used: hairpin inhibitor and DNMT1 antisense oligonucleotides. Both of these inhibitors reduced the pool of DNMT1 protein available for cellular functions, and their effects were much more rapid than previously reported effects of nucleoside analogue 5-aza-CdR. We hypothesized that the differences in the time course of action of different inhibitors were a result of inhibiting different functions of DNMT1.

In order to get further insight into different functions of DNMT1, in chapter 2 we compared the effects of DNMT1 antisense and 5-aza-CdR on cell growth, DNA methylation and gene expression. Since 5-aza-CdR traps DNMT1 during replication, we hypothesized that this drug would target mainly DNMT1 enzymatic activity, whereas knock down of DNMT1 protein would affect all DNMT1 functions. Indeed, our study was the first one to demonstrate that inhibition of different functions of DNMT1 resulted in very different cellular effects. Knock down of DNMT1 protein by antisense resulted in rapid replication arrest, limited DNA demethylation, and activation of genes in a methylation independent way. In addition, DNMT1 knock down caused a previously undescribed intra S arrest, and induction of stress response

genes. On the other hand, 5-aza-CdR caused a much slower replication arrest, massive DNA demethylation and activation of previously methylated genes. Some of the genes induced by 5-aza-CdR were previously implicated in metastasis, which pointed to the danger of using demethylating agents in anticancer therapy.

In order to further examine methylation independent roles of DNMT1, in chapter 3 we examined in detail the induction of a few candidate unmethylated genes. Based on the published DNMT1 interactions, we hypothesized that the methylation independent effects of DNMT1 require chromatin modulating activities such as HDAC and histone methyltransferase activities. Contrary to our expectations we discovered that DNMT1 can regulate gene expression in both methylation and chromatin modification independent manner. This DNMT1 activity was mediated through Sp1 transcription factor binding sites, which further expanded the role of DNMT1 in gene regulation and raised a possibility that DNMT1 plays a role in cell cycle regulation.

Based on the data presented in this thesis and previously published data, it is clear that the cell has developed mechanisms to ensure concurrent replication of DNA and the epigenome. PCNA trimer has been shown to serve as a loading platform for numerous proteins involved in DNA synthesis. In addition, PCNA has also been shown to bind DNMT1 and a chromatin assembly factor CAF-1, which suggested that it plays additional role in enabling concurrent DNA synthesis, DNA methylation and chromatin assembly. We hypothesized that PCNA could also serve as a loading platform for proteins involved in histone modifications. Since newly deposited histones are acetylated, and need to undergo deacetylation as an important step in their maturation, in chapter 4 we tested whether PCNA could also recruit HDAC activity. Our data shows that PCNA interacts directly with HDAC1 and offers a mechanism to explain how histone code could be replicated concurrently with DNA synthesis. Our data also strengthens the role of PCNA as a unifying factor for the replication of the DNA and the epigenome.

Chapter 1

DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21(WAF1/CIP1/sdi1).

Snezana Milutinovic, J. David Knox and Moshe Szyf.

This chapter has been published in the Journal of Biological Chemistry (2000) 275, 6353-6359

Previous lines of evidence have shown that inhibition of DNA methyltransferase (MeTase) can arrest tumor cell growth; however, the mechanisms involved were not clear. In this manuscript we show that out of 16 known tumor suppressors and cell cycle regulators, the cyclin-dependent kinase inhibitor p21 is the only tumor suppressor induced in the human lung cancer cell line, A549, following inhibition of DNA MeTase by a novel DNA MeTase antagonist or antisense oligonucleotides. The rapid induction of p21 expression points to a mechanism that does not involve demethylation of *p21* promoter. Consistent with this hypothesis, we show that part of the CpG island upstream of the endogenous *p21* gene is unmethylated and that the expression of unmethylated *p21* promoter luciferase reporter constructs is induced following inhibition of DNA MeTase. These results are consistent with the hypothesis that the level of DNA MeTase in a cell can control the expression of a nodal tumor suppressor by a mechanism that does not involve DNA methylation.

INTRODUCTION

DNA methylation of cytosine residues located at the dinucleotide sequence CpG can suppress expression of genes either by directly interfering with the binding of transcription factors (Becker et al., 1987) or by attracting methylated-DNA binding proteins such as MeCP2 (Jones et al., 1998; Nan et al., 1997; Nan et al., 1998). To ascertain that the epigenetic information encoded in the methylation pattern is faithfully maintained, the expression of the maintenance DNA MeTase1 enzyme (DNMT1), which catalyzes the transfer of a methyl group from *S*-adenosyl-methionine to the 5' position on cytosines residing at the dinucleotide CpG (Gruenbaum et al., 1982), is tightly coordinated with DNA replication (Araujo et al., 1998a) and the state of growth of the cell (Szyf et al., 1991). Different protooncogenic pathways can up-regulate *dnmt1* expression (Rouleau et al., 1995), and high levels of *dnmt1* mRNA have been observed in many cancer cells (el-Deiry et al., 1991; Kautiainen and Jones, 1986). It has been proposed that increased DNA MeTase levels are a downstream component of oncogenic programs (Szyf, 1994) and that they play a causal role in cellular transformation (MacLeod and Szyf, 1995; Wu et al., 1993). This hypothesis has been supported by the observation that a reduction in DNA MeTase levels by either 5-aza-deoxycytidine (Laird et al., 1995; MacLeod and Szyf, 1995), DNA MeTase antisense mRNA or antisense oligonucleotides can reverse tumor growth *in vivo* and *in vitro* (Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997).

The mechanisms responsible for cellular transformation by DNMT1 and the reversal of transformation by DNA MeTase inhibition are unknown. One attractive hypothesis is that high levels of DNA MeTase lead to ectopic methylation and inactivation of tumor suppressor genes such as *p16* (Merlo et al., 1995). Similarly, the inhibition of DNA MeTase could result in demethylation and lead to activation of *p16* (Gonzalgo et al., 1998; Otterson et al., 1995). However, because both the increase in DNA MeTase levels and its inhibition by pharmacological inhibitors are global processes, it is difficult to understand how they could predictably result in a discrete change of the

methylation state of specific sites. A more likely explanation is that ectopic methylation of tumor suppressors in tumor cells is a slow and stochastic process, but the aberrant methylation events are selected because they confer a growth advantage. This hypothesis is supported by the observation that in tumors bearing one mutant and one normal allele of p16, only the normal allele is methylated (Myohanen et al., 1998). Similarly, partial inhibition of DNA MeTase with pharmacological agents results in a stochastic demethylation of a specific site only in a fraction of the cells at each round of replication.

An alternative possibility is that the DNMT1 protein might have a more direct and immediate effect on the state of cellular growth and transformation (Szyf, 1998). To investigate this possibility we inhibited DNA MeTase and examined the expression of genes known to control the cell cycle. In the past, 5-aza-deoxycytidine has been used as the standard inhibitor of DNA MeTase (Jones, 1985a). Unfortunately, the incorporation of 5-aza-deoxycytidine into DNA and subsequent trapping of DNA MeTase enzyme unto the replicating DNA (Wu and Santi, 1985) results in pleiotropic effects that confound the interpretation of the results (Juttermann et al., 1994). Therefore, we have developed two other approaches to inhibit DNA MeTase: antisense oligonucleotides (Ramchandani et al., 1997) and oligonucleotide-based DNA MeTase antagonists that form a stable complex with DNA MeTase and inhibit its activity at an EC50 of 60 nM (Bigey et al., 1999; Szyf, 1998). We have previously shown that inhibition of DNA MeTase by DNA MeTase antagonists results in a rapid inhibition of DNA replication that is inconsistent with a stochastic model (Bigey et al., 1999). In this manuscript we demonstrate that inhibition of DNA MeTase results in the rapid induction of the known tumor suppressor and cell cycle regulator p21 by a mechanism that does not involve DNA methylation of the *p21* promoter.

EXPERIMENTAL PROCEDURES

Cell Culture, Antisense, and DNA MeTase Antagonist Treatment-- A549 cells, a human non-small cell lung carcinoma cell line (Giard et al., 1973) (ATCC: CCL 185),

were grown in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. HEK 293 cells, a human adenovirus type 5 transformed human embryonal kidney cell line (Graham et al., 1977) (ATCC, CRL 1573), were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. For oligonucleotide treatment A549 cells were plated at a concentration of 2.5 x 10^5 cells/100-mm tissue culture dish 18 h prior to treatment. The DNA MeTase antagonist (3118) used in our study is a phosphorothioate modified hemimethylated hairpin of the following sequence: 5'-CTGAA(methyl)CGGAT(methyl)CGTTTCGATCCGTTCAG-3'. The sequence of the inactive analog used in our study (3188) is identical; it is also phosphorothioate modified but has a 2'-*O*-methyl modification of the sugar backbone. The oligos 3118 and 3188 have the same structure as the DNA MeTase antagonist 3016 and the inactive analog 3060, which were previously described by us (Bigey et al., 1999), except that 3118 and 3188 bear a fluorescein tag at the 5' end. The sequence of the phosphorothioate antisense oligodeoxynucleotide used in this study is: 5'-AAGCATGAGCACCGTTCTCC-3' (as) and its mismatch control oligonucleotide (mm) has a 3-base pair mismatch. For determining *p21* mRNA stability, the 4 h oligonucleotide treatment was followed by actinomycin D treatment (10 mg/ml) for 0, 4, and 8 h. For p53 binding experiments, A549 cells were treated with actinomycin D (10 μg/ml) for 12 h in phosphate-buffered saline, following which the medium was replaced by a regular growth medium for 12 h, and the cells were harvested. For transient transfection experiments, HEK 293 cells were plated 18 h prior to transfection at a concentration of 5 x 10^5 cells/100-mm tissue culture dish. The medium was replaced 24 h after transfection, and the cells were harvested 48 h after transfection.

Multi-probe RNase Protection Assay-- Total RNA was prepared from the cells at the indicated time points after the treatment using standard protocols. 15 μg of the RNA were subjected to an RNase protection assay using two multi-probe template sets (PharMingen's RiboQuant) as recommended by the manufacturer. The first set (hCC-2) includes templates for antisense RNA probes for *p130, Rb, p107, p53, p57, p27,*

p21, p19, p18, p16, p14/15, and the controls *L32* and *GAPDH*. The second set (hTS-1) includes templates for antisense RNA probes for *p130, Rb, p107, DP1, DP2, E2F1, E2F2, E2F4, L32*, and *GAPDH*. The protected RNA fragments were resolved on a urea-polyacrylamide sequencing gel according to their size and imaged by autoradiography. The intensity of the bands was quantified by densiometric scanning (MasterScan, Scanalytics) and was normalized to the intensity of the control *GAPDH* mRNA in each lane.

Western Blot Analysis-- Total cell extracts were prepared using standard protocols and resolved on a SDS-polyacrylamide gel electrophoresis (7.5% for DNA MeTase and 12% for p21). After transferring to polyvinylidene difluoride membrane and blocking the nonspecific binding with 5% milk, p21 protein was detected using rabbit polyclonal IgG (Santa Cruz, p21 (C-19)) at 1:500 dilution, followed by peroxidase-conjugated anti rabbit IgG (Sigma) at 1:5000 dilution, and enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). DNMT1 protein was detected as described previously (Ramchandani et al., 1997).

Competitive PCR for Quantification of p21 mRNA Levels-- Total RNA (1 μg) was reverse transcribed with reverse transcriptase and random primers (Superscript/Life Technologies, Inc.) using the manufacturer's protocol in the presence of 12.5 μCi of ^{35}S-labeled dCTP (1250 Ci/mmol) to quantify the efficiency of reverse transcription. Equal amounts of reverse transcribed cDNA (70,000 cpm as determined by the incorporation of ^{35}S-labeled dCTP) were subjected to PCR amplification in the presence of increasing concentrations of a competitor DNA fragment that amplifies with the same set of primers but yields a product that is shorter by 52 base pairs. The following PCR primers and the competitor were designed using *p21* mRNA sequence (GenBankTM accession number S67388) previously described (Tao et al., 1997): 5'-GCCCAGTGGACAGCGAGCAG-3' (sense, starting at 143), 5'-GCCGGCGTTTGGAGTGGTAGA-3' (antisense, starting at 515), and 5'-CAGCGAGCAGGAGGCCCGTGAGCGATGGA-3' (primer used to create

competitor, ending at 223). PCR conditions were as follows: 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min. (40 cycles).

Electrophoretic Mobility Shift Assay-- DNA binding was analyzed by electrophoretic mobility shift assay using the ^{32}P-labeled p53 consensus sequence 5'-AGACATGCCTAGACATGCCT-3' as described (McLure and Lee, 1998), except that the binding reaction contained 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-Cl, pH 7.6, 2 μl of poly(dI-dC) (2 mg/ml), 25 μg of total cell extract, and 3 μl of polyclonal antibody, pAb 421, against p53 (Woo et al., 1998). The complexes were resolved on a nondenaturing polyacrylamide gel electrophoresis (5%).

Bisulfite Mapping of the p21 Promoter-- Bisulfite mapping was performed as described previously with minor modifications (Clark et al., 1994). 50 ng of sodium bisulfite treated A549 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 (as recommended by the manufacturer), and the clones were sequenced using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The primers used for the amplification of the *p21* genomic region (GenBankTM accession number U24170) were: 5'-GTAAAAAAAGTTAGATTTGTGG-3' (sense, starting at 4278), 5'-CTCACCTCCTCTAAATACC-3' (reverse, starting at 4640), 5'-AGGGATTGGGGGAGGAG-3' (nested sense, starting at 4342), and 5'-CAACTACTCACACCTCAAC-3' (nested reverse, starting at 4566).

Northern Blot Analysis-- RNA was isolated by the guanidinium isothiocyanate method (Rouleau et al., 1992). *p21* mRNA level was determined by Northern blot analysis using 10 μg of RNA and hybridization with ^{32}P-labeled human *p21* probe (positions 205-544, accession number U03106). The levels of expression of *p21* mRNA were quantified by densitometric scanning (MasterScan, Scanalytics) and normalized in

each lane to the amount of total RNA as determined by hybridization with ^{32}P-labeled 18 S ribosomal RNA oligonucleotide probe (Szyf et al., 1990).

Luciferase Assays-- The calcium-phosphate precipitation method was used to transiently co-transfect HEK 293 cells with either 2 μg of promoterless luciferase reporter or luciferase reporter constructs containing 2145 or 94 base pairs of the *p21* promoter upstream of the transcriptional start site (Somasundaram et al., 1997). The cells were co-transfected with 5 μg of either control pCR3.1 DNA (Invitrogen) or a *DNMT1* (base pairs 396-5066) antisense expression vector in pCR3.1 (as). 1 μg of pEGFP-C1 (CLONTECH) was included with all transfections to control for transfection efficiency by fluorescence microscopy. The luciferase activity was assayed as described previously (el-Deiry et al., 1993).

DNA MeTase Activity Assay-- DNA MeTase activity was assayed using 3 μg of HEK 293 nuclear extract protein prepared as described previously (Szyf et al., 1991). The reaction was carried out in a final volume of 30 μl containing 10 mM Tris-HCl, 25% glycerol, 5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, 1 μl of *S*-[*methyl*-^{3}H]adenosyl-L-methionine (78.9 Ci/mmol; Amersham Pharmacia Biotech) as a methyl donor, and 0.2 mM of a synthetic hemimethylated double-stranded oligonucleotide substrate as a methyl acceptor as described previously (Szyf et al., 1991). Following a 3-h incubation at 37 °C, the incorporation of methyl groups into the DNA substrate was determined by scintillation counting of tricloroacetic acid precipitable counts. The background counts reflecting methylation of other macromolecules present in the nuclear extract as well as endogenous DNA methylation were determined by incubating the nuclear extract under the same conditions in the absence of the hemimethylated substrate. The background values were subtracted from the values obtained for each point. The results are presented as the averages of three determinations.

RESULTS

p21 mRNA and Protein Levels Are Induced Following Inhibition of DNA MeTase by Either Antisense or a DNA MeTase Antagonist-- To determine whether inhibition of DNA MeTase in cancer cells results in induction of the expression of tumor suppressors, we treated A549 human lung carcinoma cells with either antisense oligonucleotides (as) directed against *DNMT1* mRNA or their mismatched control (mm), or a DNA MeTase antagonist (3118) and its inactive control (3188) for 5 days. The DNA MeTase antagonist was previously shown to inhibit DNA MeTase activity in nuclear extracts prepared from control A549 cells (*ki* of 65 nM)(Bigey et al., 1999). The antisense oligonucleotide was previously shown to prevent the production of detectable levels of DNMT1 protein at an EC50 of 60 nM (data not shown). Two different methods of inhibition of DNA MeTase were used to exclude the possibility that the changes observed represented a nonspecific side effect. RNA prepared from the treated cells was subjected to an RNase protection assay with two sets of multiple probe templates that revealed the level of expression of 16 known cell cycle regulators and tumor suppressors. As indicated in Fig. 1, *p21* was the only known tumor suppressor that was induced by both treatments. p21 protein levels are also induced upon treatment with both DNA MeTase antisense oligonucleotides and antagonists as determined by the Western blot analysis shown in Fig. 2*A*.

Time Course of p21 Induction by DNA MeTase Antagonists-- The kinetics of induction of *p21* following inhibition of DNA MeTase can provide an indication as to the mechanism involved. We chose to use the DNA MeTase antagonist (3118) because it directly interacts with DNA MeTase protein. In contrast, the antisense acts indirectly by reducing mRNA levels, which leads to the reduction of protein levels, and the turnover rate of DNA MeTase might confound the interpretation of the results. Any inhibitor of DNA methylation will have its most pronounced effect at the time of DNA replication. Therefore, the fraction of cells that are demethylated at a specific subset of regulatory sites will increase with the number of replication rounds. The level of *p21* mRNA following DNA MeTase antagonist treatment was quantified by competitive

PCR, which showed that the induction of *p21* mRNA was rapid (7-fold after 8 h) and that it decreased over the period from 8 h to 5 days (Fig. 3*B*). These results are consistent with a mechanism that does not involve demethylation of specific sites. The competitive PCR also verified the induction of *p21* following treatment with a DNA MeTase antagonist observed in the RNase protection experiments.

There is a discrepancy between the time course of induction of *p21* mRNA and protein levels. The highest level of *p21* mRNA induction is observed shortly after treatment (8 h) (Fig. 3), whereas the levels of p21 protein seem to increase up to 5 days following treatment (Fig. 2*A*). Similarly, although DNMT1 protein is undetectable within 1 day after antisense treatment (Fig. 2*B*), p21 protein levels continue to increase up to 5 days (Fig. 2*A*). A possible explanation is that the turnover rate of protein is slower than that of the mRNA, resulting in gradual accumulation of protein over time following induction of *p21* transcription. Another possibility is that inhibition of DNA MeTase induces p21 protein levels by an additional post translational mechanism as recently suggested by Fournel *et al.* (Fournel et al., 1999). Further experiments are required to clarify this question.

Part of the p21 Promoter That Overlaps with the p21 CpG Island Is Not Methylated in Untreated A549 Cells-- DNA methylation is unlikely to play a role in the modulation of p21 gene expression because the p21 gene is active even in the untreated cells (Fig. 1). DNA methylation is believed to be an on-off switch in gene expression (Yisraeli and Szyf, 1984). CpG islands present in the promoter regions have been shown to be susceptible to hypermethylation in many cancer cells (Baylin et al., 1998) and are prime candidates to be involved in regulation of their respective genes. To study the state of methylation of *p21* promoter, we performed a bisulfite mapping of the portion of the *p21* promoter that also partially overlaps with the *p21* CpG island (Fig. 4*B*). The portion tested (-243 to +1) contains no methylated CpGs in the untreated cells (Fig. 4*A*). These results are consistent with the hypothesis that DNA MeTase antagonists do not induce *p21* mRNA levels by demethylation of its promoter.

Inhibition of DNA MeTase Does Not Detectably Induce p53 Binding to Its Consensus Binding Sequence-- p21 plays a central role in the response to DNA damage and has been shown to contain p53 binding sites in its promoter region and to be a downstream effector of p53 (el-Deiry et al., 1993). Our results do not support the hypothesis that p53 mRNA is induced following treatment with DNA MeTase inhibitors (Fig. 1). An alternative possibility is that either the presence of the oligonucleotide inhibitors or the inhibition of DNA methylation are interpreted as DNA damage resulting in an activation of p53 and an increase in p53 DNA binding to p53 recognition sequences (McLure and Lee, 1998). To test this hypothesis, we performed the electrophoretic mobility gel shift assay shown in Fig. 5. Treatment of A549 cells with the DNA damaging agent actinomycin D (10 μg/ml) results in activation of p53 and binding to its consensus sequence. This produces a super-shifted complex stabilized by anti-p53 antibody pAb421, as previously shown (McLure and Lee, 1998). In contrast, treatment with DNA MeTase inhibitors does not activate p53 consensus sequence binding.

Induction of p21 Is Not Post-transcriptionally Regulated-- Several reports have indicated that the stability of the *p21* mRNA could be altered by different signals such as differentiation (Schwaller et al., 1995) and oxidative stress (Esposito et al., 1997). It has also been shown that *p21* mRNA contains a conserved element in its 3'-untranslated region that is bound by the Elav-like mRNA stabilizing proteins (Joseph et al., 1998). To determine whether DNA MeTase antagonists increase the stability of *p21* mRNA, we treated A549 cells with either the DNA MeTase antagonist (3118) or the inactive analog (3188). After 4 h, the mRNA transcription inhibitor actinomycin D (10 mg/ml) was added to the treated cultures and an untreated control, and the level of *p21* mRNA was determined by a Northern blot analysis at different time points after initiation of actinomycin D treatment. As observed in Fig. 6, DNA MeTase antagonist treatment does not considerably alter the stability of *p21* mRNA, suggesting that induction of *p21* mRNA occurs at the transcriptional level.

DNA MeTase Inhibition Induces the Expression of p21 Promoter Luciferase Reporter Construct-- *p21* luciferase reporter constructs were used to confirm that the induction

of *p21* occurred at the transcriptional level. Plasmids amplified in *Escherichia coli* cells, which do not bear a CpG MeTase, are fully unmethylated. Therefore, these experiments directly test the hypothesis that induction of *p21* transcription by inhibition of DNMT1 occurs by a mechanism that is independent of methylation of the *p21* promoter. HEK 293 cells were used in these experiments because they are easily transfected. The results presented in Fig. 7*A* show that inhibition of DNMT1 by expression of a full-length antisense *dnmt1* mRNA results in induction of both *p21* promoter luciferase reporter constructs. An assay of DNA MeTase activity confirmed that the transient transfection of the *DNMT1* antisense construct resulted in a significant reduction of DNA MeTase activity in the transfected population of HEK cells (Fig. 7*B*). To rule out the possibility that the *p21* promoter luciferase reporter constructs are *de novo* methylated in HEK cells and that antisense treatment inhibits this activity, we subjected DNA prepared from transiently transfected HEK cells to digestion with either *Hpa*II, which is sensitive to methylation at the CCmGG sequence, or *Msp*I, which is insensitive to this methylation. The digested DNA was subjected to a Southern blot analysis using the entire *p21* promoter luciferase reporter construct as a probe. Methylation in any one of the *Hpa*II sites would have resulted in partially digested fragments. The Southern blot does not reveal partially cleaved *Hpa*II fragments, thus suggesting that no significant *de novo* methylation of the studied *Hpa*II sites occurs in HEK cells (Fig. 7*C*).

The induction of *p21* by DNMT1 inhibition does not require the presence of sequences upstream to the minimal promoter (-94bp) and therefore does not require the presence of p53 recognition elements. This also confirms that the induction of *p21* promoter activity by DNA MeTase inhibition is independent of the methylation status of the promoter at sequences other than those mapped in the bisulfite sequencing experiment (Fig. 4).

DISCUSSION

Inhibition of DNA MeTase has been previously shown to arrest the growth of tumor cells (Bigey et al., 1999; Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997); however, the mechanisms involved are not clear. An attractive hypothesis is that inhibition of DNA MeTase results in the demethylation of tumor suppressors that were previously inactivated by methylation. In support of this hypothesis it has been shown that the tumor suppressor *p16*, which is methylated and inactive in many tumor cells (Merlo et al., 1995), could be activated by prolonged treatment with an inhibitor of DNA methylation, 5-aza-deoxycytidine (Gonzalgo et al., 1998; Merlo et al., 1995; Otterson et al., 1995).

In this manuscript we tested the hypothesis that alternative mechanisms are involved in the arrest of cell growth by DNA MeTase inhibitors. For this reason A549, a NSCLC cell line with a homozygous deletion of *p16INK4*, was chosen to address this question (Fukuoka et al., 1997). Two novel inhibitors of DNA MeTase that inhibit DNA MeTase by different mechanisms were used to increase the confidence that the effects observed were a consequence of DNA MeTase inhibition. Out of 16 genes known to regulate the cell cycle, only one, the housekeeping gene *p21*, was shown to be induced following inhibition of DNA MeTase.

The negative effects of DNA MeTase inhibitors on the growth of A549 cells, which we have reported previously (Bigey et al., 1999), can be explained by an induction of *p21*. The ectopic expression of p21 has been shown to arrest the growth of tumor cells (Yang et al., 1995), and through its inhibition of cyclin dependent kinases p21 is known to block entry into S and S phase progression (Luo et al., 1995; Xiong et al., 1993). These functions allow p21 to play a role in mediating stop signals such as those triggered by terminal differentiation and contact inhibition (Halevy et al., 1995; Wu and Schonthal, 1997). In addition, p21 can directly arrest DNA replication in response to DNA damage by binding to proliferating cell nuclear antigen (Waga et al., 1994).

Unlike what is expected from the p16 model, the induction of p21 expression is rapid (7-fold induction 8 h after the treatment) and points to a novel mechanism independent

of DNA methylation. Our experiments confirm that at least a portion of the *p21* promoter is not normally methylated (Fig. 4) and demonstrate the ability of DNA MeTase inhibition to induce expression of a luciferase reporter gene under the control of an unmethylated *p21* promoter (Fig. 7).

A recent publication showed that antisense inhibition of *DNMT1* mRNA in a bladder carcinoma cell line T24 results in an increase of p21 protein levels (Fournel et al., 1999). Our paper complements these results by (a) showing that the same results are produced when DNA MeTase is directly inhibited by a hemi-methylated hairpin phosphorothioate oligos as well as when inhibited by antisense oligos, (b) demonstrating the induction of p21 in a second, independent cell line, (c) providing strong evidence that the increase in p21 protein after DNA MeTase inhibition is primarily a consequence of *p21* mRNA induction, and (d) demonstrating that *p21* induction does not require a change in the methylation state (normally unmethylated) of its promoter.

It is still unclear whether p21 induction is triggered by a decline in DNA MeTase activity or DNA MeTase amount. Further experiments are required to address this question. In any case, the ability of DNA MeTase to modulate p21 levels, as well as compete with p21 for its binding site on proliferating cell nuclear antigen (Chuang et al., 1997), suggests that the increased DNA MeTase expression observed in tumor cells may override p21 dependent stop signals and result in unregulated growth.

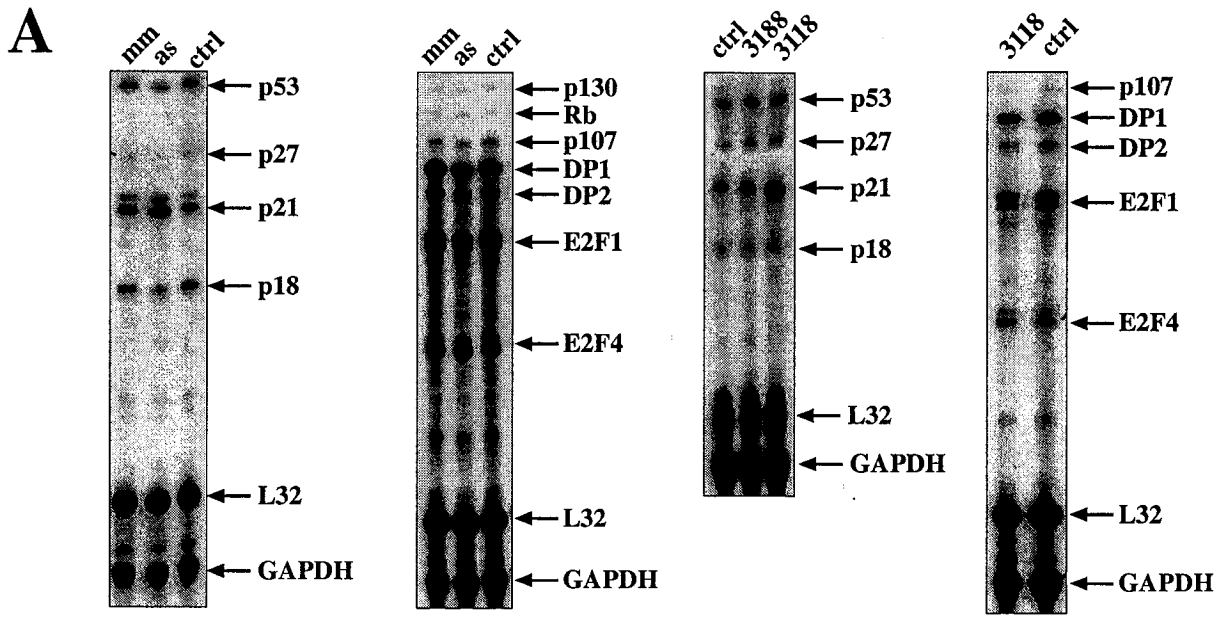
ACKNOWLEDGEMENTS

We thank Dr. W. S. El-Deiry for the luciferase reporter constructs and Dr. Patrick W. K. Lee for pAb421 antibody.

This work was supported by the Medical Research Council of Canada.

FIGURE LEGENDS

Fig. 1. Inhibition of DNA MeTase induces p21 mRNA. *A*, a multi-probe RNase protection assay was performed using two sets of probes and 15 μg of total RNA isolated from A549 cells treated for 5 days as follows. In the *left two panels* the cells were treated with antisense oligonucleotide to *DNMT1* mRNA (*as*), antisense mismatch control (*mm*), and the mock treated control (*ctrl*); in the *right two panels* the cells were treated with DNA MeTase antagonist (*3118*), inactive analog of the antagonist (*3188*), and mock treated control (*ctrl*). The RNAs protected with the probes are indicated by the *arrows*. No signals were detected with the following probes: *p16, p19, p57*, and *p14/15*. *B*, mRNA levels were quantified, normalized to L32, and presented as a fold induction relative to ctrl levels, which were assigned the value of 1.


A
mm
as
ctrl
p53
p27
p21
p18
L32
GAPDH
mm
as
ctrl
p130
Rb
p107
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DP2
E2F1
E2F4
L32
GAPDH
ctrl
3188
3118
p53
p27
p21
p18
L32
GAPDH
3118
ctrl
p107
DP1
DP2
E2F1
E2F4
L32
GAPDH

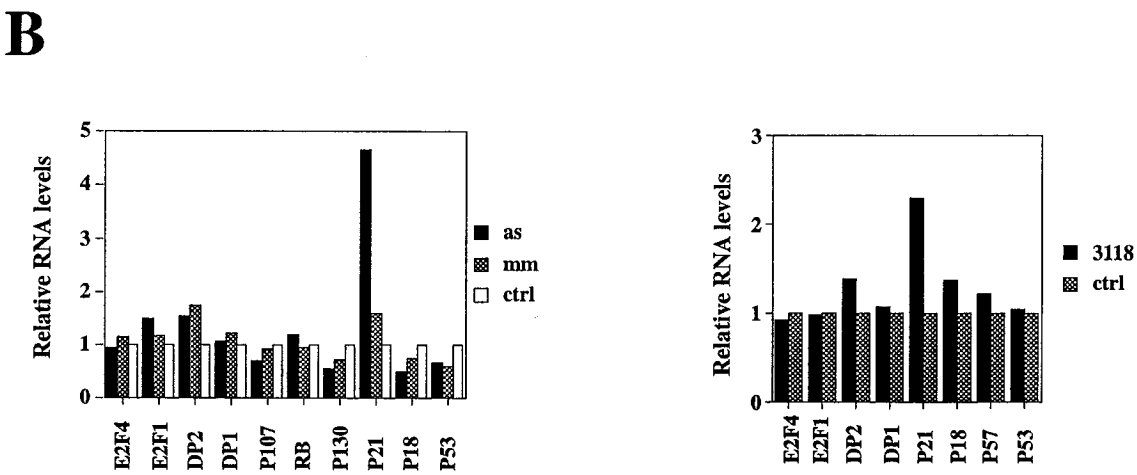
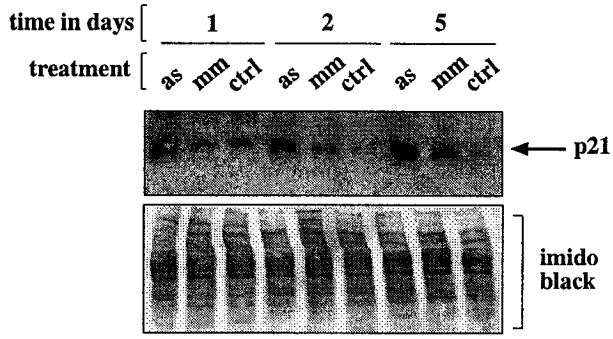
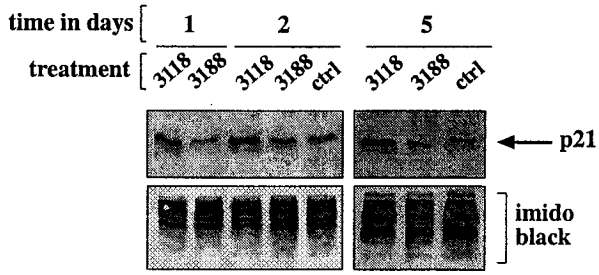

B
Relative RNA levels
0
1
2
3
4
5
as
mm
ctrl
E2F4
E2F1
DP2
DP1
P107
RB
P130
P21
P18
P53
Relative RNA levels
0
1
2
3
3118
ctrl
E2F4
E2F1
DP2
DP1
P21
P18
P57
P53

Fig. 2. Inhibition of DNA MeTase induces p21 protein. Shown is Western blot analysis of total cell extracts isolated from A549 cells treated with either antisense oligonucleotides to *DNMT1* mRNA (*as*), antisense mismatch control (*mm*), DNA MeTase antagonist (*3118*), inactive analog of the antagonist (*3188*), or the mock treated controls (*ctrl*) for 1, 2, or 5 days. The positions of p21 protein (*A*) and DNA MeTase protein (*B*) are indicated by *arrows*.

A


time in days
1
2
5
treatment
as
mm
ctrl
as
mm
ctrl
as
mm
ctrl
p21
imido
black


time in days
1
2
5
treatment
3118
3188
3118
3188
ctrl
3118
3188
ctrl
p21
imido
black

B

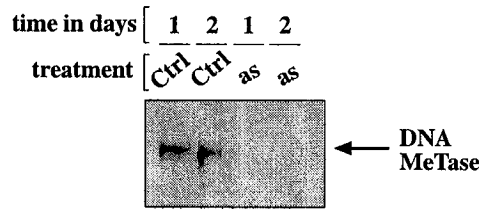
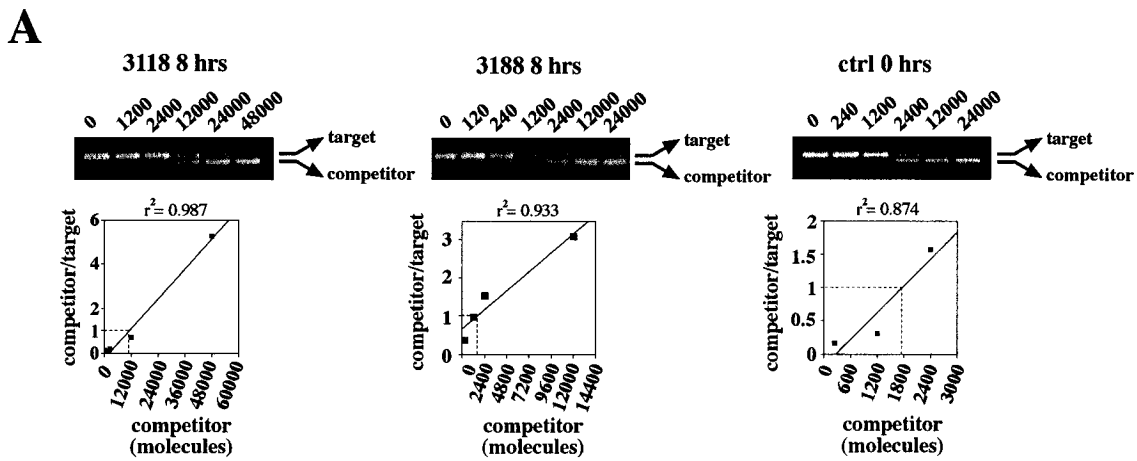

time in days
1
2
1
2
treatment
Ctrl
Ctrl
as
as
DNA
MeTase

Fig. 3. The time course of induction of p21 mRNA levels by the DNA MeTase antagonist. *A*, total RNA was isolated from control A549 cells (*ctrl*) and A549 cells treated with DNA MeTase antagonist (*3118*) or its inactive analog (*3188*) for 8 h (0.3 days). 1 μg of RNA prepared from control and treated cells was reverse transcribed into cDNA in the presence of radiolabeled nucleotides. Equal counts of labeled cDNA (*target*) and a varying number of competitor molecules were used as templates for competitive PCR reactions with primers targeted to *p21* mRNA sequence. *Dashed lines* show the number of competitor molecules needed to generate equal amount of product as the target DNA (competitor/target = 1). *B*, quantification of competitive PCRs performed as in *A* on the RNA extracted from cells treated for 0.3, 1, 2, and 5 days. The *p21* mRNA levels extracted from cells treated with DNA MeTase antagonist (*3118*) are represented relative to those treated with inactive analog (*3188*) which were assigned the value of 1.


A
3118 8 hrs
0 1200 2400 12000 24000 48000
target
competitor
r²= 0.987
competitor/target
competitor (molecules)
3188 8 hrs
0 120 240 1200 2400 12000 24000
target
competitor
r²= 0.933
competitor/target
competitor (molecules)
ctrl 0 hrs
0 240 1200 2400 12000 24000
target
competitor
r²= 0.874
competitor/target
competitor (molecules)

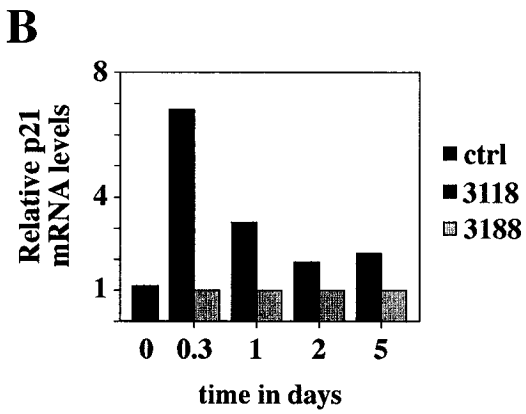
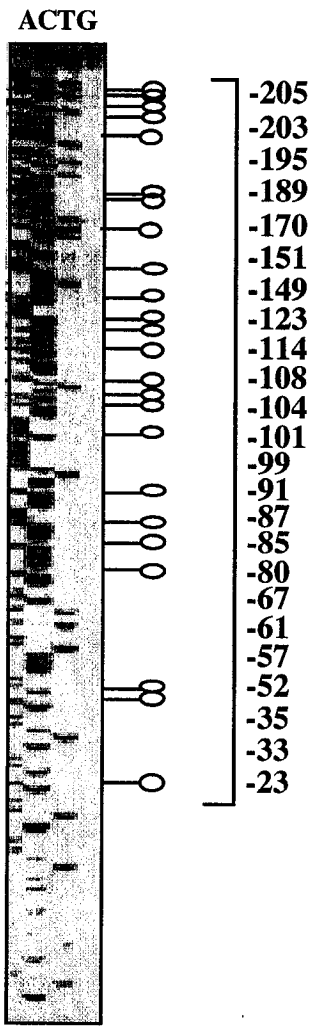

B
Relative p21 mRNA levels
ctrl
3118
3188
time in days

Fig. 4. Part of the p21 promoter which partially overlaps with the p21 CpG island is completely unmethylated. *A*, p21 promoter region between +1 and -243 was bisulfite mapped. DNA extracted from control A549 cells was treated with sodium bisulfite, which converts all the cytosines in DNA into thymidines but cannot convert methylated cytosines. One of the modified strands was amplified by PCR and cloned into the TA cloning vector as described under "Experimental Procedures." Depending on the orientation of the cloned insert, either Cs or Gs will be present only in the methylated positions on the sequencing gel. In this case the presence of a band in the *G lane* indicates the presence of a methylated cytosine on the complimentary strand. *Lollipops* indicate the potential methylation sites. *Empty lollipops* indicate unmethylated CpG sites. *B*, physical map of the partial *p21* promoter showing the density of CpG sites (*small vertical lines*). p53 binding sites and the RNA start site (+1) are indicated with *arrows* at the *bottom*. The *arrows* at the *top* indicate the primers used to bisulfite map the region shown in *A*.

A


ACTG
-205
-203
-195
-189
-170
-151
-149
-123
-114
-108
-104
-101
-99
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-85
-80
-67
-61
-57
-52
-35
-33
-23

B

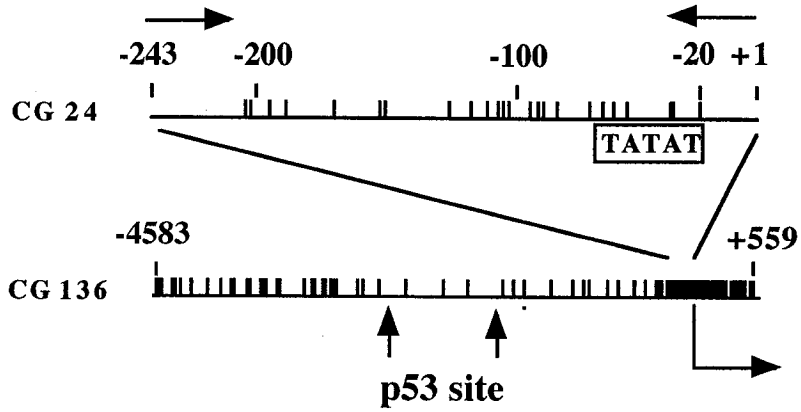

-243
-200
-100
-20
+1
CG 24
TATAT
-4583
+559
CG 136
p53 site

Fig. 5. DNA MeTase inhibitor does not induce p53 consensus sequence binding activity. A549 cells were treated with either the DNA damaging agent actinomycin D (*Act D*), antisense oligonucleotide to *DNMT1* mRNA (*as*), antisense mismatch control (*mm*), DNA MeTase antagonist (*3118*), inactive analog of the antagonist (*3188*), or mock treated control (*ctrl*) for 0.5, 1, 2, or 5 days as indicated. Total cell extracts were prepared and assayed for p53 DNA binding by electrophoretic mobility shift assay. All reactions contained the polyclonal antibody pAb421. The *arrow* indicates the position of the p53/pA421-DNA complex. The *upper band* is the position of the wells.

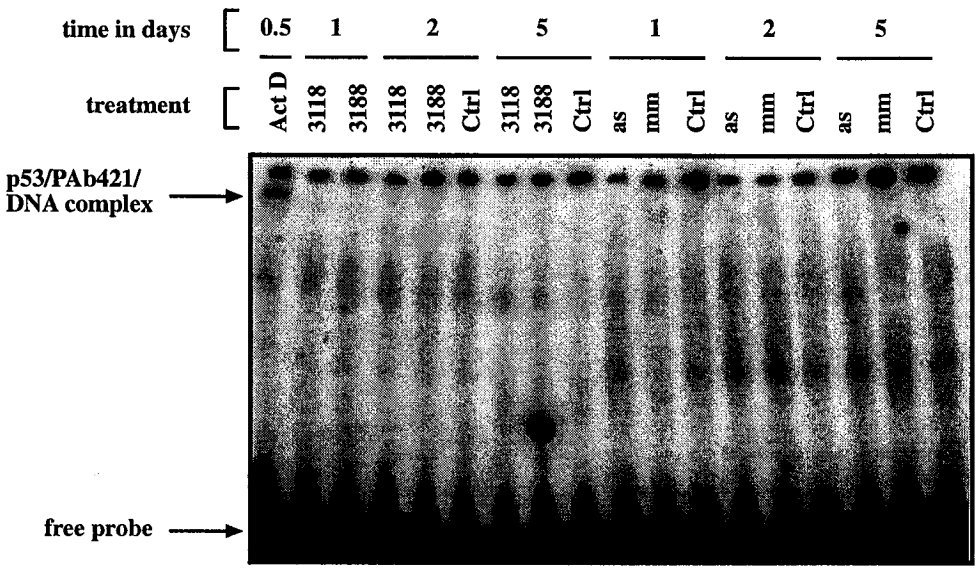

time in days
0.5
1
2
5
1
2
5
treatment
Act D
3118
3188
3118
3188
Ctrl
3118
3188
Ctrl
as
mm
Ctrl
as
mm
Ctrl
as
mm
Ctrl
p53/PAb421/
DNA complex
free probe

Fig. 6. DNA MeTase antagonist does not alter p21 mRNA stability. *A,* A549 cells were treated with DNA MeTase antagonist (*3118*), its inactive analog (*3188*), or mock treated controls (*ctrl*) for 4 h. After the treatment, the cells were exposed to 10 mg/ml of actinomycin D for 0, 4, and 8 h, after which they were harvested and total RNA was extracted. Northern blot was hybridized sequentially with *p21* (*upper panel*) and *18 s* ribosomal RNA oligonucleotide probe (*bottom panel*). *B*, quantification of the p21 mRNA levels normalized to *18 S* ribosomal RNA. Bars indicate the average of at least two independent experiments. *Error bars* indicate the range of data. *C*, the plot of the decay of *p21* mRNA. The log of the normalized *p21* mRNA levels was plotted against time following actinomycin D treatment. All the values are expressed relative to time 0 h, which was arbitrarily assigned the value of 1.

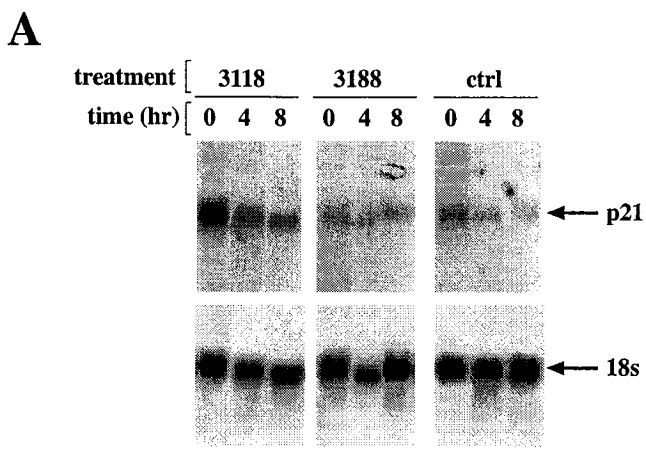
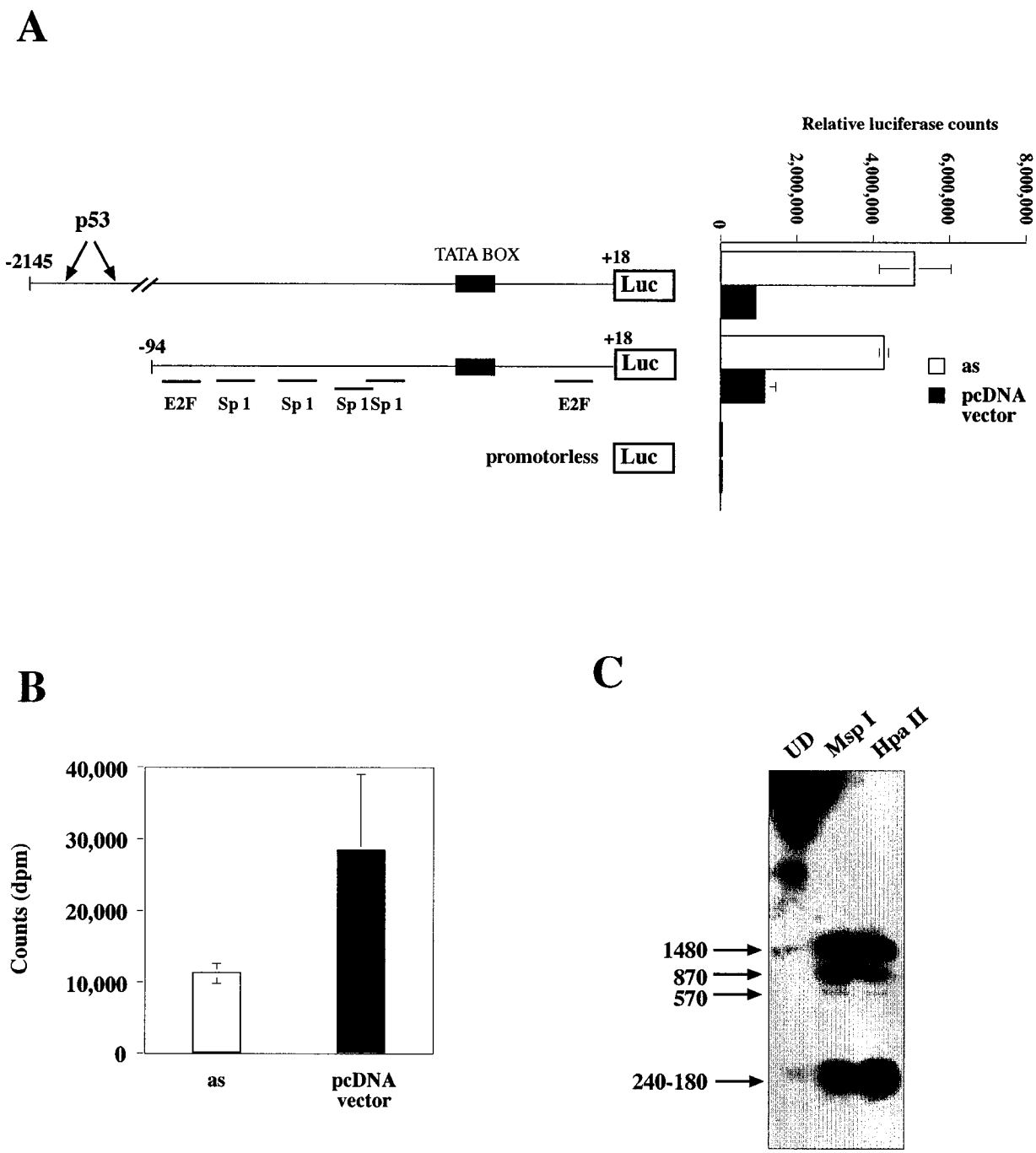

A
treatment
3118
3188
ctrl
time (hr)
0 4 8 0 4 8 0 4 8
p21
18s
B
treatment
3118
3188
ctrl
time (hr)
0 4 8 0 4 8 0 4 8
Normalized p21 mRNA levels
3
2
1
0
C
p21 mRNA remaining (arbitrary units)
1
0.5
0
0
4
8
3118
3188
ctrl

Fig. 7. Inhibition of DNA MeTase increases the p21 promoter activity. *A*, HEK 293 cells were co-transfected with the luciferase reporter constructs depicted and either a control vector (*pcDNA*) or a vector expressing *DNMT1* mRNA in the antisense orientation (*as*). The luciferase activity found in whole cell protein extracts isolated 48 h after transfection was measured. *B*, DNA MeTase activity was measured in nuclear extracts obtained from HEK 293 cells harvested 48 h following transfection with either pcDNA or as. *C*, a Southern blot of DNA isolated from HEK 293 cells transfected with the full-length *p21*-luciferase construct (*UD*), following digestion with *Msp*I (methylation insensitive) or *Hpa*II (methylation sensitive) enzymes. The whole plasmid was radiolabeled and used as a probe.


A
p53
-2145
TATA BOX
+18
Luc
-94
+18
Luc
E2F
Sp 1
Sp 1
Sp 1Sp 1
E2F
promotorless
Luc
Relative luciferase counts
0
2,000,000
4,000,000
6,000,000
8,000,000
as
pcDNA vector
B
Counts (dpm)
40,000
30,000
20,000
10,000
0
as
pcDNA vector
C
UD
Msp I
Hpa II
1480
870
570
240-180

The preceeding chapter challenges the widely accepted model of the role of DNMT1 in tumorigenesis and proposes a new mechanism of action of DNMT1 inhibitors in the arrest of cancer cell growth. Our findings, showing that the inhibition of DNMT1 results in the induction of a completely unmethylated tumor suppressor p21, unravel previously unknown role of DNMT1 in the methylation independent regulation of gene expression. This transcriptional induction of p21 was found to be much more rapid than the previously described induction of methylated tumor suppressors. Based on these data and the previous findings showing that there is not always a correlation between elevated DNMT1 levels and tumor suppressor hypermethylation, we propose that methylation independent roles of DNMT1 may be even more important in tumorigenesis than its enzymatic activity. A greater understanding of different roles of DNMT1 in cancer is required for the design of proper therapeutic strategies and the development of more specific inhibitors. The following chapter further examines different roles of DNMT1, through the comparison of the effects of different DNMT1 inhibitors on cancer cell growth and gene expression.

Chapter 2

Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes.

Snezana Milutinovic*, Qianli Zhuang*, Alain Niveleau and Moshe Szyf

*These authors have contributed equally to this work.

This chapter has been published in the Journal of Biological Chemistry (2003) 278, 14985-95.

The DNA methylation pattern is an important component of the epigenome that regulates and maintains gene expression programs. In this paper, we test the hypothesis that vertebrate cells possess mechanisms protecting them from epigenomic stress similar to DNA damage checkpoints. We show that knockdown of DNMT1 (DNA methyltransferase 1) by an antisense oligonucleotide triggers an intra-S-phase arrest of DNA replication that is not observed with control oligonucleotide. The cells are arrested at different positions throughout the S-phase of the cell cycle, suggesting that this response is not specific to distinct classes of origins of replication. The intra-S-phase arrest of DNA replication is proposed to protect the genome from extensive DNA demethylation that could come about by replication in the absence of DNMT1. This protective mechanism is not induced by 5-aza-2'-deoxycytidine, a nucleoside analog that inhibits DNA methylation by trapping DNMT1 in the progressing replication fork, but does not reduce *de novo* synthesis of DNMT1. Our data therefore suggest that the intra-S-phase arrest is triggered by a reduction in DNMT1 and not by demethylation of DNA. DNMT1 knockdown also leads to an induction of a set of genes that are implicated in genotoxic stress response such as *NF-kB*, *JunB*, *ATF-3*, and *GADD45* (growth arrest DNA damage 45 gene). Based on these data, we suggest that this stress response mechanism evolved to guard against buildup of DNA methylation errors and to coordinate inheritance of genomic and epigenomic information.

INTRODUCTION

Proper epigenomic regulation of gene expression is essential for the integrity of cell function. One critical component of the epigenome is the pattern of distribution of methylated cytosines in CG dinucleotide sequences in the genome (Razin, 1998). Methylation of CGs marks genes for inactivation by either interfering with the binding of methylated DNA-sensitive transcription factors (Prendergast and Ziff, 1991) or by recruiting methylated DNA-binding proteins such as MeCP2, which in turn recruit corepressor complexes and histone deacetylases to the chromatin associated with the gene (Nan et al., 1998). The methylation pattern can thus determine the chromatin structure and state of activity of genes. Disruption in the proper maintenance of the DNA methylation pattern results in aberrant gene expression, as is observed in tumor suppressor genes that are hypermethylated in cancer (Merlo et al., 1995). Aberrant hypomethylation can also result in improper activation of genes (Jackson-Grusby et al., 2001).

The main enzyme responsible for replicating the DNA methylation pattern is DNMT1 (DNA methyltransferase 1). This enzyme shows preference for hemimethylated DNA and is therefore believed to faithfully copy the DNA methylation pattern (Gruenbaum et al., 1982). Multiple mechanisms have been proposed to coordinate the inheritance of DNA methylation patterns with DNA replication. First, DNMT1 expression is regulated with the cell cycle (Detich et al., 2001; Szyf et al., 1991), and it is up-regulated by proto-oncogenes Ras and Jun (Bigey et al., 2000; MacLeod et al., 1995; Rouleau et al., 1995), Fos (Bakin and Curran, 1999), and T antigen (Slack et al., 1999). Second, DNMT1 is localized to the replication fork (Leonhardt et al., 1992) and is associated with the replication protein proliferating cell nuclear antigen (Chuang et al., 1997). Third, DNA methylation occurs concurrently with DNA replication (Araujo et al., 1998b). This temporal and physical association of DNMT1 with DNA replication is believed to have evolved to guarantee concordant replication of DNA and its methylation pattern. Previous studies have shown that inhibition of DNMT1 can lead to inhibition of initiation of DNA replication (Knox et al., 2000), but

it is not clear whether this response is a consequence of induction of tumor suppressor genes such as *p21* (Milutinovic et al., 2000) or p16 (Fournel et al., 1999), leading to retreat from the cell cycle. A conditional knockout of murine *dnmt1* gene was also shown to reduce the rate of cell division (Jackson-Grusby et al., 2001), but it is still unclear whether inhibition of DNMT1 leads to a change in cell cycle kinetics similar to DNA damage response checkpoints.

Multiple mechanisms have been established to guard the integrity of the genome in response to DNA damage. For example, two parallel, cooperating mechanisms, both regulated by ATM, jointly contribute to the rapid and transient inhibition of firing of origins of DNA replication in response to ionizing radiation (Falck et al., 2001; Kastan and Lim, 2000; Maser et al., 2001). This stalling of DNA synthesis is required to prevent genetic instability by coordinating replication and repair. We reasoned that similar mechanisms guard the integrity of epigenomic information in response to a disruption in the DNA methylation machinery.

In this paper, we test this hypothesis by determining the response of human cell lines to a knockdown of *DNMT1* mRNA, encoding the enzyme responsible for the replication of the DNA methylation pattern. Our data suggest that cells respond to this epigenomic stress by an intra-S-phase arrest of DNA synthesis as well as by inducing a large number of stress response genes. The slow down in DNA synthesis during S-phase protects the DNA from a global loss of the methylation pattern. This mechanism is not triggered by 5-aza-2'-deoxycytidine (5-aza-CdR), which causes an extensive loss of DNA methylation.

MATERIALS AND METHODS

Cell Culture, Antisense Oligonucleotides, and 5-aza-CdR Treatment-- Both A549, a human non-small cell lung carcinoma cell line, and T24, a human bladder transitional carcinoma-derived cell line, were obtained from the ATCC (Manassas, VA). A549 cells were grown in Dulbecco's modified Eagle's medium (low glucose) supplemented

with 10% fetal calf serum and 2 mM glutamine. T24 cells were maintained in McCoy's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. 18-24 h prior to treatment, cells were plated at a concentration of 3 x 10^5 cells/100-mm tissue culture dish or 5 x 10^4 cells/well in a six-well plate in the absence of antibiotics. The phosphorothioate oligodeoxynucleotides used in this study were MG88 (human *DNMT1* antisense oligonucleotide) and its mismatch control MG208, which has a 6-base pair difference from MG88 (Fournel et al., 1999). Oligonucleotides were transfected into cells with 6.25 μg/ml Lipofectin (Invitrogen) in serum-free Opti-MEM (Invitrogen). The oligonucleotide-containing Opti-MEM medium was removed from the cells and replaced with regular growth medium after 4 h. The treatment was repeated every 24 h. The cells were harvested 24, 48, 72, and 96 h following the first transfection. For 5-aza-CdR treatment, cells were grown in regular culture medium in the presence of 10^{-6} M 5-aza-CdR (Sigma) dissolved in Me_2SO. The 5-aza-CdR-containing medium was freshly replaced every 24 h.

DNA Methyltransferase Activity Assay and Western Blot Analysis-- To determine the level of cellular DNA methyltransferase activity, nuclear extracts were prepared, and DNA methyltransferase activity was assayed as described previously (Szyf et al., 1991). For Western blot analysis of DNMT1, 50 μg of nuclear protein was fractionated on a 5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and reacted with the polyclonal anti-DNMT1 antibody (New England Biolabs) at a dilution of 1:2000 in the presence of 0.05% Tween and 5% milk, and it was then reacted with anti-rabbit IgG (Sigma) at a dilution of 1:5000. The amount of total protein per lane was determined by Amido Black staining. The intensity of DNMT1 and total protein signal was measured by scanning densitometry, and the ratio of DNMT1/total nuclear protein was calculated.

RT-PCR-- Total RNA was extracted using the standard guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). cDNA was synthesized in a 20 μl reaction volume containing 2 μg of total RNA, 40 units of Moloney murine leukemia virus

reverse transcriptase (MBI), 5 μM random primer (Roche Molecular Biochemicals), a 1 mM concentration of each of the four deoxynucleotide triphosphates, and 40 units of RNase inhibitor (Roche Molecular Biochemicals). mRNA was denatured for 5 min at 70°C, the random primers were annealed for 10 min at 25°C, and mRNA was reverse transcribed for 1h at 37 °C. The reverse transcriptase was heat-inactivated for 10 min at 70°C, and the products were stored at -20 °C until use.

PCR was performed in a 50 μl reaction mixture containing 3μl of synthesized cDNA product, 5 μl of 10 x PCR buffer, 1.5-2.0 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of *Taq* polymerase (all from MBI) and 0.5 μM of each primer. The primer sequences that were used for the different mRNAs were *GADD45* (growth arrest DNA damage 45 gene) (sense, 5'-GTGTACGAGTCGGCCAAGTT-3'; antisense, 5'-AGGAGACAATGCAGGTCTCG-3'); *ATF-3* (sense, 5'-AAGAGCTGAGGTTTGCCATC-3'; antisense, 5'-GACAGCTCTCCAATGGCTTC-3'); *JunB* (sense, 5'-TGGAACAGCCCTTCTACCAC-3'; antisense, 5'-GGAGTAGCTGCTGAGGTTGGT-3'); *actin* (sense, 5'-GTTGCTAGCCAGGCTGTGCT-3'; antisense, 5'-CGGATGTCCACGTCACACTT-3'); *MAGEB2* (sense, 5'-AGCGAGTGTAGGGGGTGCG-3'; antisense, 5'-TGAGGCCCTCAGAGGCTTTC-3'); BCL2-interacting killer (BIK) (sense, 5'-GGCCTGCTGCTGTTATCTTT-3'; antisense, 5'-CCAGTAGATTCTTTGCCGAG-3'); *SSX2* (sense, 5'-CAGAGTACGCACGGTCTGAT-3'; antisense, 5'-GATTCCCACGGTTAGGGTCA-3'). Amplifications were performed in a Biometra T3 thermocycler (Biomedizinische Analytik GmbH) using the following programs: for *GADD45*, first cycle 94 °C for 3 min, 58 °C for 1 min, and 72 °C for 1 min, second cycle 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min followed by 37 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; for *ATF-3* and *JunB*, an initial cycle of 94 °C for 3 min 60 °C for 1 min and 72 °C for 1 min, followed by 34 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; for actin, first cycle 94 °C for 3 min, 64 °C for 1 min, and 72 °C for 1 min, second cycle 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, followed by 25 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; for *MAGEB2*, *BIK*, and *SSX2*, first cycle 94 °C for 30 s,

62 °C for 30 s, 72 °C for 30 s, second cycle 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s. The numbers of cycles were selected and tested so that the PCR amplification remained in the linear phase. 10 µl of the PCR products were applied on a 1.2% agarose gel and visualized by ethidium bromide staining. Densitometric analysis was performed using Scion Imaging Software (Scion Inc., Frederick, MD).

Competitive PCR for Quantification of DNMT1 mRNA Levels-- Total RNA (2 µg) was reverse transcribed as described above in the presence of 12.5 µCi of ^{35}S-labeled dCTP (1250 Ci/mmol) (ICN) to quantify the efficiency of reverse transcription. Equal amounts of reverse transcribed cDNA (70,000 cpm as determined by the incorporation of ^{35}S-labeled dCTP) were subjected to PCR amplification in the presence of increasing concentrations of a competitor DNA fragment that amplifies with the same set of primers but yields a product that is shorter by 48 base pairs. The following primers were used: 5'-ACCGCTTCTACTTCCTCGAGGCCTA-3' (*DNMT1* sense),5'-GTTGCAGTCCTCTGTGAACACTGTGG-3' (*DNMT1* antisense), and 5'-CGTCGAGGCCTAGAAACAAAGGGAAGGGCAAG (primer used to generate the competitor). PCR conditions were as follows: 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min (33 cycles).

Methylation-specific PCR-- Genomic DNA was extracted with DNA extraction buffer (1% SDS, 5 mM EDTA, 150 mM NaCl) followed by proteinase K digestion, phenol/chloroform extractions and ethanol precipitations. Bisulfite treatment was performed as described previously (Clark et al., 1994). The methylation status of the *p16* gene was determined by methylation-specific PCR (Herman et al., 1996a) as modified by Palmisano *et al.* (Palmisano et al., 2000).

[^{3}H]Thymidine Incorporation Assay-- Cells were plated in a six-well plate (5 x 10^4/well). For the final 4 h of incubation, 1 µCi/ml [^{3}H]thymidine (PerkinElmer Life Sciences) was added to the medium. After washing twice with PBS, the cells were incubated in 10% trichloroacetic acid for 30 min at -4 °C, washed twice with cold 10%

trichloroacetic acid, and then lysed with 1 N NaOH and 1% SDS. [^{3}H]thymidine incorporation was measured using a liquid scintillation counter (LKB Wallac).

Flow Cytometry Analysis of 5-Methylcytosine Staining-- Global DNA methylation was evaluated by staining the cells with specific monoclonal antibody against 5-methylcytidine using the protocol described previously (Habib et al., 1999) with slight modifications. Briefly, cells were washed with phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 and 1% bovine serum albumin (PBST-BSA), fixed with 0.25% paraformaldehyde at 37 °C for 10 min and 88% methanol at -20 °C for at least 30 min. After two washes with PBST-BSA, the cells were treated with 2 N HCl at 37 °C for 30 min and were then neutralized with 0.1 M sodium borate (pH 8.5). The cells were blocked with 10% donkey serum in PBST-BSA for 20 min at 37 °C, incubated with anti-5-methylcytidine antibody (1 μg/ml) for 45 min at 37 °C, followed by staining with donkey anti-mouse IgG conjugated with Rhodamine Red-X (Jackson ImmunoResearch Laboratories). Finally, the cells were washed with PBS three times and were resuspended in PBS for flow cytometry analysis.

Microarray Analysis-- A549 cells were transfected with 200 nM MG208 or MG88 or were treated with 1 μM 5-aza-CdR or Me_2SO, for 48 h. Total RNA was extracted with RNAeasy (Qiagen). Micoarray analysis was performed as previously described (Golub et al., 1999). Briefly, 20 μg of RNA was used for cDNA synthesis, followed by *in vitro* transcription with a T7 promoter primer having a poly(T) tail. The resulting product was hybridized and processed with the GeneChip system (Affymetrix) to a HuGeneFL DNA microarray containing oligonucleotides specific for ~12,000 human transcripts. Data analysis, average difference, and expression for each feature on the chip were computed using Affymetrix GeneChip Analysis Suite version 3.3 with default parameters. The gene expression analysis was performed by the Montreal Genome Center.

Double Staining of BrdUrd and Propidium Iodide-- Cells were incubated with 10 μM BrdUrd (Sigma) for the last 2 h before harvesting. Incorporated BrdUrd was stained

with anti-BrdUrd antibody conjugated with fluorescein isothiocyanate (Roche Molecular Biochemicals) following the manufacturer's protocol (Vanderlaan and Thomas, 1985). After the last washing, the cells were resuspended in PBS containing 50 μg/ml propidium iodide and 10 μg/ml RNase A for 30 min at room temperature and then analyzed with FACScan (BD Bioscience) for both fluorescein isothiocyanate and propidium iodide fluorescence.

RESULTS

Knockdown of DNMT1 mRNA by the DNMT1 Antisense Oligonucleotide MG88--DNMT1 activity is physically and temporally associated with the DNA replication machinery. The absence of DNMT1 from the replication fork could potentially lead to an epigenomic catastrophe. We have previously proposed that the coordination of DNMT1 expression and DNA replication evolved as a mechanism to protect the coordinate inheritance of genetic and epigenetic information (Szyf, 2001a; Szyf, 2001b). To test this hypothesis, we determined the cellular response to a knockdown of DNMT1 protein. We took advantage of a previously described antisense oligonucleotide, which specifically knocks down *DNMT1* mRNA (Fournel et al., 1999) MG88 and its mismatch control MG208 (see Fig. 1*A* for sequence and alignment with human and mouse DNMT1 mRNA). We first optimized the time and concentration at which MG88 specifically knocks down *DNMT1* activity in A549 cells in comparison with MG208. The results presented in Fig. 1*B* show that MG88 reduces DNA methyltransferase activity in a dose- and time-dependent manner relative to MG208. Inhibition of DNA methyltransferase activity approximates 80% after 48 h of MG88 treatment, whereas no inhibition is observed following MG208 treatment. Therefore, for our further analysis we chose to treat the cells with a 200 nM concentration of either MG88 or MG208 for 48 h. We confirmed the antisense mechanism of action of MG88 by demonstrating that *DNMT1* mRNA levels are knocked down following a 48-h treatment with this oligonucleotide in comparison with MG208 treatment using a competitive RT-PCR assay for *DNMT1* (Fig. 1, *C* and *D*). To confirm that DNMT1 inhibition results in reduction of DNMT1 protein,

nuclear extracts prepared from either MG208- or MG88-treated cells (200 nM for 48 h) were subjected to a Western blot analysis and reacted with anti-DNMT1 antibody (Fig. 1*E*). Quantification of the signal by densitometry reveals 85% reduction in protein levels. Inhibition of *DNMT1* mRNA by MG88 was also confirmed in a gene array expression analysis presented in Table II.

DNMT1 Knockdown Reduces the Fraction of Cells That Are in S-phase-- Several studies have previously demonstrated that inhibition of DNMT1 results in inhibition of cell growth (Bigey et al., 1999). One possible explanation for the reduced cell growth is that knockdown of DNMT1 results in inhibition of firing of DNA replication origins (Knox et al., 2000). We therefore addressed the question of whether this inhibition of DNA replication reflects a distinct alteration in cell cycle kinetics, similar to the DNA damage checkpoints that trigger arrest at distinct phases of the cell cycle (Bartek and Lukas, 2001).

A549 cells were treated with a 200 nM concentration of either MG88 or MG208 for 24-96 h as described under "Materials and Methods." As observed in Fig. 2, *DNMT1* knockdown results in a significant decrease in overall DNA synthetic capacity of A549 cells. This is illustrated by the reduced incorporation of [^{3}H]thymidine into DNA 24 h after the initiation of treatment, as has been previously reported (Knox et al., 2000). To exclude the possibility that inhibition of DNA synthesis by MG88 is independent of DNMT1 expression and is a toxic side effect of the sequence, we took advantage of the species specificity of MG88. As shown in Fig. 1*A*, there is a 6-base pair mismatch between MG88 and the mouse *dnmt1* mRNA. We therefore determined whether MG88 would inhibit DNA synthesis in a mouse adrenal carcinoma cell line, Y1, which was previously shown by us to be responsive to a mouse *dnmt1* antisense oligonucleotide (Ramchandani et al., 1997). The results shown in Fig. 2*B* demonstrate that 48-h treatment with 200 nM MG88 had no significant impact on the DNA synthetic capacity of Y1 cells in comparison with MG208, supporting the hypothesis that MG88 inhibition of DNA synthesis is DNMT1-dependent.

We then addressed the question of whether this inhibition in DNA synthesis represents a slowdown in the rate of DNA synthesis or a reduction in the fraction of cells that are in the synthetic phase, which would indicate a change in cell cycle phase kinetics. We pulsed MG88- and MG208-treated cells with BrdUrd 48 h after the initiation of treatment and sorted the cells that incorporated BrdUrd using fluorescence-activated cell sorting as described under "Materials and Methods." As illustrated in Fig. 2*C*, DNMT1 knockdown reduces the fraction of cells that incorporate DNA (the M1 population). However, the reduction in the fraction of cells that synthesize DNA (up to 42%) does not account for the overall reduction in DNA replication shown by the [^{3}H]thymidine incorporation assay, which is >95%. Although there is no significant cell death that can account for this disparity, this difference might reflect the fact that cell number following MG88 treatment is reduced.

DNMT1 Knockdown Results in Intra-S-phase Arrest in DNA Replication-- The reduction in the fraction of cells that incorporate DNA could be a consequence of a phase specific cell cycle arrest. However, preliminary results using flow cytometry sorting of propidium iodide-stained cells failed to show either a significant G1 or G2 arrest that could explain the reduction in S-phase cells. This raised the possibility that *DNMT1* knockdown caused an intra-S-phase arrest similar to the previously described DNA damage checkpoint (Kastan, 2001). To address this possibility, we treated A549 cells for 48 h with either MG88 or MG208 and then pulsed with BrdUrd to mark cells that are actively replicating. We then stained the cells with propidium iodide to determine their total DNA content, which is indicative of their position in the cell cycle. The cells were analyzed simultaneously for both their BrdUrd incorporation and propidium iodide staining by flow cytometry. The results of a representative analysis shown in Fig. 3*A* suggest a slight increase in sub-G1 population and a slight increase in cells found in the G2 phase of the cell cycle in response to *DNMT1* knockdown (9.76% in MG88-treated cells *versus* 7.71% in the MG208 treatment). However, an unexpected change in the cell cycle kinetics is a consequence of an intra-S-phase DNA replication arrest. This arrest results in the partition of cells found in the S phase into two distinct populations, one that incorporates BrdUrd and another comprising 30% of

the cells in the S-phase that do not incorporate BrdUrd, in comparison with less than 7% of those in the MG 208 control. Similar observations were obtained in five independent experiments. The presence of a population of cells in S-phase that does not incorporate BrdUrd in response to depletion of DNMT1 is consistent with a presence of an intra-S-phase checkpoint triggering cell cycle arrest. Cells that do not incorporate BrdUrd in S-phase are distributed throughout the S-phase of the cell cycle, indicating that the arrest in DNA replication does not occur at a specific point in S-phase. DNMT1 knockdown can lead to intra-S-phase arrest at any point in S-phase. As has been previously observed for DNA damage checkpoints, the intra-S-phase arrest is transient, since this partition of the S-phase cell population disappears after longer treatment (7 days) (Fig. 3*B*).

The Intra-S-phase Arrest Is Not Dependent on the Extent of DNA Demethylation-- The intra-S-phase arrest in DNA replication might have been triggered by either the absence of DNMT1 from some replication forks or by demethylated DNA. To address this question, we took advantage of a well characterized inhibitor of DNA methylation, 5-aza-CdR (Jones, 1985b). 5-aza-CdR is a nucleoside analogue that is incorporated into DNA following its phosphorylation to the trinucleotide form. It inhibits DNA methylation only once it is incorporated into DNA by trapping DNMT1 from the progressing replication fork. 5-aza-CdR does not inhibit either *de novo* synthesis of DNMT1 or its incorporation into the replication fork, but it inhibits DNA methylation during replication. It is dependent on DNA replication for its action, in contrast to *DNMT1* antisense, which reduces the availability of DNMT1 prior to the formation of the replication fork.

5-aza-CdR causes extensive DNA demethylation in A549 cells as well as many other cell lines and should cause a drastic change in cell cycle kinetics in S-phase if the trigger for intra-S-phase arrest in DNA replication is DNA demethylation. However, whereas 5-aza-CdR treatment, using a concentration that causes significant demethylation, results in a limited decrease in the rate of DNA synthesis as indicated by thymidine incorporation analyses shown in Fig. 4*A*, this inhibition is considerably

less than that observed with MG88 (Fig. 2*A*), and significant DNA synthesis occurs up to 96 h after treatment with 5-aza-CdR. Similarly, the analysis of the distribution of BrdUrd-incorporating cells in S (Fig. 4*B*) does not show a clear partition into two distinct populations as has been observed with MG88. 5-aza-CdR-treated cells show a gradual limited decrease in DNA replication rate as indicated by the gradient of BrdUrd-labeled cells in the S phase of the cell cycle. The continued synthesis of DNA in the presence of 5-aza-CdR can explain the ability of this agent to demethylate DNA. The main change in the cell cycle kinetics that is observed with 5-aza-CdR is an increase in the G2 population (from 9.74% in the control to 28.99% in 5-aza-CdR-treated cells). These results suggest that the intra-S-phase arrest is not correlated to the degree of inhibition of DNA methylation *per se*. Our data are consistent with the hypothesis that the intra-S-phase arrest following *DNMT1* knockdown is a response to a reduction in the availability of DNMT1 in the replication fork rather than DNA demethylation.

Intra-S-phase Arrest of DNA Replication Possibly Protects the Genome from Global Hypomethylation-- One potential role of the intra-S-phase arrest triggered by reduction of DNMT1 is to protect the epigenome from global loss of the DNA methylation pattern. Using anti-5-methylcytosine antibodies that were previously described (Piyathilake et al., 2001), we compared the state of methylation of A549 cells treated for 48 h with either 200 nM MG88, which causes intra-S-phase arrest or 5-aza-CdR which does not trigger a distinct intra-S-phase arrest. A549 cells treated with either the DNMT1 inhibitors (MG88 or 5-aza-CdR) or their respective controls (MG208 or Me_2SO) were stained with either the 5-methylcytosine antibody or the secondary antibody alone as a control and were subjected to fluorescence-activated cell sorting analysis (Fig. 5, *A* for MG88 *versus* MG208 and *B* for 5-aza-CdR *versus* Me_2SO control). MG88-treated cells are only slightly demethylated, as indicated by the slight shift in the fluorescence intensity of the MG88-treated cells (Fig. 5*A*), whereas 5-aza-CdR treatment results in extensive reduction in staining with the anti-5-methylcytosine antibody indicative of genome-wide demethylation (Fig. 5*B*). The intra-S-phase arrest of replication following MG88 treatment possibly protects A549 cells from genome-

wide loss of methylation. We have previously observed that hemimethylated inhibitors of DNMT1 that inhibit DNA replication also cause only limited demethylation of DNA (Bakin and Curran, 1999).

Comparison of the Kinetics of Demethylation of the Tumor Suppressor p16 following DNMT1 Knockdown by MG88 and 5-aza-CdR Trapping of DNMT1-- We addressed the question of whether this difference in the kinetics of global DNA demethylation between 5-aza-CdR and *DNMT1* antisense oligonucleotides is also observed when specific genes are examined. We focused on the methylated tumor suppressor gene p16 in the human bladder carcinoma cell line T24, since there is no well documented example of a methylated gene in A549 cells that is activated by pharmacological demethylation. The p16 gene is demethylated in response to both 5-aza-CdR (Bender et al., 1998) and *DNMT1* antisense (MG88) treatment (Fournel et al., 1999).

We first verified that the *DNMT1* antisense-triggered intra-S-phase arrest demonstrated above in A549 cells (Fig. 3) is also functional in T24 cells. A 48 h treatment of T24 cells with MG88 results in an intra-S-phase arrest of DNA replication (Fig. 6*A*) similar to that observed in A549 cells (Fig. 3), as indicated by the partition of cells in S-phase of the cell cycle to two distinct groups, those that incorporate BrdUrd (9.77% compared with 37.06% in the MG208 control group) and those that do not incorporate BrdUrd (12.4% *versus* 5.5% in the control). Thus, 56% of the cells found in the S-phase of the cell cycle do not synthesize DNA following MG88 treatment of T24 cells. On the other hand, 5-aza-CdR treatment results in an increase in the fraction of cells that are in G1 (72.06% in 5-aza-CdR-treated *versus* 53.55% in the control) and a slowdown of the rate of DNA synthesis in S-phase cells, as indicated by the gradient of the intensity of BrdUrd incorporation. However, there is no distinct partition of the population of S-phase cells following 5-aza-CdR to two distinct groups as is observed following MG88 treatment.

We then assessed the global state of methylation of T24 cells following either MG88 or 5-aza-CdR treatment for 48 h using fluorescence-activated cell sorting analysis of

5-methylcytosine antibody-stained cells. Fig. 6*B* demonstrates that similar to what is observed in A549 cells, MG88 treatment results in very limited global hypomethylation in T24 cells, as indicated by the slight shift to the left in the intensity of staining with 5-methylcytosine antibodies. In contrast, 5-aza-CdR results in global hypomethylation as indicated by the considerable shift to the left of the population of 5-aza-CdR-treated cells Fig. 6*C*.

We then determined the pattern of methylation of the *p16* gene following either *DNMT1* antisense or 5-aza-CdR treatments. The methylation pattern of the 5' exon of *p16* was studied by methylation-specific PCR that was previously described (Herman et al., 1996b) (Fig. 6, *D* and *E*). The results of this analysis show a dramatic difference in the kinetics of demethylation between MG88-treated (Fig. 6*D*) and 5-aza-CdR-treated (Fig. 6*E*) cells. Whereas *p16* is significantly demethylated 24 h after the initiation of 5-aza-CdR treatment and is completely demethylated after 96 h (Fig. 6*E*), *p16* remains fully methylated 48 h after MG88 treatment at the peak of the intra-S-phase DNA replication arrest (Fig. 6*D*). Demethylation initiates only at 72 h. The mechanism of this demethylation is unclear, since passive demethylation requires DNA replication in the absence of DNA methyltransferase, whereas DNA replication is inhibited in T24 cells following MG88 treatment. It is possible that the demethylation of *p16* is caused by an active demethylation mechanism or it might result from residual replication in the absence of *DNMT1*.

In summary, our data reveal that demethylation is delayed when DNA synthesis is arrested concomitantly with knockdown of *DNMT1*. The signal for the intra-S-phase arrest following *DNMT1* knockdown by MG88 is neither the extent of DNA demethylation nor the activation of *p16*, since DNA replication arrest precedes demethylation. Furthermore, 5-aza-CdR, a potent inhibitor of DNA methylation that acts by a different mechanism than MG88, does not cause an intra-S-phase arrest in DNA replication.

Knockdown of DNMT1 by Antisense, but Not DNA Methylation Inhibition with 5-aza-CdR, Induces Expression of Genotoxic Stress-responsive Genes-- Multiple genes have been shown in the past to be silenced by DNA methylation. A well accepted model is that global DNA demethylation results in misprogramming of gene expression by aberrant activation of genes that are normally silenced by methylation. A methodical analysis of genes that are induced following 5-aza-CdR treatment of a colorectal cancer cell line identified a group of genes that are silenced by DNA methylation and are demethylated by 5-aza-CdR. In addition, another group of genes that are not methylated and are activated by methylation-independent mechanisms were also shown to be induced by 5-aza-CdR (Suzuki et al., 2002). We used Affymetrix 12K gene microarrays to repeat this analysis in our system. We compared the gene expression profile of A549 cells treated with 1 μM 5-aza-CdR with the gene expression profile of A549 cells treated with Me_2SO for 48 h. The list of genes induced more than 2.5-fold by 5-aza-CdR is shown in Table I. Only genes that were induced in two separate experiments and did not show variation in expression within either the control or 5-aza-CdR replicates were included. The list of genes induced by 5-aza-CdR includes tissue-specific genes such as *smooth muscle actin 2* and genes involved in interferon response such as *interferon 2*, as well as the apoptosis promoter, *BIK*. *BIK* was previously shown to exhibit potent antitumor activity (Tong et al., 2001) and is induced by 5-aza-CdR and sodium butyrate in hepatic cancer cell lines (Wang et al., 1998). The induction of the interferon response pathway was previously proposed to be a major cellular response to 5-aza-CdR (Karpf et al., 1999). However, in addition to up-regulation of genes that are potentially antimitotic and proapoptotic, the most remarkable induction occurred in three groups of cancer/testis-specific genes residing on the X chromosome, which are exclusively expressed in testis and a wide variety of tumors but not in nontumor tissues. These are the *GAGE* (G antigen 7) family (Chen et al., 1998), the *MAGE* family of melanoma antigens (De Smet et al., 1996; Russo et al., 1995; Serrano et al., 1996; Shichijo et al., 1996; Weber et al., 1994), and the genes residing at the synovial sarcoma X breakpoint, *SSX2-4* (Chen et al., 1997; Gure et al., 1997). It is well documented that the *MAGE* (De Smet et al., 1996; Serrano et al., 1996; Shichijo et al., 1996) and *GAGE* family of genes as well as

SSX2 are controlled by DNA methylation and are induced by DNA demethylating agents (Sigalotti et al., 2002).

The up-regulation of genes identified by gene array analysis was verified by semiquantitative RT-PCR shown in Fig. 7. The cancer/testis-specific genes show a typical profile for methylated genes induced by demethylating agents. They are completely silenced in the control cells and are activated to clearly detectable levels following demethylation (Fig. 7). Methylation results in most cases in silencing of genes rather than a quantitative reduction in gene expression. In addition, expression levels are increased with time, as expected from passive demethylation kinetics. Inhibition of DNA methyltransferase during new DNA synthesis results in a time-dependent increase in the relative abundance of the population of newly replicated unmethylated DNA. On the other hand, genes such as *BIK*, which is expressed in control cells, are transiently induced by 5-aza-CdR, and their level of induction is reduced with time (Fig. 7). This profile of induction is consistent with a methylation-independent mechanism, which is also supported by the induction of *BIK* after DNMT1 knockdown before any significant global demethylation is observed (2-fold induction 24 h after antisense treatment). *BIK* and other genes induced by a methylation-independent mechanism were also induced by the deacetylase inhibitors trichostatin A (Suzuki et al., 2002) and *n*-butyrate (Wang et al., 1998). In accordance with previous studies in colorectal cancer (Suzuki et al., 2002), our data show both methylation-dependent and -independent induction by 5-aza-CdR in A549 cells (*MAGEB2, SSX2*, and *BIK*, respectively).

Based on the data presented above, we predicted that in contrast to the response to 5-aza-CdR, knockdown of DNMT1 by antisense inhibition should not result in induction of methylation-silenced genes at the early time after treatment. However, since we have previously shown that inhibition of DNMT1 induces the expression of the *p21* tumor suppressor gene by a mechanism that does not involve DNA demethylation (Milutinovic et al., 2000), and since examples of such genes were identified in the recent analysis with 5-aza-CdR (Suzuki et al., 2002), we tested the possibility that the

early response to DNMT1 knockdown results in a programmed change in gene expression that precedes global hypomethylation and is possibly involved in the stress response. We therefore compared the gene expression profile of MG88-treated A549 cells with the gene expression profile of A549 cells treated with the control MG208 oligonucleotide for 48 h using Affymetrix 12K gene microarrays.

We compared the normalized gene expression profile of the two treatment groups. 255 (2.1%) genes out of 12,000 genes were up-regulated, whereas there were just 23 (0.19%) genes that were down-regulated. The experiment was repeated with similar results. *DNMT1* expression was 75% down-regulated in two experiments, which is an internal validation of our gene expression analysis and antisense treatment. The results are presented in Table II. Only genes induced in both experiments are included. Among the genes that were induced, we did not identify genes that were previously characterized to be silenced by methylation such as *SSX2* and *MAGEB2*. These two genes were shown to be induced with 5-aza-CdR but were not induced following DNMT1 knockdown (Figs. 7 and 8). However, a distinct group of genes that stood out was a set of previously characterized genotoxic-responsive genes such as *ATF-3*, *GADD45*, and *JunB*. These three genes were not found to be induced by 5-aza-CdR treatment in the gene array analysis, and this result was confirmed by RT-PCR (Fig. 7). Their induction profiles following antisense treatment were verified using semiquantitative RT-PCR. The induction peaked at 48 h for *GADD45* and *JunB* and at 72 h for *ATF-3*. This profile of induction is consistent with the hypothesis that the cell recognizes DNMT1 knockdown as a genotoxic challenge and reacts by inducing a stress response gene expression program. The kinetics of this response, early induction followed by mitigation of the response (Fig. 8), is inconsistent with the mechanism involving passive demethylation of DNA. Consistent with this hypothesis that MG88 action at 48 h is independent of DNA methylation is the fact that the genes induced by the DNA-demethylating agent 5-aza-CdR (*MAGEB2 and SSX2*) were not induced by MG88 treatment for 48 h (Fig. 8).

DISCUSSION

Multiple mechanisms regulate expression of DNMT1 within a cell (Bigey et al., 2000; Detich et al., 2001; Szyf, 2001a; Szyf et al., 1991). In this paper, we address the question of whether mammalian cells possess a mechanism to respond to a sudden loss of DNMT1 and protect themselves from a global loss of DNA methylation during replication in the absence of DNMT1. It is well established that genotoxic challenges such as DNA damage evoke distinct cellular responses, resulting in a transient intra-S-phase arrest in DNA replication (Falck et al., 2001; Kastan and Lim, 2000; Maser et al., 2001). This intra-S-phase arrest guards against buildup of mutations during DNA replication before the other checkpoints at G2/M and G1/S could take effect. Similarly, relying on G2/M and G1/S checkpoints to respond to the absence of DNMT1 in the fork during replication could result in a significant loss of DNA methylation and a buildup of epigenomic errors.

We demonstrate here that following DNMT1 knockdown, cells found in the S phase of the cell cycle are partitioned into two groups, those that incorporate BrdUrd and those that do not incorporate BrdUrd (Fig. 3). Our data are consistent with the presence of an intra-S-phase checkpoint that arrests all of the replication forks in a cell, as illustrated by the appearance of a group of cells in S-phase that do not incorporate any BrdUrd as a response to a reduction in availability of DNMT1.

Different origins of replication replicate at discreet and well defined positions in the cell cycle. Origins that replicate early in the cell cycle are associated with genomic regions that are hypomethylated and are actively transcribed (Antequera and Bird, 1999), whereas origins that replicate late in S-phase are associated with inactive genes, which are also known to be hypermethylated (Mostoslavsky et al., 2001). The results presented in Figs. 3 and 6 show that DNA replication is arrested at any point in the S-phase of the cell cycle. This is inconsistent with the hypothesis that DNMT1 knockdown affects only specific classes of origins.

What is the signal that triggers a stress response to antisense *DNMT1* knockdown? It is possible that the emergence of demethylated DNA caused by replication in the absence of DNMT1 triggers the intra-S-phase arrest. Alternatively, the signal is the inhibition of *de novo* synthesis of DNMT1, leading to its absence from DNA replication factories. The fact that 5-aza-CdR, which causes a far more extensive demethylation than MG88, does not trigger the same magnitude of intra-S-phase arrest of replication (Fig. 3), suggests that it is not the demethylation that triggers the intra-S-phase arrest. Rather, our data are consistent with the hypothesis that it is the reduction in DNMT1 protein that triggers the intra-S-phase arrest observed after MG88 treatment.

We propose that the intra-S-phase arrest guarantees that no DNA is synthesized in the absence of DNMT1. However, this is a transient and incomplete protection, and delayed demethylation is observed following extended MG88 treatment (Fig. 6). 5-aza-CdR bypasses this checkpoint to a large extent, since it does not reduce DNMT1 synthesis but traps DNMT1 only once the replication fork has formed in the presence of DNMT1.

These differences in the mechanisms of action of these two inhibitors have important implications on the design and therapeutic utility of different DNA methylation inhibitors (Szyf, 2001b). Agents such as MG88, that reduce the availability of DNMT1 at the replication fork, are strong inhibitors of cell growth and should be effective in inhibiting tumor growth but will not cause extensive demethylation (Bigey et al., 1999). There might be an advantage for therapeutic agents that do not cause extensive demethylation, since extensive hypomethylation has been previously associated with metastasis (Ormerod et al., 1986) and possibly induction of silenced repetitive elements (Barbot et al., 2002; Bender, 1998). The data presented in Table I illustrate the risks inherent in using DNA-demethylating agents. In addition to induction of antimitotic and proapoptotic genes, 5-aza-CdR induces three families of testis/cancer-specific antigens that were previously implicated in tumor progression and potentially tumor invasion and metastasis. It is interesting to note that expression

of the *GAGE* antigen family has been associated with poor prognosis in some cancers. *G antigen 7* is expressed in prostate cancer (Chen et al., 1998), and *G antigen 7c* was proposed to be an antiapoptotic gene (Cilensek et al., 2002). Similarly, *MAGE* expression is associated with metastasis (Mostoslavsky et al., 2001; Ormerod et al., 1986). *SSX2* was shown to be expressed in a wide variety of tumors (Gure et al., 1997) and was identified as one of 13 antigens that react exclusively with sera from colon cancer patients but not with sera from normal patients (Scanlan et al., 2002).

In addition to the change in cell kinetics, the cells respond to DNMT1 knockdown by a change in the gene expression program. A significant fraction of the induced genes is known to be involved in genotoxic stress responses (Table II). We have previously proposed that DNMT1 controls the expression of certain genes by a direct repression function that does not involve DNA methylation (Milutinovic et al., 2000). DNMT1 was previously shown to interact with HDAC1 (Fuks et al., 2000), HDAC2 (Rountree et al., 2000), and Rb-E2F1 (Robertson et al., 2000a). We propose that some of the early genes induced by DNMT1 knockdown are similarly controlled by the DNA methylation-independent gene repression activities of DNMT1.

It remains to be seen whether the genes induced by DNMT1 knockdown are also involved in the intra-S-phase arrest or whether they are parallel responses that augment the protection against epigenomic loss. Recent studies have identified some of the players involved in the intra-S-phase checkpoint in response to ionizing DNA damage. ATM is activated by ionizing radiation, which in turn activates two signaling pathways, one leading to inactivation of Cdk2 and intra-S-phase arrest and the other leading to activation of *p21* and G1 arrest (Kastan, 2001). We have previously shown that DNMT1 inhibition can lead to transcriptional induction of *p21*, and here we show induction of *GADD45*. The most established function of p21 is at the G1/S boundary (Harper et al., 1993), and GADD45 has been shown to play an important role in the G2/M checkpoint in response to DNA damage (Jin et al., 2000), but their involvement in the intra-S-phase checkpoint is unclear. However, it is possible that inhibition of Cdk2 by p21 can lead to S-phase arrest. An additional possibility is that the assembly

of the replication fork requires the presence of DNMT1 and that its absence from the fork is what signals arrest of DNA replication.

Although the precise mechanism by which reduction of DNMT1 causes intra-S-phase arrest is unknown, our data describe a new class of putative checkpoints that react to epigenomic stress caused by reduction of DNMT1 levels. We propose that this mechanism has evolved to protect the genome from unscheduled demethylation and to maintain the coordination of replication of the genome and the epigenome.

ACKNOWLEDGEMENTS

We thank Dr. Hudson and the Montreal Genome Center for support and help in performing and analyzing the microarrays. We thank Gula Sadvakassova for technical assistance and Nancy Detich for critical review of the manuscript.

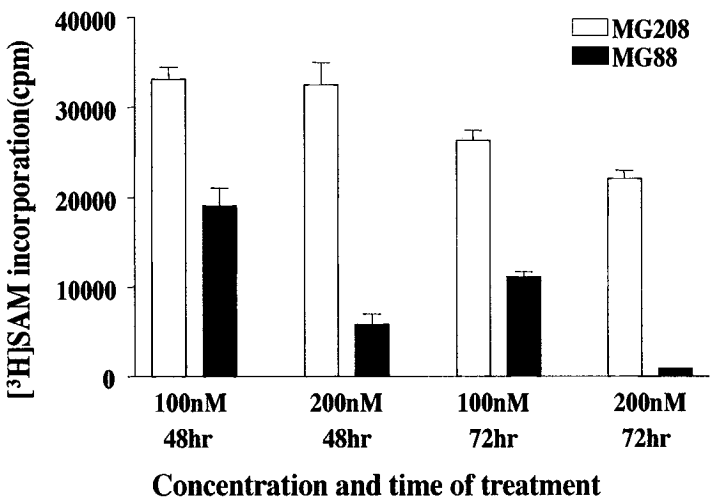
This work was supported by the Canadian Institute of Health Research.

Fig. 1. Time- and dose-dependent inhibition of DNMT1 mRNA and DNA methyltransferase activity by DNMT1 antisense oligonucleotide MG88. *A*, the sequence of *DNMT1* antisense oligonucleotide MG88 and its mismatch control oligonucleotide MG208 are aligned to the human and mouse *DNMT1* mRNA sequence. Mismatches with the human *DNMT1* mRNA are in boldface italic type. *B*, A549 cells were incubated with either 100 or 200 nM MG88 or MG208 for either 48 or 72 h. DNA methyltransferase activity was determined for 3 μg of nuclear extracts using a hemimethylated substrate and ^{3}H-labeled *S*-adenosylmethionine as a methyl donor. The results presented are an average of three determinations ± S.E. of triplicate determinations from three independent experiments. *C* and *D*, representative experiments from five similar experiments using competitive RT-PCR for quantification of *DNMT1* mRNA. 2 μg of RNA isolated from A549 cells treated with either MG88 or MG208 at 200 nM for 48 h was reverse transcribed into cDNA in the presence of [^{35}S]dCTP. Equal counts of labeled cDNA (*target*) and increasing amounts of competitor molecules (from 10^{-16} to 10^{-12} M) were used as templates for PCRs with primers targeted to *DNMT1* mRNA sequence. The PCR products were run on 1.2% agarose gel and quantified by densitometry, the logarithm of the ratio of target to competitor products were plotted against the log of competitor concentration (*D*). *E*, Western blot analysis of DNMT1 expression in nuclear extracts prepared from either MG88- or MG208-treated A549 cells (200 nM for 48 h). In the *right panel*, the membrane was stained with Amido Black to visualize the total protein transferred onto the membrane.

A

human mRNA	5'	G	G	A	G	A	A	C	G	G	T	G	C	T	C	A	T	G	C	T	-	T	3'
MG88	3'	C	C	T	C	T	T	G	C	C	A	C	G	A	G	T	A	C	G	A	-	A	5'
MG208	3'	C	C	T	***G***	T	T	***C***	C	C	A	***G***	G	A	***C***	T	A	***G***	***C***	A	-	A	5'
mouse mRNA	5'	G	G	A	G	A	A	C	G	G	***A***	***A***	C	***A***	C	A	***C***	***A***	C	T	***C***	T	3'

B



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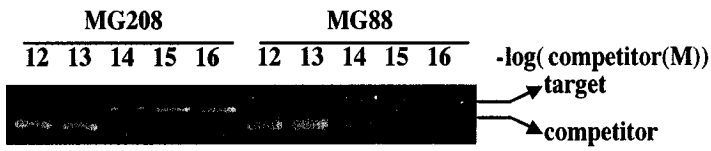
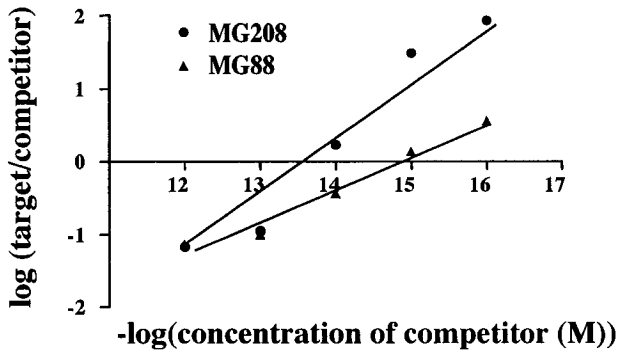


D



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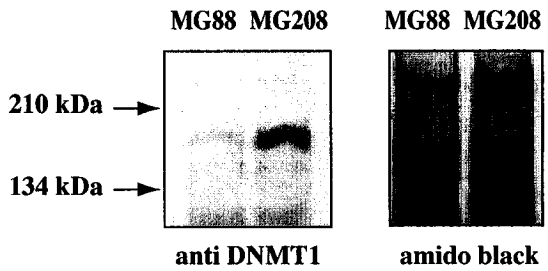
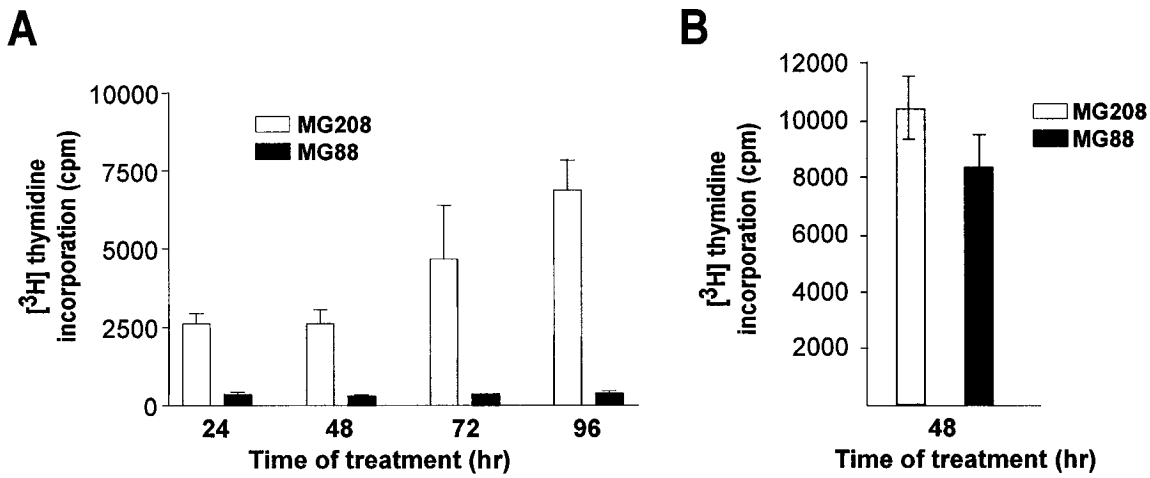


Fig. 2. MG88 knockdown of DNMT1 inhibits DNA synthesis and reduces the fraction of cells that synthesize DNA in A549 cells. *A*, A549 cells were treated with MG208 and MG88 at 200 nM for the indicated time intervals. [^{3}H]thymidine incorporation into DNA was quantified as described under "Materials and Methods." The results presented are mean ± S.D. of triplicate determinations from one of three independent experiments that resulted in similar results. *B*, Y1 cells were treated with either MG208 or MG88 at 200 nM for 48 h. [^{3}H]thymidine incorporation into DNA was quantified as in *A*. *C*, A549 cells were incubated with either MG208 or MG88 at 200 nM for 48 h. In the last 2 h of the experiment, the cells were pulsed with 10 μM BrdUrd and were stained with anti-BrdUrd Ab and sorted by flow cytometry. The *left peak* represents cells that are negative for anti-BrdUrd staining, and the right peak (*M1*) represents cells that are positive for anti-BrdUrd staining. Similar profiles were obtained for three other independent experiments.


A
10000
7500
5000
2500
0
[3H] thymidine incorporation (cpm)
MG208
MG88
24
48
72
96
Time of treatment (hr)
B
12000
10000
8000
6000
4000
2000
[3H] thymidine incorporation (cpm)
MG208
MG88
48
Time of treatment (hr)

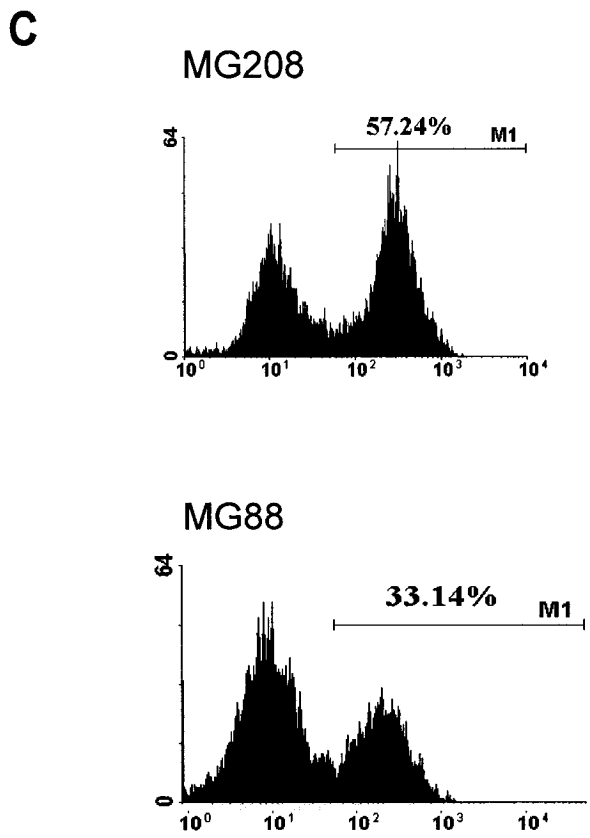
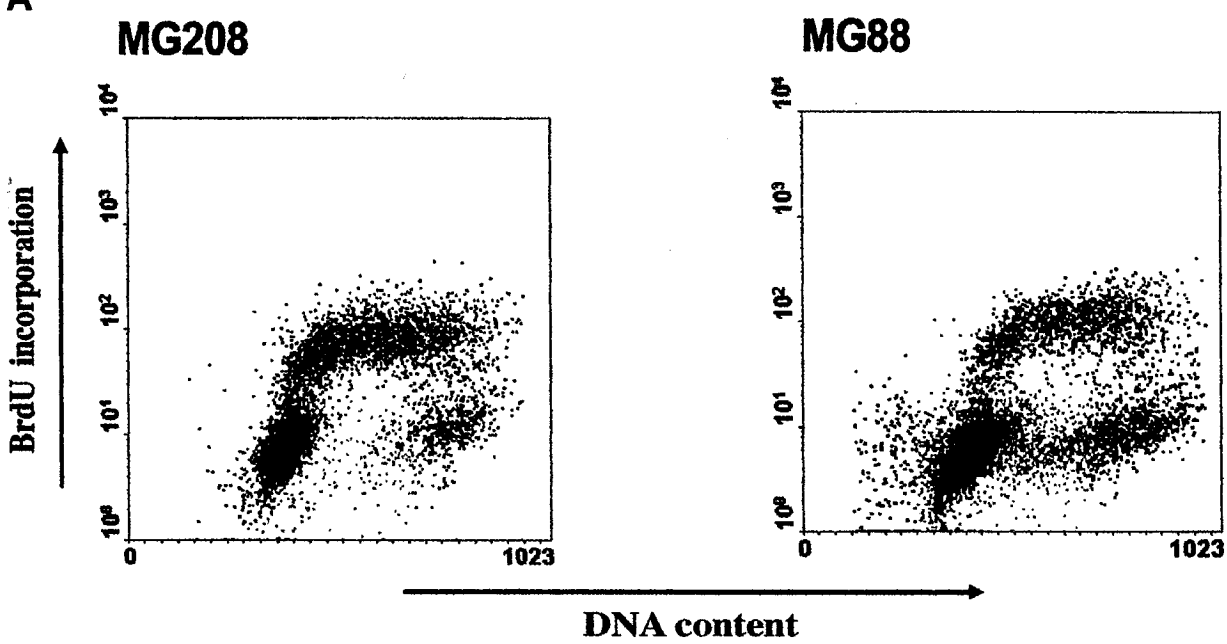

C
MG208
57.24%
M1
64
0
10^0
10^1
10^2
10^3
10^4
MG88
33.14%
M1
64
0
10^0
10^1
10^2
10^3
10^4

Fig. 3. Knockdown of DNMT1 triggers intra-S-phase arrest of DNA replication. *A*, A549 cells were treated with 200 nM of either MG208 or MG88 for 48 h. Cells pulsed with BrdUrd (10 μM) for 2 h were stained with anti-BrdUrd antibody and propidium iodide and then analyzed with flow cytometry. The profiles of BrdUrd staining fluorescence *versus* propidium iodide staining fluorescence are depicted. The *dots* representing population of cells in the different phases of the cell cycle are *color-coded* (*green*, G0/G1; *pink*, S-phase with BrdUrd incorporation; *orange*, S phase without BrdUrd incorporation; *blue*, G2/M). A representative experiment of five independent experiments is presented. *B*, A549 cells treated with either MG88 or MG208 for 7 days were pulsed for 2 h with BrdUrd, stained with anti BrdUrd antibodies and propidium iodide, and subjected to flow cytometry. The profiles of BrdUrd staining fluorescence *versus* propidium iodide staining fluorescence are depicted.

A


MG208
MG88
BrdU incorporation
DNA content
0
1023

B

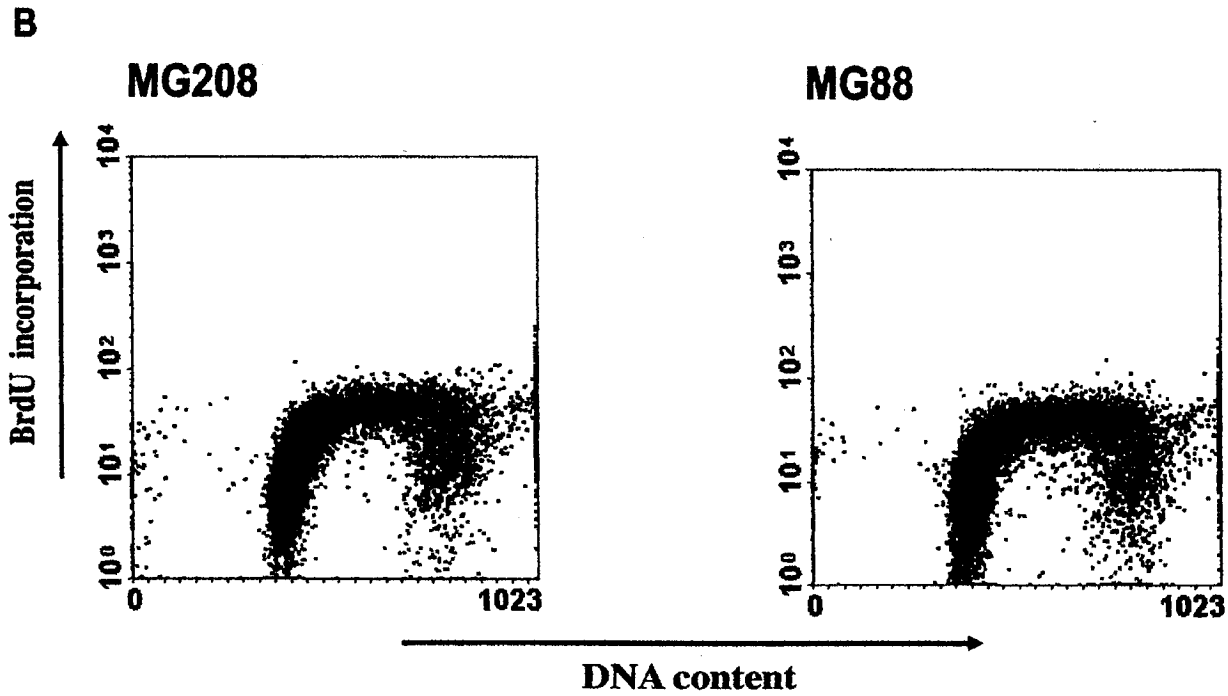
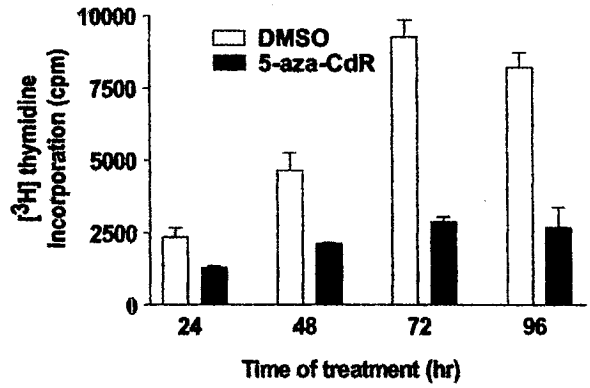

MG208
MG88
BrdU incorporation
DNA content
0
1023

Fig. 4. The effects of 5-aza-CdR on DNA replication and cell cycle kinetics in A549 cells. *A*, A549 cells were treated with 1μM 5-aza-CdR (*solid bar*) and the vehicle control Me_2SO (*DMSO*; *open bar*) for 24-96 h. [^{3}H]Thymidine incorporation into DNA was quantified as described under "Materials and Methods." The results presented are mean ± S.D. of triplicate determinations from one of three independent experiments with similar results. *B*, A549 cells treated with either 1 μM 5-aza-CdR or Me_2SO for 48 h were pulsed with BrdUrd (10 μM) for 2 h and stained with both anti-BrdUrd Ab and propidium iodide and then analyzed with flow cytometry. The profiles of BrdUrd staining fluorescence *versus* propidium iodide staining fluorescence are depicted. Different cell populations are *color-coded* as in Fig. 3. This experiment is a representative of three similar experiments.

A


10000
7500
5000
2500
0
[3H] thymidine Incorporation (cpm)
DMSO
5-aza-CdR
24
48
72
96
Time of treatment (hr)

B

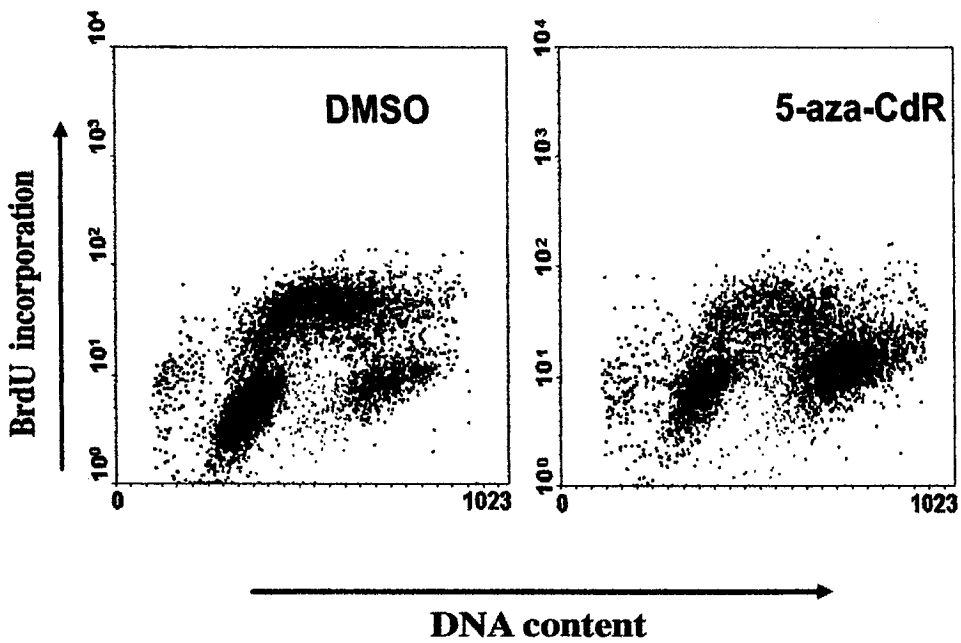
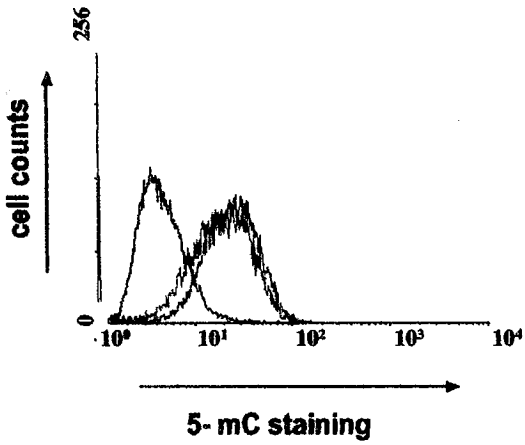

DMSO
5-aza-CdR
BrdU incorporation
DNA content
0
1023
10⁰
10¹
10²
10³
10⁴

Fig. 5. The state of methylation of A549 cells following DNMT1 antisense or 5-aza-CdR treatment. *A*, A549 cells were treated with a 200 nM concentration of either MG208 (*red*) or MG88 (*green*) for 48 h. Cells were harvested, stained with anti-5-mC antibody and anti-mouse IgG conjugated with Rhodamine Red-X, and subjected to flow cytometry. Cells stained with secondary Ab only are used as a blank control (*black line*). *B*, A549 cells were treated with either Me_2SO (*red*) or 5-aza-CdR (*green*) for 48 h and subjected to flow cytometry as in *A*.

A


256
cell counts
0
10⁰
10¹
10²
10³
10⁴
5- mC staining

B

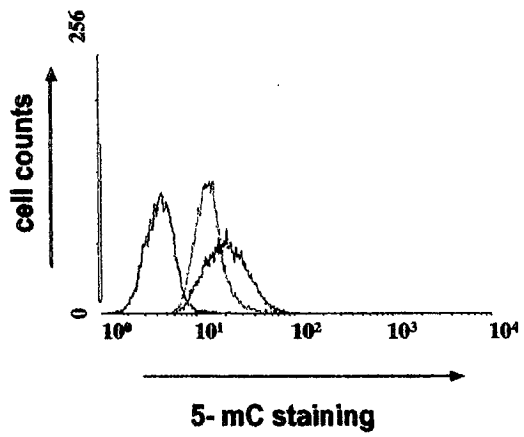

256
cell counts
0
10⁰
10¹
10²
10³
10⁴
5- mC staining

Fig. 6. Time course of global and p16 DNA demethylation in T24 cells treated with either DNMT1 antisense or 5-aza-CdR. *A*, DNMT1 antisense treatment causes an intra-S-phase arrest in T24 cells. T24 cells were treated with a 200 nM concentration of either MG208 or MG88, Me_2SO, or 1 μM 5-aza-CdR for 48 h and were then pulsed for 2 h with BrdUrd. The cells were stained with anti-BrdUrd Ab and propidium iodide and subjected to flow cytometry. The profile of BrdUrd staining *versus* propidium iodide staining is shown for each of the treatment groups as described in the legend to Fig. 3. *B*, T24 cells were treated with 200 nM of either MG208 (*red*) or MG88 (*green*) for 48 h. Cells were harvested, stained with anti-5-mC Ab and anti-mouse IgG conjugated with Rhodamine Red-X and subjected to flow cytometry. Cells stained only with secondary antibody are used as a blank control (*black line*). *C*, T24 cells were treated with either Me_2SO (*DMSO*; *red*) or 5-aza-CdR (*green*) for 48 h and subjected to flow cytometry as in *B*. T24 cells were treated for 48 h with a 200 nM concentration of either MG208 or MG88 (*D*) or 1 μM 5-aza-C or Me_2SO (*E*). DNA was extracted and treated with sodium bisulfite. Two-stage PCR was performed as described under "Materials and Methods." The PCR products were separated on 2% agarose gel. One of two independent experiments with identical results is shown. *L*, ladder; *208*, MG208-treated cells; *88*, MG88-treated cells; *Con*, Me_2SO-treated cells; *Aza*, 5-aza-CdR-treated cells; *U*, unmethylated; *M*, methylated.

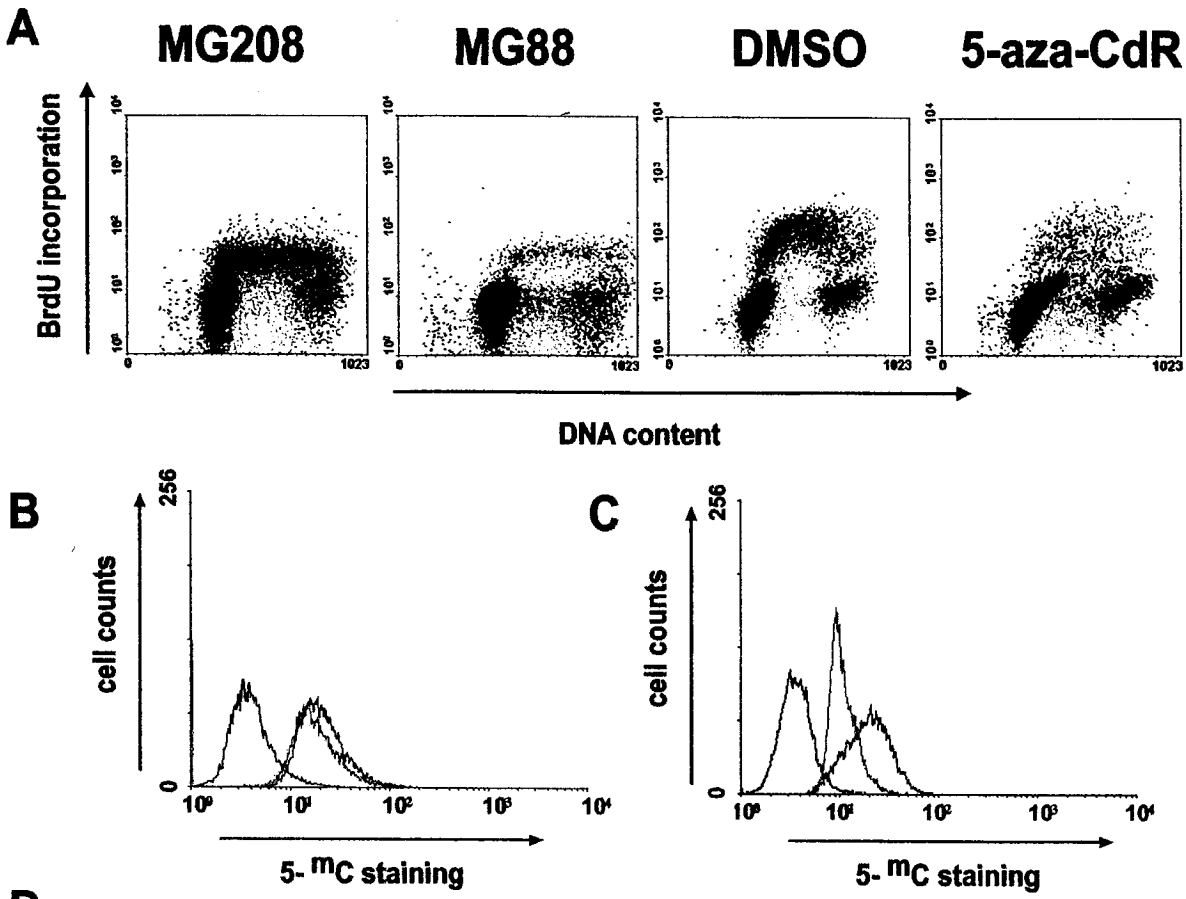

A
MG208
MG88
DMSO
5-aza-CdR
BrdU incorporation
DNA content
B
cell counts
5- mC staining
C
cell counts
5- mC staining
D
24hr
48hr
72hr
96hr
208
88
L U M U M U M U M U M U M U M U M
E
24hr
48hr
72hr
96hr
Con
Aza
L U M U M U M U M U M U M U M U M

Fig. 7. 5-aza-CdR treatment induces expression of the proapoptotic gene BIK and cancer/testis-specific genes MAGEB2 and SSX2. *A*, total RNA was isolated from A549 cells treated with either 5-aza-CdR (1 μM) or Me_2SO (*DMSO*) as a control for 24-96 h. RT-PCR was performed with primers for the indicated genes as described under "Materials and Methods." 10 μl of PCR products were run on 1.2% agarose gel. *B*, PCR products were quantified by densitometry, normalized to actin, and presented as arbitrary units for *SSX2* and *MAGEB2* or as a ratio of 5-aza-CdR (*Aza*) to Me_2SO for *BIK*, *ATF-3*, *GADD45*, and *JunB*. Representative data from two separate experiments are shown.

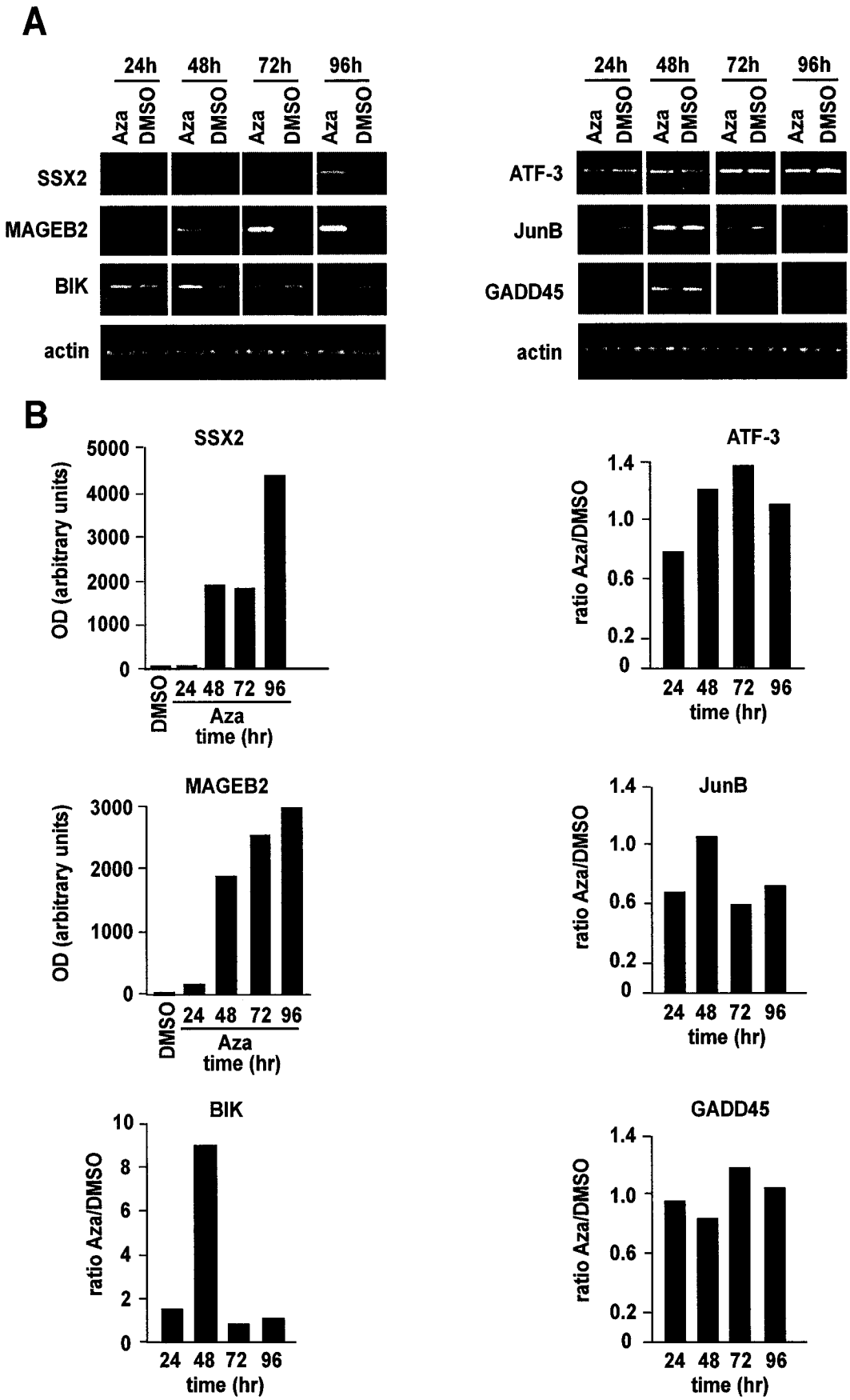

A
24h
48h
72h
96h
Aza
DMSO
SSX2
MAGEB2
BIK
actin
ATF-3
JunB
GADD45
B
OD (arbitrary units)
ratio Aza/DMSO
DMSO
24 48 72 96
Aza
time (hr)

Fig. 8. DNMT1 antisense knockdown induces expression of the genotoxic stress response genes ATF-3, JunB, and GADD45. *A*, total RNA was isolated from A549 cells treated with 200 nM of either MG208 or MG88 for 24-96 h. RT-PCR was performed with primers for the indicated genes as described under "Materials and Methods." 10 μl of PCR products were run on 1.2% agarose gel. In the case of *MAGEB2, SSX2*, and *BIK*, RT-PCR was also performed on A549 cells treated with 5-aza-CdR (1 μM) for 72 h as an expression control. *B*, PCR products were quantified by densitometry and normalized to actin product. The relative ratio of MG88-treated to MG208-treated cells were then calculated and presented in *B*. Representative data from 2-4 separate experiments are shown.

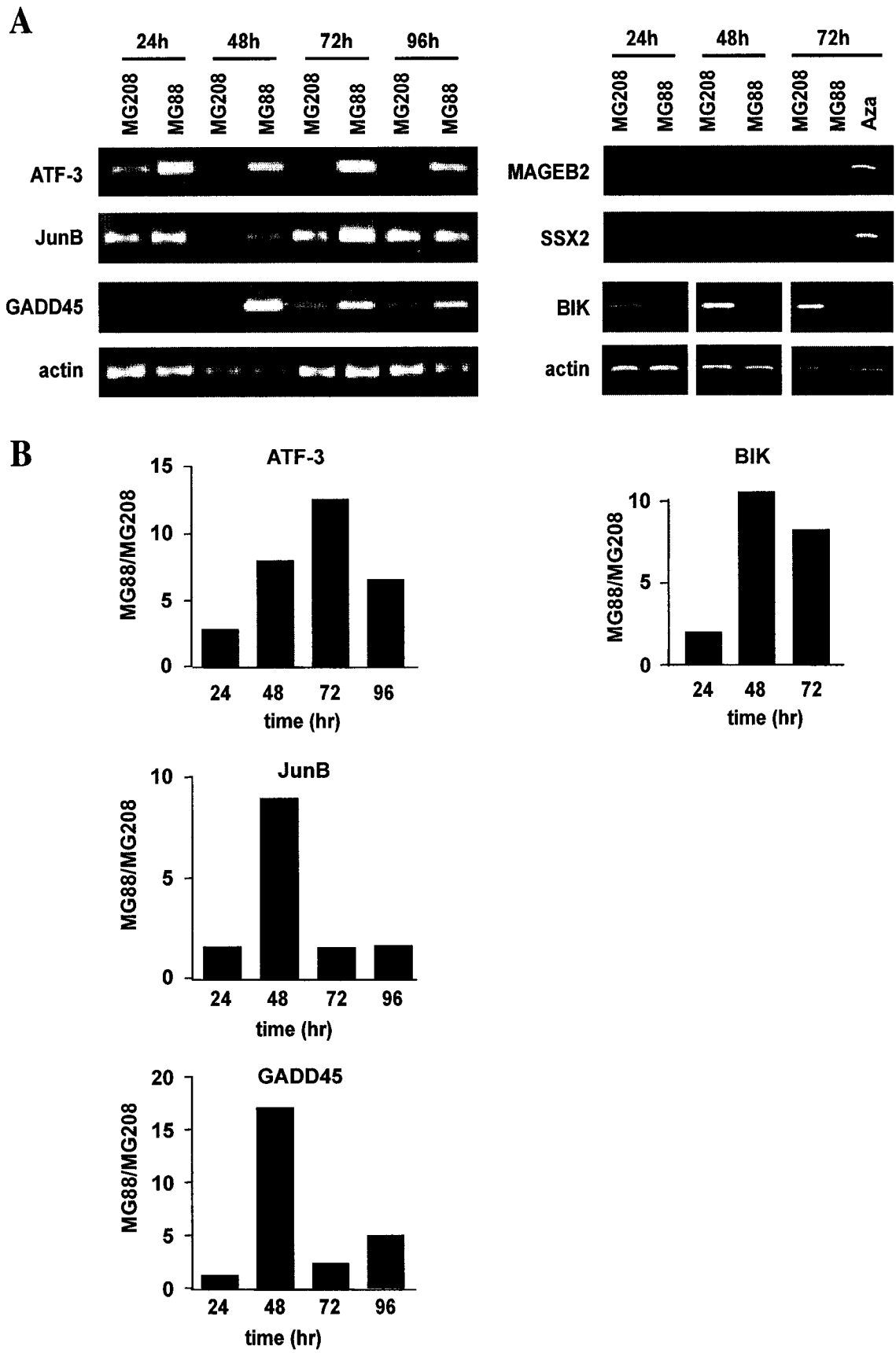

A
24h
48h
72h
96h
MG208
MG88
ATF-3
JunB
GADD45
actin
24h
48h
72h
MG208
MG88
Aza
MAGEB2
SSX2
BIK
actin
B
ATF-3
MG88/MG208
time (hr)
BIK
JunB
GADD45

Table I

Genes up-regulated by 5-aza-CdR after 48 h of treatment.

A549 cells were treated with 1 μM 5-aza-CdR or Me_2SO for 48 h. Total RNA was subjected to a differential expression micoarray analysis using HuGeneFL DNA microarrays containing oligonucleotides specific for approximately 12,000 human transcripts as described under "Materials and Methods." The first column indicates the fold difference of the normalized expression of the indicated genes in 5-aza-CdR-, *versus* Me_2SO-treated A549 cells. The second column lists the accession numbers of the genes. The third column lists the names of the genes, and the last column provides their abbreviated names.

Ratio	Accession #	Title	Name
268.8	X79200	Synovial sarcoma, X breakpoint 2	SSX2
11.0	U90840	Synovial sarcoma, X breakpoint 3	SSX3
3.8	U90841	Synovial sarcoma, X breakpoint 4	SSX4
53.8	U66078	Deleted in azoospermia like	DAZL
9.7	M77481	Melanoma antigen, family A, 1	MAGEA1
5.2	L18920	Melanoma antigen, family A, 2	MAGEA2
14.2	U10688	Melanoma antigen, family A, 4	MAGEA4
4.4	U10689	Melanoma antigen, family A, 5	MAGEA5
3.9	L18877	Melanoma antigen, family A, 12	MAGEA12
62.5	U93163	Melanoma antigen, family B, 2	MAGEB2
9.8	U34584	BCL2-interacting killer (apoptosis-inducing)	BIK
4.6	AA131149	S100 calcium-binding protein P	S100P
7.9	X04430	Interleukin 6 (interferon, beta 2)	IL6
4.4	AJ223280	Linker for activation of T cells	LAT
3.2	U05875	Intrerferon gamma receptor 2 (interferon gamma transducer 1)	IFNGR2
8.9	L24564	Ras-related associated with diabetes	RRADα
10.1	M94250	Midkine (neurite growth-promoting factor 2)	MDK
2.7	AI985272	Neuromedin B	NMB
5.7	M20469	Clathrin, light polypeptide (Lcb)	CLTB
5.2	AB000714	Claudin 3	CLDN3
3.3	X13839	Actin, alpha 2, smooth muscle, aorta	ACTA2
2.7	AFOO1691	periplakin	PPL
6.0	J03040	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC
3.7	X53463	Glutathione peroxidase 2 (gastrointestinal)	GPX2
4.0	D00632	Glutathione peroxidase 3 (plasma)	GPX3
5.6	S79854	Deiodinase, iodothyronine, type II	DIO3
5.8	N74607	Aquaporin 3	AQP3
6.4	U36610	RNA binding motif protein, Y chromosome, family 1, member A1	NULL
2.7	C20962	ATP-binding casette, sub-family B (MDR/TAP), member 6	ABCB6
2.5	Y18504	DNA segment on chromosome 6 (unique) 2654 expressed sequence	D6S2654E
13.0	AF042498	Cyclic nucleotide gated channel beta 1	CNGB1
5.0	W68830	HSPC022	HSPC022
44.2	U58096	TSPYq1	TSPY
-1.4	X63692	DNA (cytosine-5-)-methyltransferase 1	DNMT1

Table II

Stress-responsive genes up-regulated by MG88 after 48 h of treatment.

A549 cells were transfected with 200 nM of either MG208 or MG88 for 48 h. Total RNA was subjected to a differential expression microarray analysis using HuGeneFL DNA microarrays containing oligonucleotides specific for approximately 12,000 human transcripts as described under "Materials and Methods." The first column indicates the fold difference of the normalized expression of the indicated genes in MG88 *versus* MG208 treated A549 cells. The second column lists the accession numbers of the genes. The third column lists the names of the genes, and the last column provides their abbreviated names.

Ratio	Accession #	Title	Name
17.6	AF078077	growth arrest and DNA-damage-inducible, beta	GADD45β
15.1	M59465	tumor necrosis factor, alpha-induced protein 3	TNFAIP3
12.1	S76638	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2
11.6	L19871	activating transcription factor 3	ATF3
9.1	J04111	v-jun avian sarcoma virus 17 oncogene homolog	JUN
9.1	U19261	TNF receptor-associated factor 1	TRAF1
7.4	U72206	rho/rac guanine nucleotide exchange factor (GEF) 2	ARHGEF2
7.1	J04111	v-jun avian sarcoma virus 17 oncogene homolog	JUN
6.9	U10550	GTP-binding protein overexpressed in skeletal muscle	GEM
6.9	V01512	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS
6.2	M58603	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1
6.0	L49169	FBJ murine osteosarcoma viral oncogene homolog B	FOSB
5.8	M59287	CDC-like kinase1	CLK1
5.6	M60974	growth arrest and DNA-damage-inducible, alpha	GADD45α
5.5	L36463	ras inhibitor	RIN1
5.3	M69043	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA
5.2	X51345	jun B proto-oncogene	JUNB
4.8	D87116	mitogen-activated protein kinase kinase 3	MAP2K3
4.5	AF059617	serum-inducible kinase	SNK
4.1	S82240	ras homolog gene family, member E	ARHE
3.8	AB014551	rho/rac guanine nucleotide exchange factor (GEF) 2	ARHGEF2
3.7	X61498	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2
3.6	U83981	growth arrest and DNA-damage-inducible 34	GADD34
3.6	AF050110	TGFB inducible early growth response	TIEG
3.2	AB011093	Rho-specific guanine nucleotide exchange factor p114	P114-RHO-GEF
3.1	S81439	TGFB inducible early growth response	TIEG
3.1	AI304854	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B
2.9	M62831	immediate early protein	ETR101
2.9	U03106	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A
2.9	L08246	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1
2.8	X52560	CCAAT/enhancer binding protein (C/EBP), beta	CEBPB
2.8	M36820	GRO2 oncogene	GRO2
2.8	AF093265	Homer, neuronal immediate early gene, 3	HOMER-3
2.7	AI038821	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	HRAS
2.7	AF016266	tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B
2.6	X82260	Ran GTPase activating protein 1	RANGAP1
2.6	D87953	N-myc downstream regulated	NDRG1
2.5	X54489	GRO1 oncogene (melanoma growth stimulating activity, alpha)	GRO1
2.5	L20320	cyclin-dependent kinase 7 (homolog of Xenopus MO15 cdk-activating kinase)	CDK7
-3.4	X63692	DNA (cytosine-5-)-methyltransferase 1	DNMT1

The data shown in the previous chapter further supports the hypothesis that DNMT1 plays alternative roles in tumorigenesis independent of its methyltransferase activity. Our findings that 5-aza-CdR and oligonucleotide antisense produce very different effects on cell growth and gene expression reflect multiple functions of DNMT1 in different cellular processes. Whereas knock down of DNMT1 protein by antisense resulted in rapid replication arrest, limited DNA demethylation, and activation of genes in a methylation independent way, inhibition of DNMT1 methyltransferase activity by 5-aza-CdR produced limited cell cycle arrest, massive demethylation and activation of genes silenced by methylation. In addition, only knock down of DNMT1 resulted in the intra-S-phase arrest and the activation of stress response genes. Since these effects were not seen with the demethylating agent 5-aza-CdR, we propose that these effects result from the activation of the epigenetic check point that senses the presence of DNMT1 protein rather than DNA methylation status. Our findings also suggest that agents causing global demethylation should be avoided because of their potential to induce genes involved in metastasis. Therefore, we propose that the methylation independent functions of DNMT1 should be targeted in cancer, since this would lead to a rapid cell cycle arrest and limited global demethylation. In the following chapter we further explore the methylation independent roles of DNMT1 in the regulation of gene expression. We found that DNMT1 may function independent of its methyltransferase activity and its recently identified HDAC-recruiting activity. In addition, the regulation of the Sp1 responsive promoters by DNMT1 levels suggests that DNMT1 may also play a role in the regulation of the cell cycle.

Chapter 3

DNA methyltransferase 1 knock down induces gene expression by a mechanism independent of DNA methylation and histone deacetylation.

Snezana Milutinovic, Shelley E. Brown, Qianli Zhuang and Moshe Szyf

This chapter has been published in the Journal of Biological Chemistry (2004) 279, 27915-27

DNA methyltransferase 1 (DNMT1) catalyzes the post-replication methylation of DNA and is responsible for maintaining the DNA methylation pattern during cell division. A long list of data supports a role for DNMT1 in cellular transformation and inhibitors of DNMT1 were shown to have antitumorigenic effects. It was long believed that DNMT1 promoted tumorigenesis by maintaining the hypermethylated and silenced state of tumor suppressor genes. We have previously shown that DNMT1 knock down by either antisense oligonucleotides directed at DNMT1 or expressed antisense activates a number of genes involved in stress response and cell cycle arrest by a DNA methylation-independent mechanism. In this report we demonstrate that antisense knock down of DNMT1 in human lung carcinoma A549 and embryonal kidney HEK293 cells induces gene expression by a mechanism that does not involve either of the known epigenomic mechanisms, DNA methylation, histone acetylation, or histone methylation. The mechanism of activation of the cell cycle inhibitor *p21* and apoptosis inducer *BIK* by DNMT1 inhibition is independent of the mechanism of activation of the same genes by histone deacetylase inhibition. We determine whether DNMT1 knock down activates one of the nodal transcription activation pathways in the cell and demonstrate that DNMT1 activates Sp1 response elements. This activation of Sp1 response does not involve an increase in either Sp1 or Sp3 protein levels in the cell or the occupancy of the Sp1 elements with these proteins. The methylation-independent regulation of Sp1 elements by DNMT1 unravels a novel function for DNMT1 in gene regulation. DNA methylation was believed to be a mechanism for suppression of CG-rich Sp1-bearing promoters. Our data suggest a fundamentally different and surprising role for DNMT1 regulation of CG-rich genes by a mechanism independent of DNA methylation and histone acetylation. The implications of our data on the biological roles of DNMT1 and the therapeutic potential of DNMT1 inhibitors as anticancer agents are discussed.

INTRODUCTION

DNA modification by methylation of cytosines residing at the dinucleotide sequence CG plays an important role in epigenomic programming of gene expression (Razin, 1998). Not all CGs are methylated, and the pattern of distribution of methylated and unmethylated CGs is cell type specific (Razin and Szyf, 1984). DNA methylation in regulatory regions of genes plays a role in silencing genes either by directly inhibiting the interaction of transcription factors with their regulatory sequences (Comb and Goodman, 1990; Inamdar et al., 1991) or by attracting methylated DNA-binding proteins, which in turn recruit histone deacetylases and histone methyltransferases, resulting in an inactive chromatin structure (Jones et al., 1998; Nan et al., 1998). DNA methylation is catalyzed by DNA methyltransferases DNMTs, which transfer the methyl moiety from the methyl donor *S*-adenosylmethionine to 5th position on the cytosine ring (Wu and Santi, 1985). DNMT1 is responsible for maintaining the DNA methylation pattern during embryonal development and cell division (Li et al., 1992; Szyf et al., 1991). DNMT1 deregulation was proposed to play a critical role in cellular transformation (Szyf et al., 2000). Forced expression of DNMT1 was shown to transform NIH 3T3 cells (Wu et al., 1993), DNMT1–/– knockouts are resistant to colorectal tumorigenesis (Laird et al., 1995), and antisense knock down of DNMT1 reverses tumorigenesis *in vitro* (Fournel et al., 1999; MacLeod and Szyf, 1995) and *in vivo* (Ramchandani et al., 1997). The mechanisms through which DNMT1 causes cellular transformation and through which inhibition of DNMT1 reverses cellular transformation are unknown (Szyf et al., 2000). The most obvious mechanism is that aberrant expression of DNMT1 causes methylation and silencing of tumor suppressor genes (Herman et al., 1995; Merlo et al., 1995). This hypothesis is supported by numerous documentations of methylated tumor suppressor genes in tumors (Baylin et al., 2001). In accordance with this hypothesis, knock down of DNMT1 by either antisense or siRNA results in demethylation and activation of tumor suppressor genes such as *p16* (Fournel et al., 1999; Robert et al., 2003).

However, it was surprisingly previously shown that knock down of DNMT1 results in induction of the unmethylated tumor suppressor gene *p21* by a mechanism that does not involve DNA methylation (Milutinovic et al., 2000). More recently it was shown that DNMT1 interacts with histone deacetylases (HDACs) 1 (Fuks et al., 2000) and 2 (Rountree et al., 2000) as well as histone methyltransferase SUV39H1 (Fuks et al., 2003a), suggesting that DNMT1 silences gene expression by recruiting chromatin-modifying enzymes. It was also shown that ectopic expression of DNMT1 could suppress exogenous genes bearing E2F1 sites by recruiting Rb·E2F1·HDAC1 complex (Robertson et al., 2000a). However, it is not clear whether DNMT1 regulates endogenous genes by these mechanisms.

We have previously shown that DNMT1 expression is regulated with the cell cycle (Detich et al., 2001; Szyf et al., 1991; Szyf et al., 1985b) and that antisense knock down of DNMT1 results in an intra-S-phase arrest of DNA replication (Milutinovic et al., 2003). This intra-S-phase arrest requires knock down of the DNMT1 protein rather than inhibition of DNA methylation, suggesting that DNMT1 protein plays an important role in the regulatory circuitry independent of its methyltransferase activity (Szyf, 2003). We and others have previously shown that antisense ODNs directed at DNMT1 exhibit sequence-specific down-regulation of DNMT1 and that they could be utilized to delineate the immediate consequences of knock down of the DNMT1 protein on gene expression and cellular regulation (Fournel et al., 1999; Knox et al., 2000; Milutinovic et al., 2003; Robert et al., 2003; Szyf, 2002). In difference from agents such as 5-azacytidine that inhibit the catalytic activity of DNMT1 and, thus, mainly measure the DNA methylation-dependent roles of this protein (Jones et al., 1983), antisense knock down of DNMT1 causes an immediate arrest of DNA replication (Milutinovic et al., 2003), resulting in limited passive demethylation. This allows us to study mainly DNA methylation-independent regulatory functions of DNMT1. We have previously shown that a number of stress response-related genes are activated by DNMT1 knock down (Milutinovic et al., 2003). In this report we dissect the transcription factor pathway activated by DNMT1 knock down and show that surprisingly DNMT1 regulates gene expression by a new mechanism that does not

involve either DNA methylation or chromatin modification. We show that DNMT1 knock down and histone deacetylation inhibition activate gene expression by independent mechanisms. These results have important implications on understanding the regulatory roles of DNMT1 in the cell cycle and cellular transformation, as well as on future approaches to targeting DNMT1 in anticancer therapy.

MATERIALS AND METHODS

Cell Culture, Antisense Oligonucleotides, and TSA Treatment—A549 cells, a human non-small cell lung carcinoma-derived cell line (Giard et al., 1973) (ATCC:CCL 185), were grown in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. 18–24 h before treatment, cells were plated at a density of 3 x 10^5 cells/100-mm tissue culture dish, or 4 x 10^4 cells/well in a six-well plate in the absence of antibiotics. The phosphorothioate oligodeoxynucleotides used in this study were MG88 (human *DNMT1* antisense) and its mismatch control MG208, which has a 6-bp difference from MG88 (Fournel et al., 1999). Oligonucleotides were transfected into cells at a concentration of 120 nM with 3.75 μg/ml Lipofectin (Invitrogen) in serum-free Opti-MEM (Invitrogen). The oligonucleotide-containing Opti-MEM was removed from the cells and replaced with regular growth media after 4 h. The treatment was repeated after 24 h. The cells were harvested 12, 18, 24, 30, 36, 42, or 48 h after the first treatment. For TSA treatment, cells were grown in regular culture media in the presence of 1 μM of trichostatin A (TSA). The cells were harvested 2, 6, or 12 h after the initiation of treatment. HEK293 cells, a human adenovirus type 5-transformed human embryonal kidney cell line (ATCC, CRL 1573), were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum and 2 mM glutamine.

Western Blot Analysis—50 μg of nuclear protein was fractionated on a 5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and reacted with the polyclonal anti-DNMT1 antibody (New England Biolabs) at a dilution of 1:2,000 in the presence of 0.05% Tween and 5% milk, and it was then reacted with

anti-rabbit IgG (Sigma) at a dilution of 1:5,000 in the presence of 0.05% Tween and 5% milk. As a control for protein loading, actin protein levels were examined in the same nuclear extracts. 50 μg of protein was fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and reacted with the monoclonal anti-actin antibody (Sigma, A5316) at a dilution of 1:5000 in the presence of 0.05% Tween and 5% milk, and it was then reacted with anti-mouse IgG (Jackson Immunochemicals) at a dilution of 1:20,000 in the presence of 0.05% Tween.

RT-PCR—Total RNA was extracted using an RNeasy kit (Qiagen). cDNA was synthesized in a 20 μl reaction volume containing 2 μg of total RNA, 40 units of Moloney murine leukemia virus reverse transcriptase (MBI), 5 μM random primer (Roche Applied Science), 1 mM of each of the deoxynucleotide triphosphates, and 40 units of RNase inhibitor (Roche Applied Science). mRNA was denatured for 5 min at 70°C, the random primers were annealed for 10 min at 25°C, and the mRNA was reverse-transcribed for 1 h at 37 °C. The reverse transcriptase was heat-inactivated for 10 min at 70 °C, and the products were stored at –20 °C until use. PCRs were performed in a 40 μl reaction mixture containing 2 μl of synthesized cDNA product, 4 μl of 10 x PCR buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 unit of *Taq* polymerase (all from MBI), and 0.4 μM of each primer. Amplifications were performed in a Biometra T3 thermocycler (Biomedizinische Analytik GmbH). The primer sequences and the amplification programs were as follows: *p21* (*cyclin-dependent kinase inhibitor 1*) (sense, 5'-GCCCAGTGGACAGCGAGCAG-3'; antisense, 5'-GCCGGCGTTTGGAGTGGTAGA-3') 95°C 5 min, one cycle (95 °C, 68 °C, and 72 °C for 30 s each), one cycle (95 °C, 66 °C, 72 °C for 30 s each), 24 cycles (95 °C, 65 °C, and 72 °C 30 s each), 72 °C 5 min; *BIK* (*BCL2-interacting killer*) (sense, 5'-GGCCTGCTGCTGTTATCTTT-3'; antisense, 5'-CCAGTAGATTCTTTGCCGAG-3') 95 °C 5 min, one cycle (95 °C, 62 °C, and 72 °C for 30 s each), one cycle (95 °C, 60 °C, and 72 °C for 30 s each), 29 cycles (95 °C, 58 °C, and 72 °C 30 s each), 72 °C 5 min; *HSPA2* (*heat shock protein 70-kDa 2*) (sense, 5'-CTTTCGCCTTCTGCCGTGA-3'; antisense, 5'-ACTGCACTGTGGCATCCTC-3') 95 °C 5 min, one cycle (95 °C, 58 °C, and 72 °C for 30 s each), one cycle (95 °C, 56

°C, and 72 °C for 30 s each), 26 cycles (95 °C, 54 °C, and 72 °C for 30 s each), 72 °C 5 min; *actin* (sense, 5'-GTTGCTAGCCAGGCTGTGCT-3'; antisense, 5'-CGGATGTCCACGTCACACTT-3') 95 °C 5 min, one cycle (95 °C, 66 °C, and 72 °C for 30 s each), one cycle (95 °C, 64 °C, and 72 °C for 30 s each), one cycle (95 °C, 62 °C, and 72 °C for 30 s each), and 17 cycles (95 °C, 60 °C, and 72 °C 30 s each), 72 °C 5 min. The number of cycles was tested and selected so that the PCR amplification remained in the linear phase. 20 μl of the PCR products were run on a 1.2% gel and visualized by ethidium bromide staining. Densitometric analysis was performed using MCID software (Imaging Research Inc.).

Bisulfite Mapping of the BIK Promoter—A549 cells were treated with 120 nM MG88 or MG208 for 24 h. Bisulfite mapping was performed as described previously with minor modifications (Clark et al., 1994). 50 ng of sodium bisulfite-treated DNA samples were subjected to PCR amplification using the first set of primers (sense, 5'-GTAAAAAAAGTTAGATTTGTGG-3'; antisense, 5'-CTCACCTCCTCTAAATACC-3'). PCR products were used as templates for the nested PCR using the second set of primers (sense, 5'-AGGGATTGGGGGAGGAG-3'; antisense, 5'-CAACTACTCACACCTCAAC-3'). The PCR products of the second reaction were subcloned into a TA cloning vector (Invitrogen), and the clones were sequenced using the T7 Sequencing kit (Amersham Biosciences).

Reporter Constructs and Luciferase Assay—The calcium phosphate precipitation method was used to transiently co-transfect HEK293 cells, plated in six-well dishes, with 2 μg of luciferase reporter constructs and 5 μg of either control pcDNA3.1 vector (Invitrogen) or a *DNMT1* antisense (bp 396–5066) subcloned into pcDNA3.1 vector (as *DNMT1*). The reporter constructs that were used contained either full p21 promoter (–2326 bp) or four different 5' deletions (–1481, –883, –291, and –94) upstream from the luciferase gene. In addition, Sp1 responsive reporters were constructed using basic luciferase reporter containing only TATA box and initiator sequence (pGL2 T+I). An oligonucleotide containing consensus Sp1 binding site corresponding to the bases –71 to –86 of the *p21* promoter was synthesized and inserted either once (1 x Sp1) or four

times (4 x Sp1) into the pGL2 T+I vector. 48 h after transfection, the cells were lysed and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

A549 cells plated in six-well dishes were transfected using 3.75 μg/ml Lipofectin (Invitrogen) and 0.5 μg of the reporter vectors containing different *cis*-acting enhancer elements upstream from the firefly luciferase gene. All the reporters, except for the 4 x Sp1, belong to the Mercury Pathway Profiling System (Clontech). 24 h after transfection, the cells were either treated with 120 nM MG88 and MG208 for 24 h or with 1 μM TSA for 6 h. The luciferase activity was assayed using the Luciferase Assay System (Promega).

The full *p21* promoter-luciferase reporter construct (–2326 p21) was used to create a construct with 2-bp mutations (CCGA) within the Sp1 site corresponding to bases between –71 and –86 of the *p21* promoter (–2326 p21 CCGA). A Quick-Change XLII site-directed mutagenesis Kit (Stratagene) was used with the following mutagenic primers: 5'-GCGCGGGTCCCGGATCCTTGAGGCGGG-3' and 5'-CCCGCCTCAAGGATCCGGGACCCGCGC-3'. The amplification cycles were as follows: 98 °C 3 min, 18 cycles (98 °C 50 s, 68 °C 17 min), 68 °C 7 min. A549 cells plated in six-well dishes were transfected using 3.75 μg/ml Lipofectin (Invitrogen) and 0.5 μg of either wild type or Sp1-mutated full *p21* promoter construct. 24 h after transfection, the cells were treated with 120 nM MG88 and MG208 for 24 h, and the luciferase activity was assayed using the Luciferase Assay System (Promega).

Chromatin Immunoprecipitation Assay—A549 cells were treated with 120 nM MG88 and MG208 for 24 h or 48 h, or with 1 μM TSA for 6 h. Formaldehyde was added to a final concentration of 1%, and the cells were incubated at 37 °C for 10 min. The cells were washed and harvested in cold phosphate-buffered saline containing protease inhibitors (Complete mini, Roche Applied Science), were pelleted, resuspended in 300 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. Lysates were sonicated with six 10-s bursts. Debris was

removed by centrifugation for 10 min at 13,000 rpm at 4 °C. Supernatants were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and incubated with 80 μl of agarose G mix (50% agarose G, 0.2 mg/ml sonicated herring sperm, 0.5 mg/ml bovine serum albumin, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 30 min at 4 °C. Beads were pelleted for 5 min at 3,000 rpm at 4°C. 100 μl of the supernatant was saved as input, and the rest was divided into equal aliquots and incubated by rocking with either no antibody (control), or with 10 μl of specific antibodies o/n at 4 °C. The following antibodies were used: anti-acetylated histone H3 (Upstate Biotechnology #06-599), anti-acetylated histone H4 (Upstate Biotechnology #06–866), anti-dimethylhistone H3 (Lys-9) (Upstate Biotechnology #07-212), anti-Sp1 (Santa Cruz Biotechnology, #sc-59), and anti-Sp3 (Santa Cruz Biotechnology, #sc-644X). 60 μl of agarose G mix was added, and the samples were rocked for 2 h at 4 °C. The bead complexes were washed for 5 min at 4 °C with the following buffers: low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl wash (0.25 M LiCl, 1% Nonident P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and six washes of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Immune complexes were eluted twice with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature. The input (volume adjusted to 500 μl with elution buffer) and combined eluates were de-cross-linked by adding 20 μl of 5 M NaCl and incubating for 4 h at 65 °C. EDTA, Tris-HCl, pH 6.5, and proteinase K were then added to the final concentrations of 10 mM, 40 mM, and 0.1 μg/μl, respectively, and the samples were incubated at 45 °C for 1 h. Immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation and was analyzed by PCR. For amplifying 255 bp region of *p21* promoter, *BIK* promoter, and enhancer region of 4 x Sp1 luciferase reporter, PCR was performed in a 40 μl reaction mixture containing 2 μl of ChIP product, 4 μl of 10 x PCR buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 unit of *Taq* polymerase (all from MBI), and 0.2 μM of each primer. For the 324-bp region of the *p21* promoter, and promoter and exon regions of *HSPA2*, the FailSafe PCR system (Epicenter) was used with pre-mixes D, A, and E

(respectively), 0.5 μM of each primer, 0.5 μl of FailSafe polymerase, and 2 μl of ChIP product. The primer sequences and the amplification programs were as follows: *p21* (324 bp region) (sense, 5'-ACCAACGCAGGCGAGGGACT-3'; antisense, 5'-CCGGCTCCACAAGGAACTGA-3'), 95 °C 5 min, one cycle (95 °C, 64 °C, and 72 °C for 30 s each), one cycle (95 °C, 62 °C, and 72 °C for 30 s each), 32 cycles (95 °C, 60 °C, and 72 °C 30 s each), 72 °C 5 min; *p21* (255 bp region) (sense, 5'-GGTGTCTAGGTGCTCCAGGT-3'; antisense, 5'-GCACTCTCCAGGAGGACACA-3'), 95 °C 5 min, one cycle (95 °C, 64 °C, 72 °C for 30 s each), one cycle (95 °C, 62 °C, and 72 °C for 30 s each), 32 cycles (95 °C, 60 °C, and 72 °C 30 s each), 72 °C 5 min; 4 x sp1 (sense, 5'-AGGTACCGAGCTCTTACGC-3'; antisense, 5'-CGCCGGGCCTTTCTTTATG-3'), 95 °C 5 min, one cycle (95 °C, 60 °C, and 72 °C for 30 s each), one cycle (95 °C, 58 °C, and 72 °C for 30 s each), 24 cycles (95 °C, 56 °C, and 72 °C 30 s each), 72 °C 5 min; *BIK* (sense, 5'-GCACACTCTTTGAACGCTG-3'; antisense, 5'-GCTACAAAGTGCAGCCCGTTG-3') 95 °C 5 min, one cycle (95 °C, 62 °C, 72 °C for 30 s each), one cycle (95 °C, 60 °C, and 72 °C for 30 s each), 32 cycles (95 °C, 58 °C, and 72 °C 30 s each), 72 °C 5 min; promoter of *HSPA2* (sense, 5'-TGCTTGGTTCTCATTCTCTCT-3'; antisense, 5'-AGGTGTACCAGCATTTCAGC-3'), 95 °C 5 min, one cycle (95 °C, 60 °C, and 72 °C for 30 s each), one cycle (95 °C, 58 °C, and 72 °C for 30 s each), 32 cycles (95 °C, 56 °C, and 72 °C 30 s each), 72 °C 5 min; exon region of *HSPA2* (sense, 5'-GGGGCAAGATTAGCGAGCAGGA-3'; antisense, 5'-TTCGATGGTGGGTCCCCCGGAG-3'), 95 °C 5 min, one cycle (95 °C, 60 °C, and 72 °C for 30 s each), one cycle (95 °C, 58 °C, and 72 °C for 30 s each), 32 cycles (95 °C, 56 °C, and 72 °C 30 s each), 72 °C 5 min. 20 μl of the PCR products was run on a 1.4% gel and visualized by ethidium bromide staining. Densitometric analysis was performed using MCID software (Imaging Research Inc.).

To assess the effect that Sp1 mutation might have on Sp1 occupancy of the *p21* promoter in living cells, the wild type (–2326 p21) and Sp1-mutated (–2326 p21 CCGA) *p21* promoter luciferase reporter construct, were transiently transfected into A549 cells using 3.75 μg/ml Lipofectin (Invitrogen) and 1.5 μg/10 cm plate of the

reporter construct. 48 h after transfection, the cells were cross-linked, and the chromatin was immunoprecipitated using 10 μg of Sp1 antibody (Upstate Biotechnology, #07-124) as described above. The precipitated wild type and Sp1-mutated reporter constructs were PCR amplified using the same primers (sense, 5'-ACCAACGCAGGCGAGGGACT-3'; antisense, 5'-CGCCGGGCCTTTCTTTATG-3'). The 5' primer corresponds to the bases between –256 and –236 of the *p21* promoter, whereas the 3' primer corresponds to the luciferase sequence. Thus our primers will selectively amplify the exogenous *p21* promoter luciferase reporter and not the endogenous wild type *p21* promoter. The FailSafe PCR system (Epicenter) was used with the pre-mix D, 0.5 μM of each primer, 0.5 μl of FailSafe polymerase, and 2 μl of ChIP product with the following program: 95 °C 5 min, one cycle (95 °C, 60 °C, and 72 °C for 30 s each), one cycle (95 °C, 58 °C, and 72 °C for 30 s each), 23 cycles (95 °C, 56 °C, and 72 °C 30 s each), 72 °C 5 min. 20 μl of the PCR products was run on a 1.4% gel and visualized by ethidium bromide staining. The intensity of the bands was quantified by densitometric analysis using MCID software (Imaging Research Inc.). The intensities of the *p21*-amplified bands immunoprecipitated with the anti-Sp1 antibody were normalized to the intensities of the bands amplified from the input material.

Electrophoretic Mobility Shift Assays—Complementary oligonucleotides carrying either the Sp1 binding site corresponding to bases –71 and –86 of the wild type *p21* promoter (5'-GGTCCCGCCTCCTTGA-3' and 5'-TCAAGGAGGCGGGACC-3') or to a mutated Sp1 site containing 2-bp mismatch (CCGA) (5'-GGTCCCGGATCCTTGA-3' and 5'-TCAAGGATCCGGGACC-3') were synthesized and annealed. 20 ng of the annealed oligonucleotides was end-labeled with [^{32}P]ATP and T4 polynucleotide kinase (MBI) and gel-purified from a 5% non-denaturing polyacrylamide gel. To measure the binding of either the Sp1 site or its mutated version to Sp1 protein 1 x 10^5 counts of the labeled oligonucleotides were used in a gel shift reaction containing 400 ng of the purified human recombinant Sp1 protein (Promega, catalog number E6391), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)/(dI-dC), and the reaction was

incubated for 20 min at 23 °C. To demonstrate the presence of Sp1 in the DNA·protein complex, the Sp1·DNA reaction mixtures were preincubated with anti-Sp1 antibody (Santa Cruz Biotechnology, #sc-59) for 10 min before the addition of the labeled oligonucleotides. The complexes were resolved on a 5% non-denaturing polyacrylamide gel, and the gel was dried and analyzed by autoradiography.

RESULTS

DNMT1 Antisense and HDAC Inhibitor TSA Induce p21 and BIK Gene Expression; p21 and BIK Are Regulated by HDAC Activity—DNMT1 knock down was previously shown using a microarray gene expression analysis to induce expression of a cluster of genes involved in stress response (Milutinovic et al., 2003). The kinetics of induction indicated that some of these genes were induced by a methylation-independent mechanism. All of the methylation-independent pathways of gene repression by DNMT1 that have been proposed to date are based on the ability of DNMT1 to interact with and possibly recruit histone deacetylases HDAC1 and HDAC2 (Fuks et al., 2000; Robertson et al., 2000a; Rountree et al., 2000) and histone methyltransferase SUV39H1 to promoters (Fuks et al., 2003a). However, the data supporting these interactions are based on ectopic expression experiments, and there is no evidence as of yet that an endogenous unmethylated gene is regulated by DNMT1-HDAC1 interaction through a histone acetylation-dependent mechanism. We therefore tested the hypothesis that DNMT1 knock down induces gene expression by a histone acetylation-dependent mechanism.

We focused on two genes that were demonstrated to be induced by DNMT1 knock down, the cell cycle inhibitor *p21* and apoptosis inducer, *Bcl-2 interacting killer*, *BIK*. We have previously shown that the induction of *p21* is methylation-independent (Milutinovic et al., 2000), and the kinetics of *BIK* induction suggested that it is also induced by a methylation-independent mechanism (Milutinovic et al., 2003).

We first tested the hypothesis that *p21* and *BIK* genes are regulated by the state of histone acetylation by taking advantage of the general HDAC inhibitor trichostatin A (TSA). A549 cells were treated with 120 nM of either antisense to DNMT1 MG88, or its mismatch control MG208, for 12–42 h as well as with 1 μM TSA for 2–12 h. We show that both DNMT1 knock down and HDAC inhibition by TSA induce these genes to a comparable degree (4- to 5-fold). MG88 induces almost complete knock down of DNMT1 mRNA and protein 24 h after treatment (Fig. 1, A and B) at which point we observe peak induction of *p21* and *BIK* mRNA (Fig. 1, B and C). TSA causes peak induction somewhat earlier, between 6 and 12 h (Fig. 2) as previously demonstrated (Sowa et al., 1999). The slight delay in induction of *BIK* and *p21* by DNMT1 knock down reflects the different mechanisms of action of these two inhibitors. TSA inhibits the catalytic activity of HDACs, whereas MG88 inhibits *de novo* synthesis of *DNMT1* mRNA and protein. The delay in induction probably reflects the turnover rate of DNMT1 protein. The induction of *p21* and *BIK* with TSA illustrates that these genes are down-regulated by histone deacetylation in their basal state, and it is therefore possible that knock down of DNMT1 affects the state of histone acetylation of these genes. We now address the question of whether induction of these genes by DNMT1 knock down and histone deacetylation inhibition act through identical or independent pathways.

Knock Down of DNMT1 Does Not Cause Demethylation of BIK Promoter and Induces Its Expression by a Methylation-independent Pathway—CG methylation is believed to be an on-off switch in gene expression. Hypermethylation of promoter regions is associated with compact chromatin inaccessible to the transcriptional machinery, which results in a stable suppression of gene expression (Razin, 1998). Hence, DNA methylation is unlikely to play a role in the regulation of *p21* and *BIK* genes, because they are expressed even in the untreated cells (Figs. 1 and 2). In accordance with this hypothesis, we have previously shown that the regulatory region of the *p21* promoter upstream of transcription start site is unmethylated in A549 cells (Milutinovic et al., 2000).

To rule out the possibility that induction of the *BIK* expression by DNMT1 knock down involves demethylation of its promoter, we examined the state of methylation of the *BIK* promoter after treatment with either MG88 or MG208. *BIK* gene bears a CG island around the transcriptional start site, which renders it susceptible to control by methylation (Fig. 3A). We performed bisulfite mapping of the proximal part of this CG island and found that it is predominantly unmethylated, except for the sporadically methylated CG sites in both MG88- and MG208-treated A549 cells (Fig. 3B). These results suggest that DNMT1 knock down does not result in demethylation of the *BIK* promoter. Thus, similar to *p21* (Milutinovic et al., 2000; Szyf et al., 2000), *BIK* seems to be induced by a methylation-independent mechanism.

Histone Acetylation of Promoters of p21 and BIK Is Induced by TSA Treatment but Remains Unchanged after DNMT1 Antisense Knock down; DNMT1 and HDAC Control p21 and BIK Expression by Independent Pathways—If DNMT1 knock down induces *p21* and *BIK* through either a histone deacetylase-dependent pathway (Fuks et al., 2000) or a histone methyltransferase-dependent pathway (Fuks et al., 2003a), as predicted by the published protein-protein interactions of DNMT1, MG88 treatment should cause a change in either the state of acetylation or methylation of histones associated with these genes. To test this hypothesis we performed chromatin immunoprecipitations (ChIP) with antibodies against acetylated H3 or acetylated H4 histones, followed by PCR amplification of the *p21* and *BIK* promoters. As shown in Fig. 4, there was no detected difference in histone acetylation at either 24 or 48 h after MG88 treatment. We then tested whether the knock down of DNMT1 reduces K9 H3 histone methylation. We performed ChIP analysis on A549 cell treated with 120 nM MG88 or MG208 for 48 h, using anti-dimethyl K9 H3 antibody. We did not detect any changes in histone methylation at either *p21* or *BIK* promoters (Fig. 4B), which suggested that the effects of DNMT1 knock down are not mediated through the disruption of DNMT1·SUV39H1 complex. To verify that histones associated with these regions are subject to a change, we tested whether HDAC inhibitor TSA, which produced comparable induction of *p21* and *BIK* mRNA (Fig. 2), induced a change in acetylation of histones associated with these regions. As shown in Fig. 4, C and D, 1

μM TSA treatment for 6 h causes a 5–7 fold increase in the state of acetylation of both histones H3 and H4 associated with promoter regions of *p21* and *BIK*. We conclude that the state of acetylation of histones associated with these regions is regulated by HDACs, but DNMT1 knock down does not affect either HDAC or histone methyltransferase activity on these histones. HDAC inhibitors and DNMT1 knock down induce *p21* and *BIK* by independent pathways.

The Sp1 Site Corresponding to Bases between –71 and –86 in the p21 Promoter Is Sufficient to Mediate the Induction by DNMT1 Knock down—Since we showed that DNMT1 knock down does not induce *p21* and *BIK* by modifying their chromatin, we tested whether this induction is mediated by the activation of any of the principal transcription activation pathways. We first used *p21* promoter deletion constructs and identified a minimal promoter (94 bp) responsive to reduction of DNMT1 levels. We transiently co-transfected HEK cells with either control vector or vector expressing full-length antisense to *DNMT1* and five *p21* deletion constructs. We identified that the 94-bp region of the *p21* promoter is equally induced with DNMT1 antisense as the entire 2.3 kbp of *p21* promoter. The analysis of this region for transcription factor binding sites revealed that this region is rich in Sp1 and E2F consensus sites (Fig 5A). Previous studies using mutation analysis of this region identified the Sp1 site corresponding to bases between –71 and –86 of the *p21* promoter to be essential for its activation by histone deacetylation inhibition (Sowa et al., 1999). It was also shown that the luciferase reporter containing four tandem copies of this Sp1 site is as capable in mediating transforming growth factor- effects on *p21* gene expression, as is the luciferase reporter containing the full *p21* promoter (Datto et al., 1995). We tested whether this 4 x Sp1 reporter construct is induced by *DNMT1* antisense and found that this Sp1 site is also sufficient to mediate the effects of DNMT1 inhibition (Fig. 5B). This data demonstrates that both antisense ODN (Fig. 6) and expressed antisense to DNMT1 (Fig. 5) induce 4 x Sp1 and *p21* promoter activity suggesting that the effects observed in this report with ODN are a true consequence of DNMT1 knock down rather than a nonspecific effect of the ODNs. In addition, this experiment shows that

DNMT1 knock down can induce gene expression in a different cell line than A549, the human embryonal kidney, HEK293 cell line (Fig. 5).

Mutation of the Sp1 Site Corresponding to Bases between –71 and –86 of the p21 Promoter Abolishes Induction of Expression by DNMT1 Knock Down—The data presented in the previous section illustrated that the Sp1 site at –71 to –86 of the *p21* promoter could mediate induction of transcription in response to DNMT1 knock down. To test whether this Sp1 site is required for induction of *p21* within the context of the full *p21* promoter, we generated a construct containing CCGA mutation of this site within the full-length *p21* promoter luciferase reporter construct (–2326 CCGA). We transfected A549 cells with either the wild type (–2326 p21) or the Sp1 mutated (–2326 p21 CCGA) reporter construct and then treated the cells with either 120 nM MG88 or MG208 for 24 h. The construct bearing the mutated Sp1 site showed a marked reduction in luciferase expression suggesting that this site is important for the basal activity of the *p21* promoter. In addition, this Sp1 site is essential for the effects of DNMT1 knock down by MG88, because its mutation abolishes the induction of expression observed with the wild type *p21* promoter construct (Fig. 6B). To confirm that CCGA mutation precludes Sp1 binding, we performed gel shift assays using either wild type or mutated synthetic oligonucleotides corresponding to the bases between –71 and –86 of the *p21* promoter. Purified recombinant human Sp1 protein formed a DNA·protein complex exclusively with the wild type Sp1 oligonucleotide but not with the CCGA mutated Sp1 oligonucleotide (Fig. 6C). To determine that Sp1 is present in the complex, we demonstrated that an anti-Sp1 antibody supershifted the complex (Fig. 6C). To test whether this mutation precludes the binding of Sp1 to the *p21* promoter in living cells, we transfected A549 cells with either wild type (–2326 p21) or Sp1-mutated (–2326 CCGA p21) full *p21* promoter reporter construct and performed a ChIP assay using anti-Sp1 antibody 48 h after transfection. The binding of the endogenous Sp1 protein to the Sp1-mutated construct was markedly reduced compared with the wild type p21 promoter construct (Fig. 6D). Some minute Sp1 binding to the –2326 CCGA construct was detected suggesting that other putative Sp1 elements found in the *p21* promoter might also mediate Sp1 protein binding in A549

cells. However, our results suggest that the occupancy of the *p21* promoter with Sp1 is mostly determined by the Sp1 site at –71 to –86.

DNMT1 Antisense and TSA Affect Different Transcription Activator Pathways—Our previously published differential microarray gene expression analysis revealed that DNMT1 knock down results in induction of multiple genes, including many stress response genes (Milutinovic et al., 2003). The downstream response of a cell to different physiological and toxic signals involves activation of distinct transcription factors, which in turn activate genes bearing their cognate response elements. To test which nodal transcription factor pathway in the cell is responsive to DNMT1 knock down, we utilized the Mercury Pathway Profiling System (Clontech). This system consists of a set of reporter constructs containing distinct enhancer elements upstream from the firefly luciferase gene. The description of the enhancer elements used in our study is shown in Fig. 7A. To further determine whether DNMT1 knock down acts through an HDAC-independent pathway, we compared the pathway profile induced by DNMT1 knock down to the profile elicited by HDAC inhibition. 24 h after transient transfection of A549 cells with each of these reporter vectors, we treated cells with 120 nM MG88 or MG208 for 24 h, or with 1 μM TSA for 6 h. We then determined the luciferase activity in these cells and found that the profiles of signal transduction pathway activation by MG88 and TSA were different. HDAC inhibition resulted in a general induction of 9 out of the 11 pathways tested, which is consistent with its general effects on histone acetylation. On the other hand out of 11 pathways tested, 4 were induced by MG88 treatment by at least 2-fold (Fig. 7B). These different profiles of responses to the two treatments are consistent with our results showing that MG88 induces unmethylated promoters independent of histone acetylation (Fig. 3). Two of the response elements activated by DNMT1 knock down, NF-B and AP-1, were expected, because our gene array analysis revealed the induction of transcription factors binding to these elements, NF-B and c-Jun, respectively (Milutinovic et al., 2003). The induction of the heat shock elements (HSEs) was strong, and this is also in accordance with our gene array data showing that DNMT1 knock down induces stress response genes (Milutinovic et al., 2003). However, one transcription factor that

consistently stood out as the highest responder to DNMT1 knock down was Sp1. Based on our analysis of *p21* and *BIK* promoters, which revealed the presence of multiple Sp1 elements (Fig. 4A), and based on our deletion analysis of the *p21* promoter, we conclude that these elements are primarily responsible for mediating the effects of DNMT1 knock down. Interestingly, the Sp1 family of proteins contains zinc finger motifs that primarily bind to the CG-rich *cis*-elements, and it is well established that CG-rich promoters are silenced by DNA methylation. One excellent example of this silencing is the tumor suppressor p16. It has also been shown that binding of the methylated DNA-binding protein MeCP2 to methylated CG-rich promoters inhibits transactivation by Sp1 (Kudo, 1998). Also, methyl-binding domain protein 1 can repress transactivation of a methylated promoter by Sp1 through its interactions with methyl-binding domain protein 1-containing chromatin-associated factor (Fujita et al., 2003a). Methylation of CGs adjacent to Sp1/Sp3 binding elements inhibits Sp1 binding and the activity of the *p21* promoter (Zhu et al., 2003). It is therefore important to note that all the reporters used in this profiling experiment were unmethylated, because they were all amplified in *Escherichia coli*, which does not express a CG DNA methyltransferase. We have also previously demonstrated that plasmids do not undergo *de novo* methylation in the cell (Milutinovic et al., 2000). Thus, our assay detects exclusively the methylation-independent effects of DNMT1 knock down. The fact that DNMT1 regulates Sp1 responsiveness in the absence of DNA methylation might explain how DNMT1 regulates the expression of multiple housekeeping genes rich in unmethylated CG sites.

MG88 Activates Sp1 Firing by a Mechanism, Which Is Independent of Histone Acetylation—It has been previously demonstrated that induction of *p21* by TSA requires the presence of Sp1 response elements (Sowa et al., 1999). We examined whether the induction of 4 x Sp1 reporter vector by DNMT1 knock down is acetylation-dependent or -independent, like the induction of endogenous *p21* and *BIK* genes (Fig. 4). A549 cells were transiently transfected with 4 x Sp1 construct, and the cells were treated with either MG88 or MG208 for 24 h. The cells were then subjected to a ChIP assay using antibodies against acetylated H3 or acetylated H4 histones,

followed by PCR amplification of the region containing four tandem Sp1 copies of this luciferase reporter. The results indicate that MG88 treatment does not cause a change in acetylation of either H3 or H4 histones associated with the transiently transfected 4 x Sp1 reporter construct, similar to the endogenous *p21* and *BIK* promoters (Figs. 4 and 8). On the other hand, HDAC inhibitor TSA, which induces 4 x Sp1 reporter construct to the similar extent as the MG88 treatment (6- and 8-fold, respectively, Fig. 7), produces a marked induction of acetylation of both histones H3 and H4 associated with this reporter (Fig. 8). Thus, Sp1 element firing could be induced by either inhibition of histone deacetylation or by knock down of DNMT1 through independent mechanisms.

Knock down of DNMT1 Does Not Increase the Occupancy of Either p21 or BIK or the 4 x Sp1 Reporter Gene Promoter with Sp1/Sp3—One possible explanation for the increase in Sp1 promoter firing is that DNMT1 knock down results in an increase in either *Sp1* mRNA transcription or Sp1 binding activity. The microarray gene expression analysis did not reveal an induction of any of the Sp1-binding proteins upon MG88 treatment (Milutinovic et al., 2003). It was previously reported that Sp1 binding activity is increased after treating cells with a DNA methylation inhibitor 5-aza-2'-deoxycytidine (DAC) (Shin et al., 1992). We performed gel shift assays but did not detect any increase in either Sp1 or Sp3 binding to Sp1 recognition elements upon MG88 treatment (data not shown).

We then tested whether Sp1 or Sp3 bind to *p21, BIK*, and the 4 x Sp1 promoter, which is expected if DNMT1 knock down activates these promoters through Sp1 elements. Because it was previously shown that Sp3 binding might negatively affect promoters regulated by Sp1 (Ammanamanchi and Brattain, 2001; Hagen et al., 1994), we determined the occupancy of the *p21* promoter with both transcription factors. ChIP assays using either anti-Sp1 or anti-Sp3 antibodies was performed on either MG88- or MG208-treated A549 cells, followed by PCR amplification of *p21, BIK*, and 4 x Sp1 promoters. The results in Fig. 7B show that both Sp1 and Sp3 interact with these promoters in A549 cells, which is consistent with either Sp1 or Sp3 mediating the

effects of DNMT1 knock down. However, DNMT1 knock down does not change the occupancy of these promoters by either Sp1 or Sp3. In summary, DNMT1 knock down induces the promoters containing Sp1 recognition elements by a mechanism that does not involve increasing the occupancy of these promoters by Sp1 or Sp3. DNMT1 regulates CG-rich Sp1-containing promoters by a mechanism that does not involve DNA methylation.

DNMT1 Knock Down Induces Heat Shock 70-kDa Protein 2 by a Histone Acetylation-independent Mechanism—Our pathway profiling revealed that MG88 and TSA induce the firing of a promoter bearing the heat shock element (HSE) (Fig. 7). HSEs are found within the promoters of heat shock proteins (HSPs), and they bind heat shock factors resulting in gene activation (Voellmy, 1994). HSPs are a family of molecular chaperons that are involved in response to heat shock and a range of other cellular insults and might be involved in the epigenomic stress response, which is launched by DNMT1 knock down (Milutinovic et al., 2003). To validate that the induction of firing of HSEs by DNMT1 knock down applies also to an endogenous HSP gene, we determined whether the gene encoding the nodal *heat shock 70-kDa protein 2* (*HSPA2*) is induced by this treatment. The results presented in Fig. 9A illustrate that DNMT1 knock down induces *HSPA2* 3-fold. Induction of *HSPA2* by DNMT1 knock down was also observed in the microarray gene expression analysis (data not shown).

We then addressed the question whether the acetylation-independent effect that DNMT1 knock down has on Sp1 elements is specific to these elements, or whether it applies to other nodal transcriptional regulatory pathways such as the heat shock response. We first demonstrated that the *HSPA2* gene is partially suppressed by histone deacetylation in our system, as indicated by its induction with the HDAC inhibitor TSA (Fig. 9B). It has been previously shown that TSA induces Hsp70 gene expression in *Drosophila* (Chen et al., 2002). As expected, ChIP analysis of the *HSPA2* promoter on A549 cells treated with 1 μM TSA for 6 h resulted in an increase in the state of acetylation of both H3 and H4 histones associated with this promoter. However, as is the case with Sp1 elements, DNMT1 knock down did not change the

state of acetylation of *HSPA2* promoter (Fig. 10B). To test whether these effects on histone acetylation are carried through the gene downstream of the enhancer elements, we analyzed a region of the second exon of *HSPA2* gene. We found that, unlike TSA treatment, which produces an increase in acetylation of both histones H3 and H4, DNMT1 knock down does not cause any change in histone acetylation associated with exon 2 (Fig. 10C).

In summary, DNMT1 knock down results in induction of two nodal transcriptional regulatory pathways in the cell by a mechanism that is surprisingly independent of both DNA methylation and histone acetylation. We propose a new function of DNMT1 that is independent of both its methyltransferase activity and its histone deacetylase-recruiting activity.

DISCUSSION

This report addresses the question of whether DNMT1 regulates gene expression by a DNA methylation independent pathway and whether this regulation is mediated by the state of chromatin modification through histone acetylation and methylation. We took advantage of well characterized second generation DNMT1 antisense ODNs (MG88) and their mismatch controls (MG208). We first show that our ODNs achieve a complete knock down of DNMT1 protein 24 h after initiation of treatment. In the same time frame, DNMT1 knock down induces the expression of critical cell-cycle regulatory genes such as *p21* and *BIK*. The regulation of expression of either *p21* or *BIK* does not involve a change in their DNA methylation state (Fig. 3) and previous data (Milutinovic et al., 2000). Moreover, DNMT1 knock down results in activation of transiently transfected unmethylated reporter genes directed by tandem copies of distinct transcription factor recognition elements. Because it was previously shown that DNMT1 interacts with histone-modifying enzymes (Fuks et al., 2000; Fuks et al., 2003a), we tested the hypothesis that *DNMT1* knock down effects are mediated by a change in histone acetylation. Both *BIK* and *p21* are partially suppressed by HDACs in our system, because the inhibition of HDACs by TSA increases the state of

acetylation of their promoters and induces their expression. Nevertheless, we demonstrate that DNMT1 regulates the expression of these genes without changing their state of acetylation (Fig. 4). Our data therefore unravel a third novel mechanism of regulation of gene expression by DNMT1 that does not involve either DNA methylation or histone modification, the two most established fundamental principles of epigenomic regulation.

Knock down of DNMT1 results in induction of many genes, which includes a class of genes encoding stress response proteins (Milutinovic et al., 2003) suggesting that DNMT1 acts on a common transcriptional regulatory pathway. Our analysis of the *p21* promoter revealed that the Sp1 element corresponding to the bases between –71 and –86 mediates the effects of DNMT1 knock down, because four copies of this site (4 x Sp1) were sufficient to mediate the induction of the luciferase reporter produced by DNMT1 knock down (Fig. 5B). In addition, 2-bp mutation (CCGA) of this Sp1 site in the full-length *p21* promoter abolished Sp1 binding and the induction by DNMT1 knock down. *p21* promoter responds to both antisense ODN as well as a plasmid expressed *DNMT1* antisense mRNA, demonstrating that this induction is not a consequence of nonspecific effects of the modified ODNs (Figs. 5 and 6). We further tested a number of basic *cis*-acting elements that are known to respond to major regulatory pathways in the cell. Among these, E2F elements were previously shown to play an important role in regulating *p21* gene expression (Ammanamanchi and Brattain, 2001; Hagen et al., 1994; Shin et al., 1992; Zhu et al., 2003). Our data exclude the possibility that DNMT1 acts on a pathway triggering E2Fs, because the E2F recognition element was unresponsive. This is surprising, because DNMT1 was shown to suppress exogenous E2F-responsive elements by recruiting HDACs into E2F1·Rb·DNMT1 complex (Robertson et al., 2000a). Our data, showing that histone deacetylation is not changed by DNMT1 knock down, further support our conclusion that E2F elements are not involved. Sp1 therefore appears to be the main transcription regulatory element triggered by DNMT1 knock down.

Induction of Sp1 response by DNMT1 knock down is not mediated by increased Sp1 expression or increased occupancy of the *p21* and *BIK* promoters with either Sp1 or Sp3 (Fig. 8). DNMT1 must therefore be acting either directly or indirectly on proteins interacting with Sp1 or Sp3 and modulating their trans-activation activity. Identifying these proteins will require extensive future experiments. Sp1 protein contains zinc finger motifs that primarily bind to the GC-rich *cis*-elements that are widely distributed in the promoters, enhancers, and locus-control regions of housekeeping genes and some cell-specific genes (Kaczynski et al., 2003). It was generally accepted that these genes escape regulation by DNMT1, because their promoters are especially enriched in unmethylated CG sites and are usually not methylated. The fact that DNMT1 regulates Sp1 responsiveness in the absence of DNA methylation might explain how DNMT1 regulates the expression of multiple house keeping cell cycle regulatory genes (Milutinovic et al., 2003). Whereas ectopic DNA methylation is known to suppress Sp1 responsive genes by attracting methylated DNA-binding proteins that suppress Sp1 activation (Fujita et al., 2003a; Kudo, 1998; Zhu et al., 2003), our data suggest an entirely distinctive mechanism through which DNMT1 suppresses Sp1-responsive genes.

The observation that DNMT1 regulates Sp1-responsive genes suggests that it may play a role in the regulation of the cell cycle. Sp1 elements are found in genes that perform contradictory cellular roles such as, *thymidine kinase*, which stimulates the cell cycle, and *p21*, which causes cell cycle arrest (Kivinen et al., 1999; Li et al., 1998; Pagliuca et al., 2000). Because these two classes of genes are expressed at different phases of the cell cycle, there must be regulatory factors that switch their responsiveness to Sp1. Such factors are responsible for coordinating cell arrest and cell division during the different stages of the cell cycle. It stands to reason that DNMT1 acts on a regulatory pathway, which inhibits Sp1 activation of a distinct subclass of Sp1-regulated genes involved in cell cycle arrest. DNMT1 is therefore proposed to play a role in orchestrating the cell program toward DNA synthesis and cell division. Such a mechanism possibly evolved to coordinate DNMT1 expression and the inheritance of the DNA methylation pattern with the cell cycle. Unraveling of

the Sp1 regulatory pathway that DNMT1 is acting upon is therefore extremely important for our understanding of the cell cycle regulation.

The fact that DNMT1 can act on unmethylated elements found in most housekeeping genes obliges us to revisit the biological roles of DNMT1 and its possible roles in cancer (Szyf, 2001b; Szyf, 2003). The common thinking is that DNA methylation is the main function of DNMT1 and that its downstream effectors are methylated genes. Although it was previously shown that DNMT1 could interact with HDACs, it was still believed that the main role of such an interaction is to enhance epigenomic silencing through a mechanism that involves both histone deacetylation and DNA methylation. Our data suggests that DNMT1 acts on genes independent of these common mechanisms of epigenomic regulation, DNA methylation, and histone acetylation.

If DNMT1 functions through methylation-independent mechanisms, we must be cautious in our interpretation of the effects of DNMT1 inhibition, knock out, and knock down data. It is possible that many of the effects seen in either normal or cancer cells should be attributed to methylation-independent roles of DNMT1 rather than the loss of DNA methylation. This has obvious therapeutic implications. There are currently several attempts to develop DNA methylation inhibitors as anticancer agents. However, inhibition of DNA methylation has been also shown to induce genes that promote metastasis (Guo et al., 2002; Gupta et al., 2003; Rosty et al., 2002). Hypomethylation was also shown to promote T cell lymphoma in mice hypomorphic for DNMT1 (Gaudet et al., 2003). However, if DNMT1 is involved in transformation through methylation-independent mechanisms, then it stands to reason that these functions of DNMT1 should be targeted. Such an approach would circumvent the unwanted effects of inhibition of DNA methylation (Szyf, 2001b; Szyf, 2003). The fact that DNMT1 and histone deacetylase inhibitors act on Sp1-bearing sequences by an independent mechanism suggests that a combination of DNMT1 knock down and histone deacetylase inhibition would have an additive effect on genes involved in the cell cycle arrest. It was previously shown that DNMT1 inhibitors and histone

deacetylase inhibitors have synergistic effects on activation of methylated genes. Our data suggest that a combination of TSA and DNMT1 inhibitors might be advantageous for inducing the DNA-methylation independent effects of DNMT1 knock down as well.

ACKNOWLEDGMENTS

We thank Dr. W. S. El-Deiry for the *p21* deletion constructs and Dr. X.-F. Wang for the Sp1 reporter constructs.

This research was supported by a grant from the Canadian Institute of Health Research (to M. S.).

FIGURE LEGENDS

Fig. 1. DNMT1 antisense knocks down DNMT1 protein and induces expression of the tumor suppressor p21 and apoptosis inducer BIK. *A*, Western blot analysis of DNMT1 expression in nuclear extracts prepared from A549 cells treated with 120 nM of antisense MG88 or its mismatch control MG208 for 24 h or 48 h. In the *bottom panel*, actin Western blot is shown as a control. *B*, RT-PCR was performed on total RNA isolated from A549 cells treated with MG88 or MG208 for 12–42 h. The primers for the indicated genes are described under "Materials and Methods." *C*, PCR products were quantified by densitometry and normalized to actin. The induction of genes in MG88-treated cells was shown as a percentage of MG208-treated cells for each time point. Duplicates are shown, and the *error bars* indicate their range.

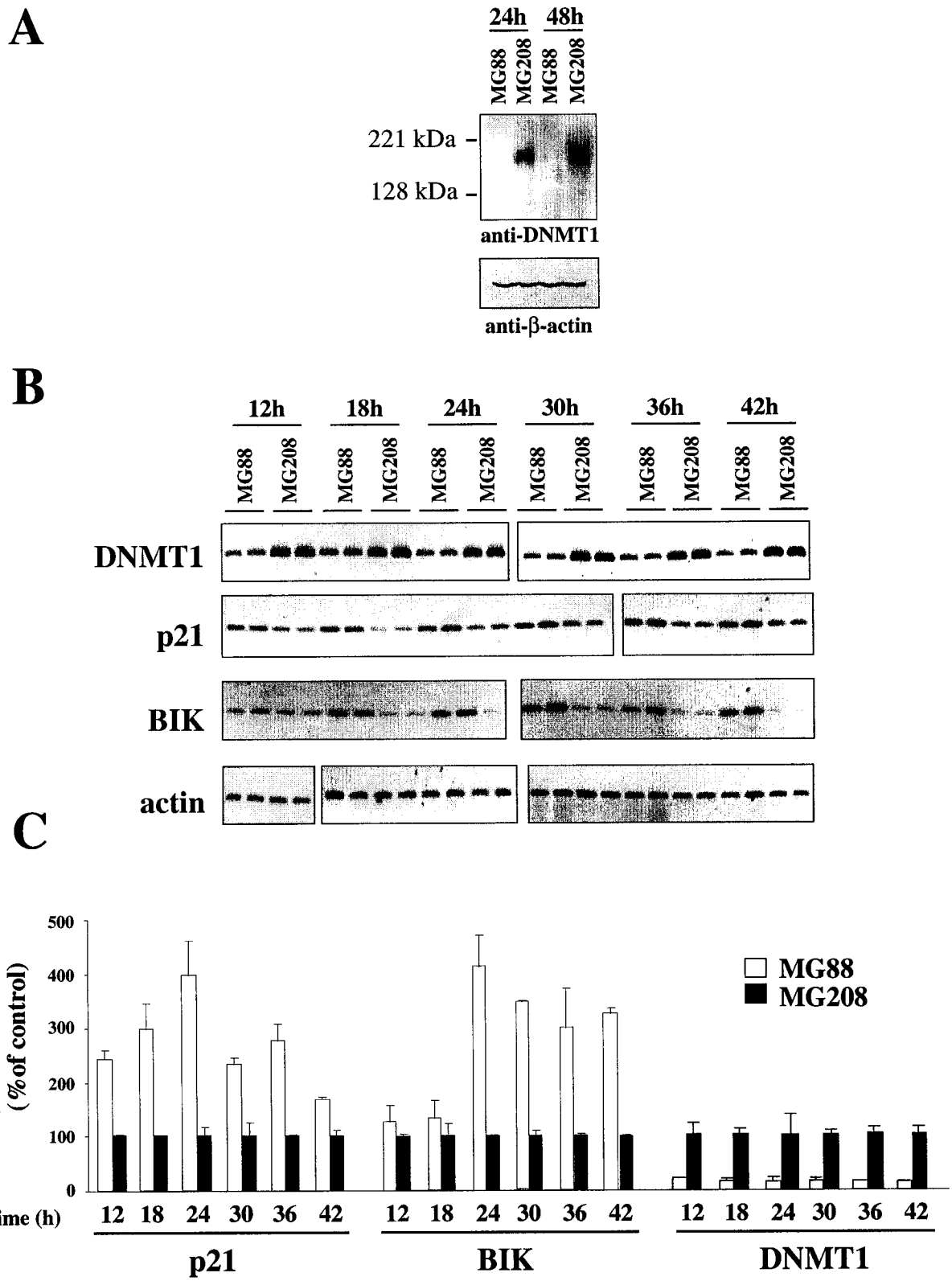
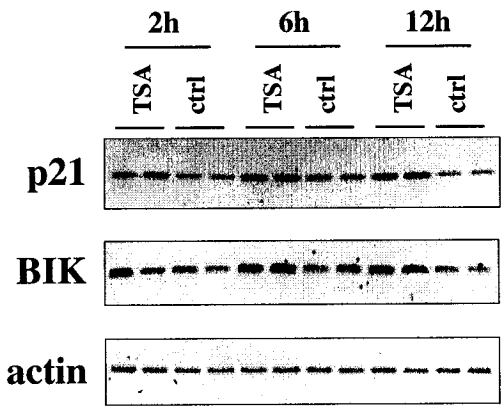

A
24h
48h
MG88
MG208
MG88
MG208
221 kDa
128 kDa
anti-DNMT1
anti-β-actin
B
12h
18h
24h
30h
36h
42h
MG88
MG208
DNMT1
p21
BIK
actin
C
normalized RNA
(%of control)
500
400
300
200
100
0
MG88
MG208
time (h)
12 18 24 30 36 42
p21
BIK
DNMT1

Fig. 2. TSA induces the expression of the tumor suppressor p21 and apoptosis inducer BIK. Total RNA was isolated from A549 cells treated with 1 μM TSA for 2–12 h. *A*, RT-PCR was performed for the indicated genes as described under "Materials and Methods." *B*, PCR products were quantified by densitometry and normalized to actin. The induction of genes in TSA-treated cells was shown as a percentage of control cells. Duplicates are shown, and the *error bars* indicate their range.

A


2h
6h
12h
TSA
ctrl
TSA
ctrl
TSA
ctrl
p21
BIK
actin

B

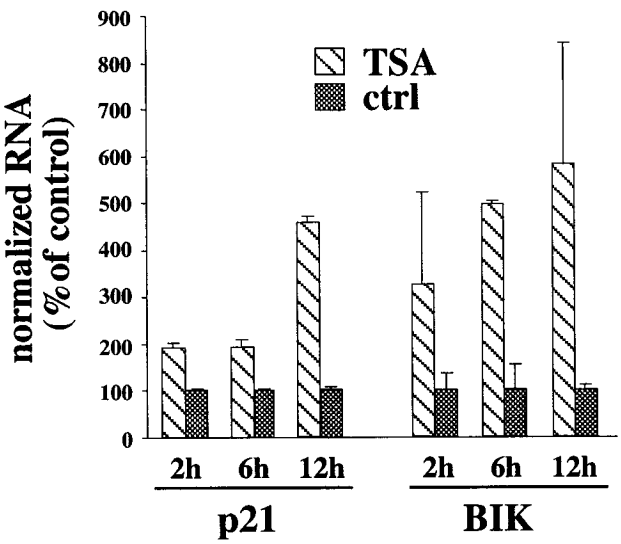
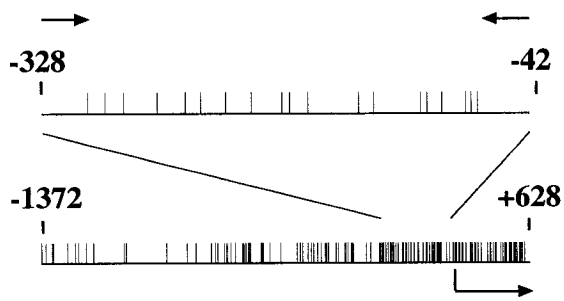

normalized RNA
(% of control)
900
800
700
600
500
400
300
200
100
0
TSA
ctrl
2h
6h
12h
2h
6h
12h
p21
BIK

Fig. 3. Bisulfite mapping of CG island in the BIK promoter. *A*, physical map of the *BIK* promoter showing the density of CG sites (*small vertical lines*). The transcription start site is indicated with the *bottom arrow*. The primers used to amplify the bisulfited DNA are shown as *arrows* on the top of the promoter. *B*, A549 cells were treated with 120 nM MG88 or MG208 for 24 h. DNA was isolated and treated with sodium bisulfite, which converts all unmethylated cytosines into thymidines while methylated cytosines remain intact. The specific methylation pattern is revealed by PCR amplification, subcloning, and sequencing of the region of interest. The methylation pattern of 19 CG sites in the *BIK* promoter is shown (10 clones for each treatment). *Open* and *closed circles* indicate unmethylated and methylated sites, respectively.

A



B

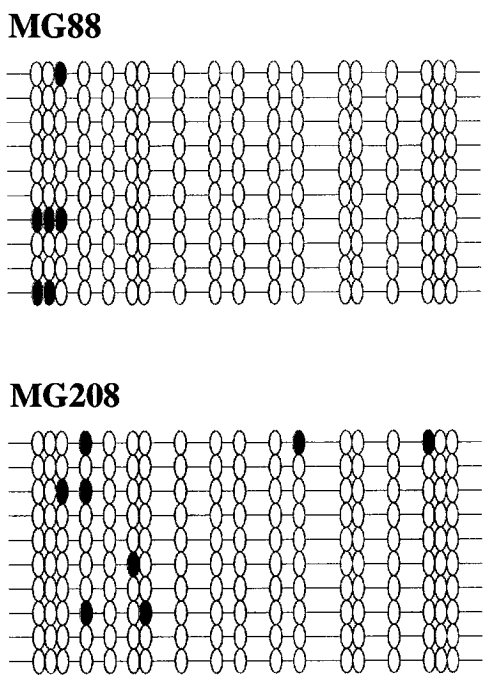


Fig. 4. Histone acetylation of promoters of p21 and BIK is induced by TSA treatment but remains unchanged after DNMT1 antisense knock down. *A*, the map of *p21* and *BIK* promoters is shown, indicating the primers and the sizes of regions that they amplify. The analysis of promoter sequences for Sp1 binding sites (*gray circles*) was done using the SignalScan program of the BioBase web site (www.gene-regulation.com). *B*, A549 cells were treated with either 120 nM MG88 or MG208 for 24 or 48 h. Chromatin was isolated and immunoprecipitated with antibodies specific for acetylated histones H4 and H3, as well as dimethyl Lys-9 of histone H3. Associated DNA was analyzed by PCR using primers that amplify 255- and 307-bp regions of *p21* and *BIK* promoters, respectively. Triplicates are shown for each experiment. The regions of *p21* and *BIK* rich in Sp1 elements (324 and 307 bp, respectively) were tested for Sp1 and Sp3 binding in Fig. 7. *C*, similar chromatin immunoprecipitations were performed on control cells or cells treated with 1 μM TSA for 6 h. Anti-acetylated H4 and H3 antibodies were used. Triplicates are shown for each experiment. *D*, the relative occupancy of the promoters with each of the studied histone states is determined by densitometric quantification of PCR products and normalization to the input DNA. The rate of induction following MG88 and TSA treatment is shown as a percentage of MG208 and control cells, respectively. The *error bars* are standard deviations of the triplicates. There was no PCR amplification in no antibody control immunoprecipitations (data not shown).

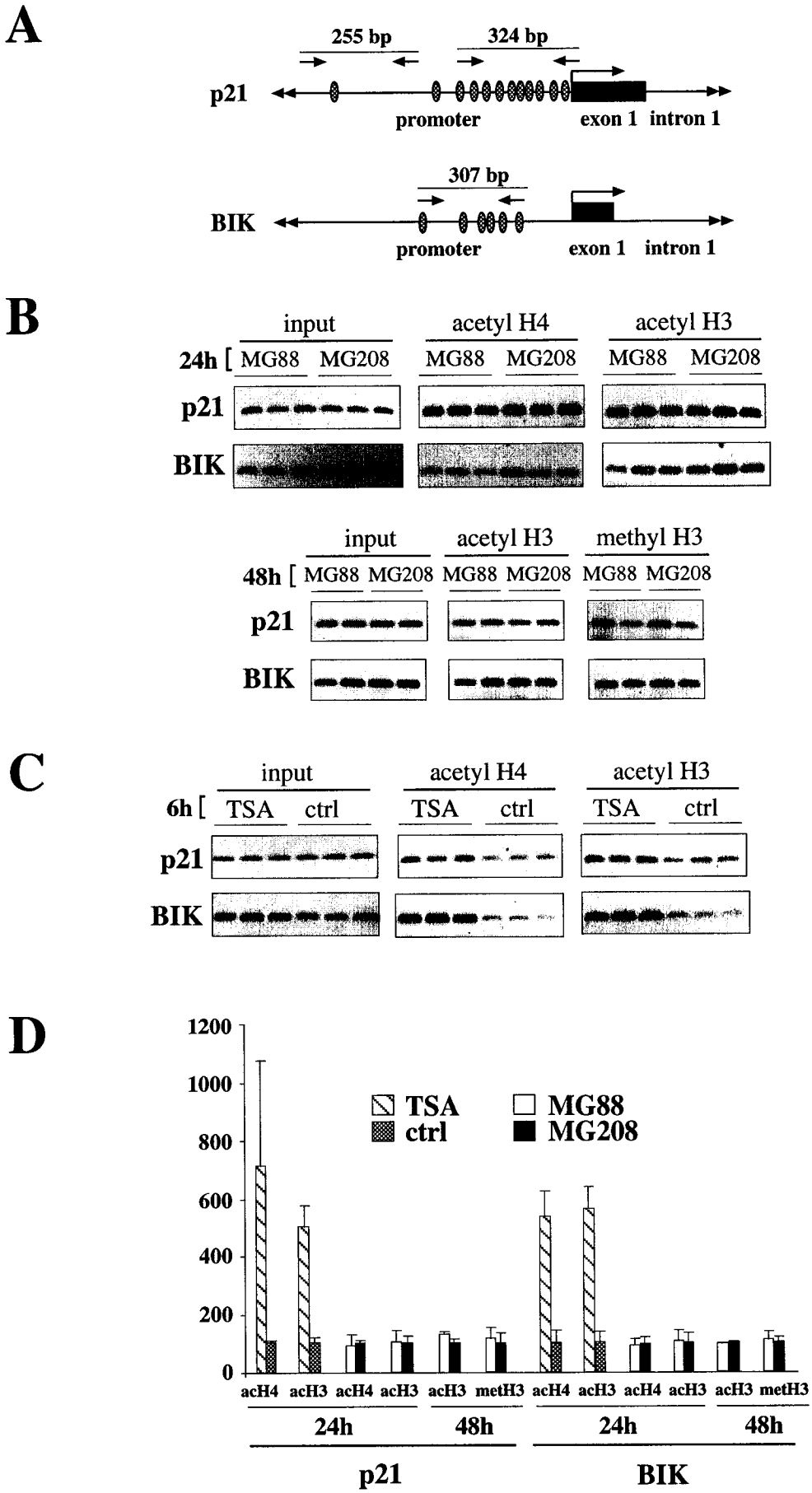

A
255 bp
324 bp
p21
promoter
exon 1
intron 1
307 bp
BIK
promoter
exon 1
intron 1
B
input
acetyl H4
acetyl H3
24h [
MG88
MG208
p21
BIK
input
acetyl H3
methyl H3
48h [
C
input
acetyl H4
acetyl H3
6h [
TSA
ctrl
D
1200
1000
800
600
400
200
0
TSA
ctrl
MG88
MG208
acH4
acH3
metH3
24h
48h
p21
BIK

Fig. 5. Inhibition of DNMT1 induces the activity of p21 promoter through Sp1 sites. HEK 293 cells were co-transfected with either a control vector (pcDNA3.1), or the same vector expressing *DNMT1* mRNA in the antisense orientation (as *DNMT1*), as well as the luciferase reporter constructs depicted. *A*, serial deletions of the *p21* promoter inserted upstream from the firefly luciferase gene and their activities are shown. The presence of the Sp1 and E2F sites in the first 94 bp of *p21* promoter are shown (*gray* and *black circles*). The sequence of the Sp1 site crucial for the *p21* activation is shown. *B*, basic luciferase expression vector containing only TATA box and the initiator sequence (pGL2 T+I) was used to create 1 x Sp1 and 4 x Sp1 constructs by inserting either one or four copies of the Sp1 response element (shown in *A*) upstream from TATA box. The luciferase activity from the whole cell lysate was assayed 48 h after the transfection. The induction of the luciferase reporters by antisense to *DNMT1* is expressed as a percentage of the empty vector controls. The *error bars* are S.E. values of triplicate experiments.

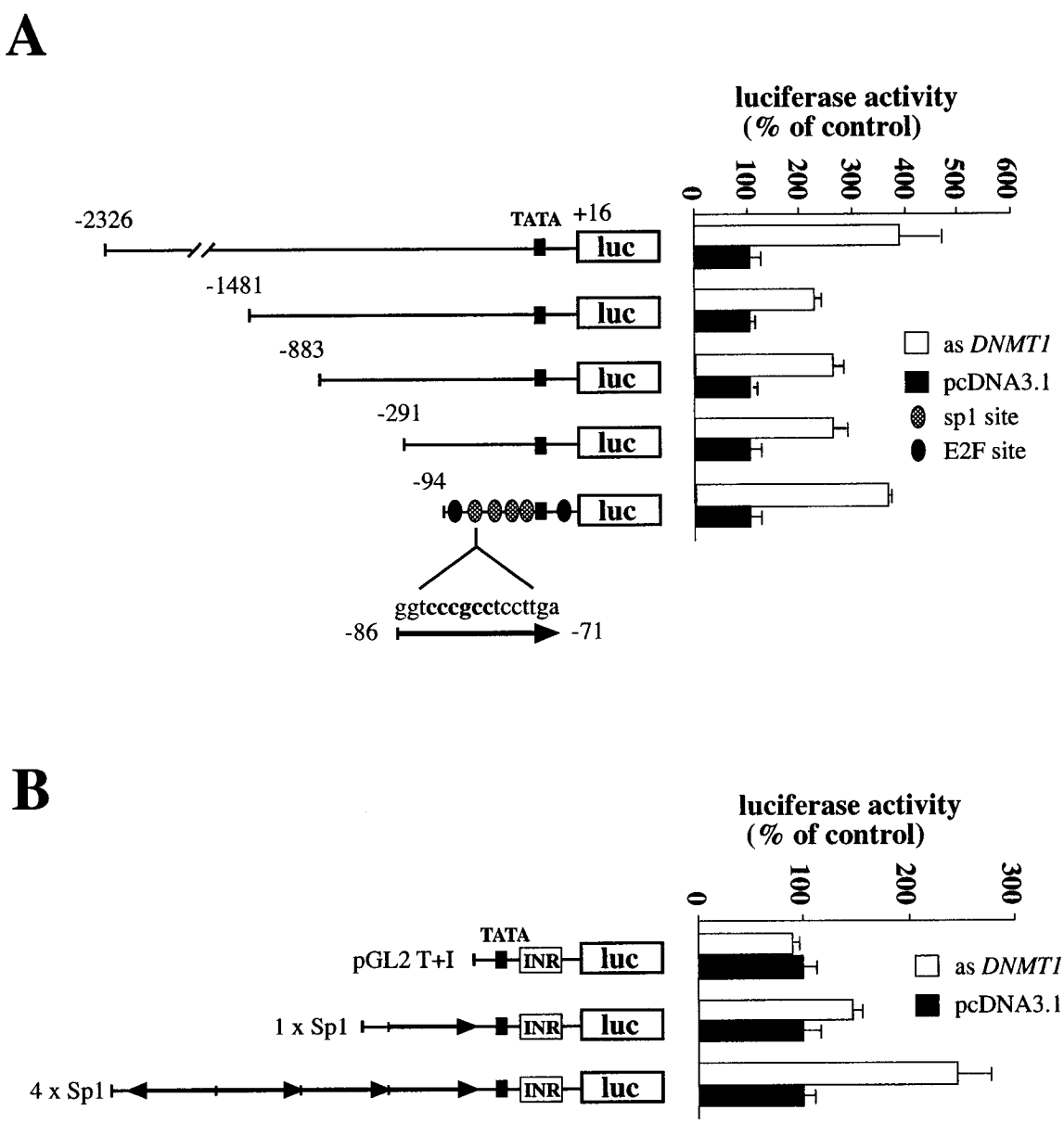
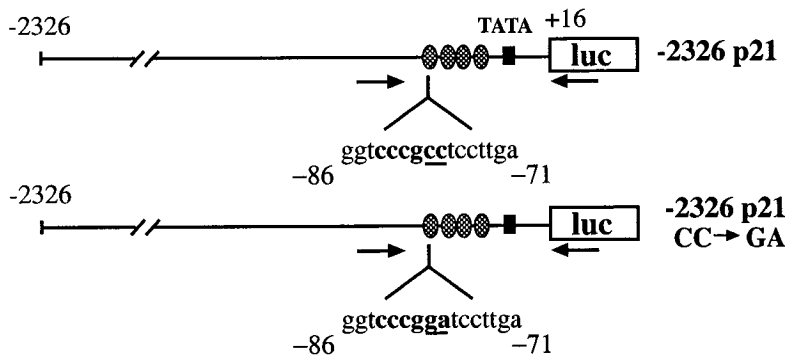
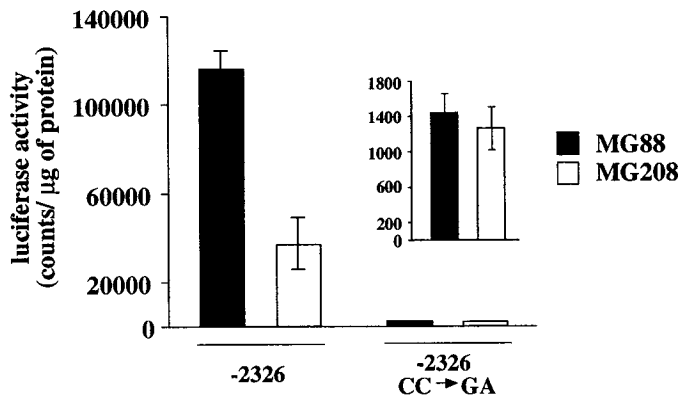

A
luciferase activity
(% of control)
0
100
200
300
400
500
600
-2326
TATA
+16
luc
-1481
luc
-883
luc
-291
luc
-94
luc
ggtcccgcctccttga
-86
-71
as DNMT1
pcDNA3.1
sp1 site
E2F site
B
luciferase activity
(% of control)
0
100
200
300
TATA
pGL2 T+I
INR
luc
1 x Sp1
INR
luc
4 x Sp1
INR
luc
as DNMT1
pcDNA3.1

Fig. 6. Mutation of the Sp1 site corresponding to bases between –71 and –86 of the p21 promoter precludes Sp1 binding and abolishes the activation produced by DNMT1 knock down. *A*, the physical maps of wild type (–2326 p21) and Sp1 mutated (–2326 p21 CCGA) full *p21* promoter luciferase reporter construct are shown. The sequence of the *p21* promoter Sp1 site used for the gel shifts (*C*) is indicated, with the *boldface letters* representing the Sp1 recognition element and the *underlined letters* indicating the mutated bases. The *arrows* indicate the primers used for ChIP (*D*). *B*, A549 cells were transfected with 0.5 μg of either wild type (–2326 p21) or Sp1-mutated (–2326 p21 CCGA) full *p21* promoter luciferase reporter construct. 24 h post-transfection, the cells were treated with 120 nM MG88 and MG208 for 24 h, and the luciferase activity was measured and normalized as described under "Materials and Methods." *C*, 400 ng of recombinant human Sp1 protein (*hrSp1*) was incubated with end-labeled oligonucleotides corresponding to bases between –71 and –86 of either a wild type or CCGA mutated *p21* promoter. A supershift was generated by incubating the reaction with 1 μg of anti-Sp1 antibody. The positions of the Sp1·DNA complex and the anti-Sp1-Sp1-DNA supershift are indicated with arrows. *D*, A549 cells were transfected with 1.5 μg of either –2326 *p21* or –2326 p21 CCGA construct. Chromatin was isolated and immunoprecipitated with anti-Sp1 antibody, followed by PCR amplification of the 256 bp of the proximal *p21* promoter of the transfected luciferase reporters. The relative occupancy of the promoters with Sp1 is determined by densitometric quantification of PCR products in the anti-Sp1 immunoprecipitate and normalization to the input DNA. The normalized Sp1 occupancy of the wild type construct (–2326 p21) and the mutated construct (–2326 p21 CCGA) is represented in arbitrary units. The *error bars* are S.E. values of the triplicates. There was no PCR amplification in no antibody control immunoprecipitations (data not shown).

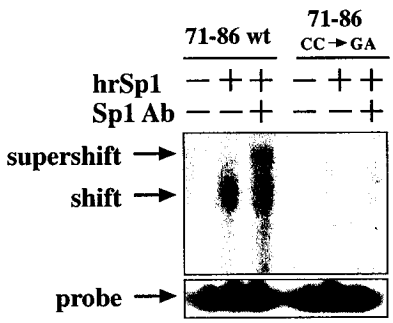
A


-2326
TATA
+16
luc
-2326 p21
ggtcccgcctccttga
-86
-71
-2326
luc
-2326 p21
CC→GA
ggtcccggatccttga
-86
-71

B


luciferase activity
(counts/ µg of protein)
140000
100000
60000
20000
0
1800
1400
1000
600
200
0
MG88
MG208
-2326
-2326
CC→GA

C


71-86 wt
71-86
CC→GA
hrSp1 − + + − + +
Sp1 Ab − − + − − +
supershift
shift
probe

D

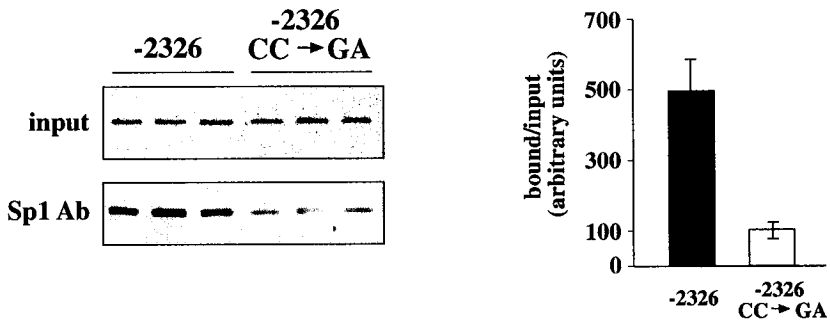
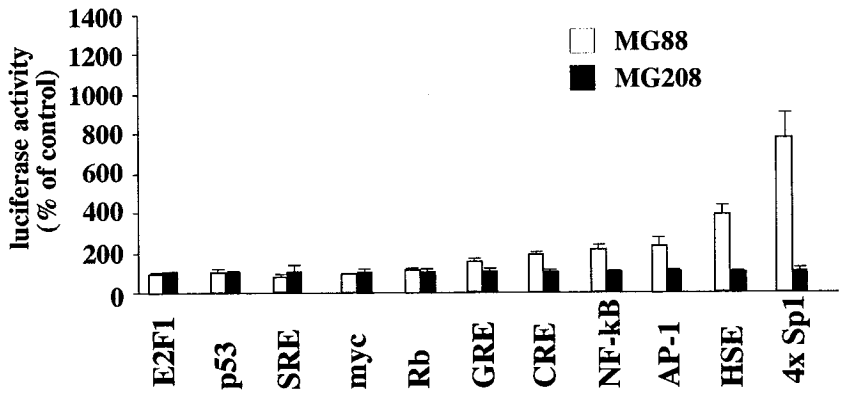

-2326
-2326
CC→GA
input
Sp1 Ab
bound/input
(arbitrary units)
700
500
300
100
0
-2326
-2326
CC→GA

Fig. 7. Profile of the transcription factor pathways affected by TSA and DNMT1 antisense knock down. A549 cells were transfected with 0.5 μg of luciferase reporter vectors containing *cis*-acting enhancer elements upstream from the TATA box and the firefly luciferase gene. The description of the enhancer elements is shown in *A*. 24h after the transfection of the luciferase reporters, the cells were treated with either 120 nM MG88 and MG208 for 24 h (*B*) or with 1 μM TSA for 6 h (*C*). Luciferase activity was measured and normalized as described under "Materials and Methods." The induction of luciferase activity in MG88- and TSA-treated cells is shown as a percentage of MG208-treated or control cells, respectively. *Error bars* are standard deviations of triplicates.

A

E2F = 1 x E2F transcription factor consensus sequence
p53 = 1 x p53 response element
SRE = 3 x serum response element
Rb = 1 x Rb response element
myc = 6 x E-box consensus sequence
GRE = 3 x glucocorticoid response elements
CRE = 1 x cAMP response element
NF-kB = 4 x NF-kB consensus sequence
AP-1 = 4 x activator protein enhancers
HSE = 3 x heat shock element
Sp1 = 4 x Sp1 transcription factor consensus sequence

B



C

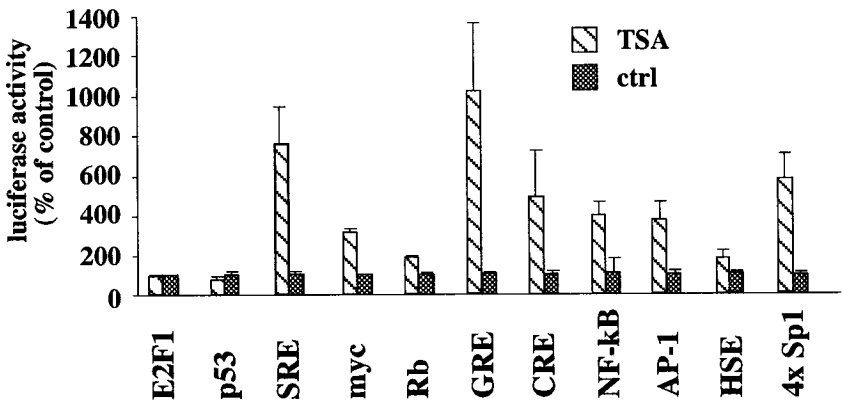
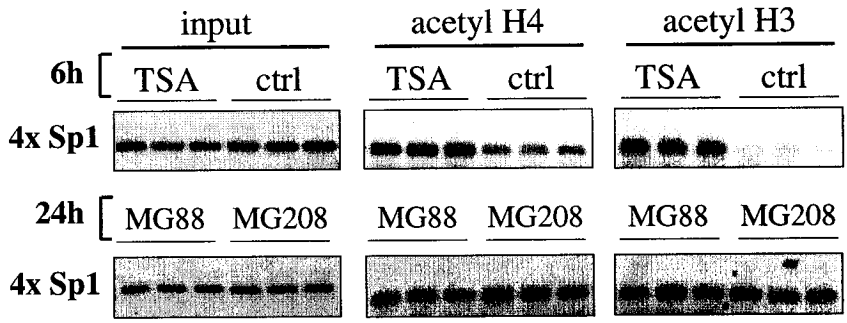
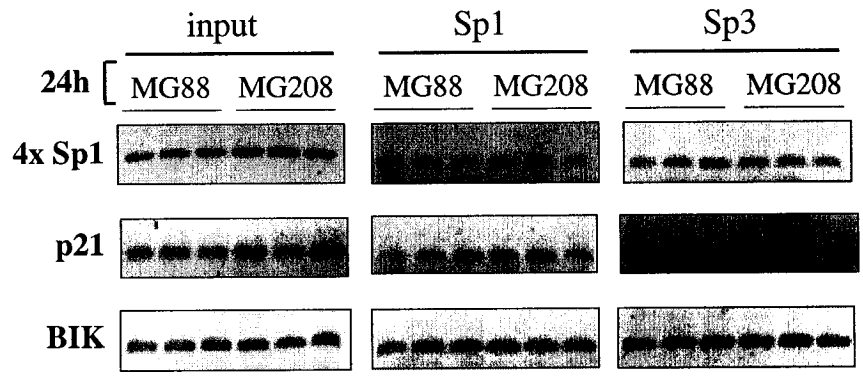


Fig. 8. The state of acetylation of histones associated with the Sp1 elements in the 4 x Sp1 luciferase reporter is induced by TSA but remains unchanged after DNMT1 antisense knock down. A549 cells were transfected with 0.5 μg of 4 x Sp1 luciferase reporter construct. 24 h later, the cells were treated with 120 nM MG88 and MG208 for 24 h, or with 1 μM TSA for 6 h. Chromatin was isolated and immunoprecipitated with anti-acetylated histone H3 and H4 (*A*), or anti-Sp1 and -Sp3 antibodies (*B*). The precipitated DNA was used to amplify the 324- and 307-bp regions of *p21* and *BIK* promoters (the positions are shown in Fig. 4A). For the amplification of 4 x Sp1 luciferase reporter construct, the primers were designed within the pGL2 T+I vector on both sides of tandem Sp1 copies (Fig. 5B). The enrichment in specific chromatin is calculated after densitometric quantification of PCR products and normalization to the input DNA. The induction in MG88- and TSA-treated cells is shown as a percentage of MG208-treated and control cells, respectively. The *error bars* are standard deviations of the triplicates. There was no PCR amplification in any of the antibody control immunoprecipitations (data not shown).

A


input
acetyl H4
acetyl H3
6h
TSA
ctrl
TSA
ctrl
TSA
ctrl
4x Sp1
24h
MG88
MG208
MG88
MG208
MG88
MG208
4x Sp1

B


input
Sp1
Sp3
24h
MG88
MG208
MG88
MG208
MG88
MG208
4x Sp1
p21
BIK

C

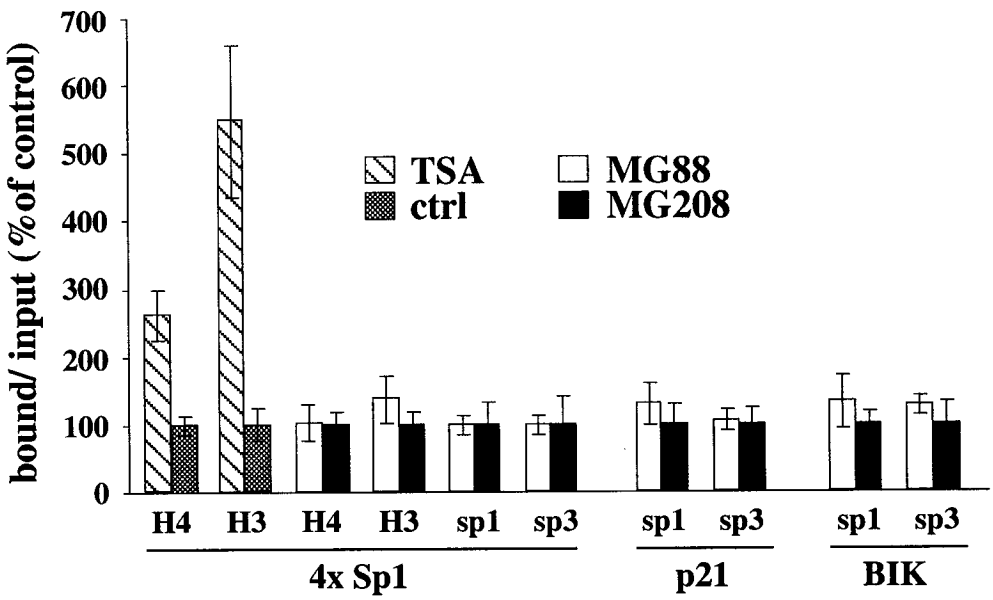

700
600
500
400
300
200
100
0
bound/ input (%of control)
TSA
ctrl
MG88
MG208
H4
H3
H4
H3
sp1
sp3
sp1
sp3
sp1
sp3
4x Sp1
p21
BIK

Fig. 9. DNMT1 antisense knock down and TSA induce expression of heat shock 70-kDa protein 2 (HSPA2). Total RNA was isolated from A549 cells treated with 120 nM of either MG88 or MG208 for 12–42h (*A*), or with 1 μM TSA or control cells for 2–12 h (*B*). RT-PCR was performed with primers for *HSPA2*, and PCR products were quantified by densitometry and normalized to actin. The induction in MG88- and TSA-treated cells is shown as a percentage of MG208-treated and control cells, respectively. Duplicates are shown, and the *error bars* indicate their range.

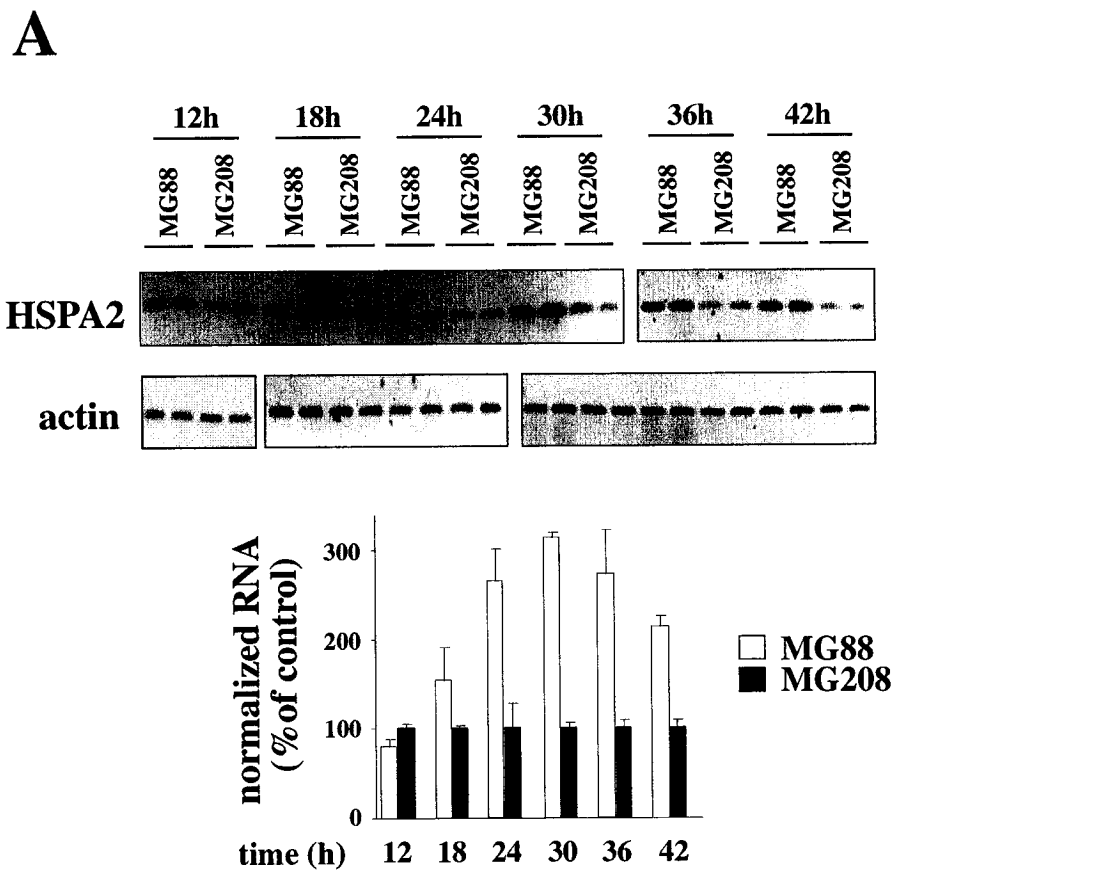
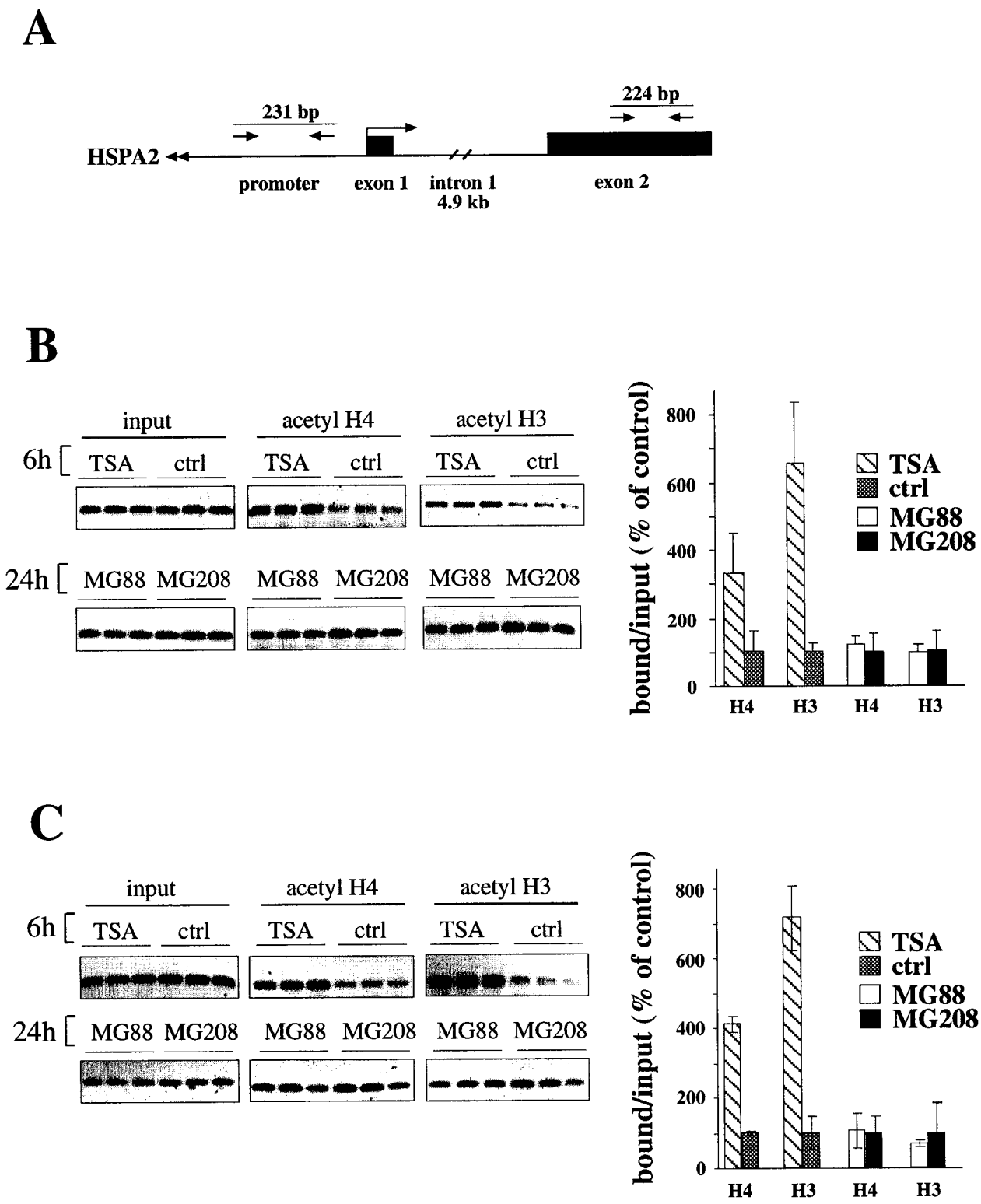

A
12h
18h
24h
30h
36h
42h
MG88
MG208
HSPA2
actin
normalized RNA
(% of control)
300
200
100
0
time (h)
12
18
24
30
36
42
MG88
MG208
B
2h
6h
12h
TSA
ctrl
HSPA2
actin
normalized RNA
(% of control)
500
400
300
200
100
0
2h
6h
12h
TSA
ctrl

Fig. 10. Histone acetylation of the promoter and exon regions of HSPA2 gene is induced by TSA treatment but remains unchanged after DNMT1 antisense knock down. *A*, the structure of the *HSPA2* gene is shown, indicating the primers and the sizes of regions that they amplify. *B* and *C*, A549 cells were treated with 120 nM of either antisense MG88 or its mismatch control MG208 for 24 h, or with 1 μM TSA or control cells for 6 h. Chromatin was isolated and immunoprecipitated with antiacetylated H3 and H4 antibodies. Associated DNA was used to amplify the promoter (*B*) and exon 2 (*C*) regions of *HSPA2*. The enrichment in specific chromatin is calculated after densitometric quantification of PCR products and normalization to the input DNA. The induction in MG88- and TSA-treated cells is shown as a percentage of MG208 treated and control cells, respectively. The *error bars* are standard deviations of the triplicates. There was no PCR amplification in any of the antibody control immunoprecipitations (data not shown).


A
231 bp
224 bp
HSPA2
promoter
exon 1
intron 1
4.9 kb
exon 2
B
input
acetyl H4
acetyl H3
6h
TSA
ctrl
24h
MG88
MG208
bound/input (% of control)
800
600
400
200
0
H4
H3
C

The preceding chapter further strengthens the role of DNMT1 in the regulation of gene expression independent of its methyltransferase activity. The recent advances in the field have established that this function of DNMT1 is dependent on its ability to recruit histone modifying activities such as HDAC and histone methyltransferase. However, our data demonstrates that DNMT1 can play yet another role which is both methylation and histone modification independent. Our findings that DNMT1 regulates Sp1 responsive genes suggest that DNMT1 may be involved in the regulation of the cell cycle by repressing tumor suppressors while promoting replication fork assembly and S phase progression. It is possible that this role of DNMT1 has evolved to ensure that DNMT1 is always present during DNA synthesis in order to maintain methylation pattern on the newly synthesized DNA. Any loss of the epigenetic modifications would result in the aberrant gene expression and improper cell function. The cell has therefore developed mechanisms to ensure concurrent replication of the genome and the epigenome. The following chapter examines the mechanism of the inheritance of the histone code during DNA replication. Our data demonstrates that PCNA, which serves as a loading platform for many proteins involved in DNA synthesis, also recruits HDAC activity. This data offers a novel mechanism for the maturation of newly deposited histones after DNA synthesis, and establishes PCNA as a universal factor in coordinating the replication of the genome and the epigenome.

Chapter 4

Proliferating Cell Nuclear Antigen Associates with Histone Deacetylase Activity, Integrating DNA Replication and Chromatin Modification

Snezana Milutinovic, Qianli Zhuang and Moshe Szyf

This chapter has been published in the Journal of Biological Chemistry (2002) 277, 20974-20978

Faithful inheritance of the chromatin structure is essential for maintaining the gene expression integrity of a cell. Histone modification by acetylation and deacetylation is a critical control of chromatin structure. In this study, we test the hypothesis that histone deacetylase 1 (HDAC1) is physically associated with a basic component of the DNA replication machinery as a mechanism of coordinating histone deacetylation and DNA synthesis. Proliferating cell nuclear antigen (PCNA) is a sliding clamp that serves as a loading platform for many proteins involved in DNA replication and DNA repair. We show that PCNA interacts with HDAC1 in human cells and *in vitro* and that a considerable fraction of PCNA and HDAC1 colocalize in the cell nucleus. PCNA associates with histone deacetylase activity that is completely abolished in the presence of the HDAC inhibitor trichostatin A. Trichostatin A treatment arrests cells at the G2-M phase of the cell cycle, which is consistent with the hypothesis that the proper formation of the chromatin after DNA replication may be important in signaling the progression through the cell cycle. Our results strengthen the role of PCNA as a factor coordinating DNA replication and epigenetic inheritance.

INTRODUCTION

Epigenetic markings play an essential role in regulating the gene expression program of vertebrate cells. One of the fundamental challenges of cell division is therefore coordinating the processes of genetic and epigenetic inheritance. The cell must possess multiple mechanisms to coordinate these processes (Szyf and Detich, 2001). For example, DNA methylation is coordinated with DNA replication (Araujo et al., 1998a) by physical association of maintenance DNA methyltransferase 1 with the DNA replication fork protein PCNA (Chuang et al., 1997). Inhibition of DNA methyltransferase 1 leads to inhibition of initiation of DNA replication (Knox et al., 2000).

Similar to DNA methylation, DNA replication-coupled chromatin assembly is essential for the inheritance of the epigenetic code. The specific targeting of nucleosome assembly to the newly synthesized DNA is achieved by direct interaction of histone chaperone CAF-1 with PCNA (Shibahara and Stillman, 1999). PCNA is a homotrimeric protein that forms a sliding clamp around DNA and functions as a DNA polymerase processivity factor during replication and nucleotide excision repair. Through its multiple protein-protein interactions, PCNA coordinates events in replication, epigenetic inheritance, repair, and cell cycle control (Zhang et al., 2000). A recent study in *Saccharomyces cerevisiae* has shown that several mutations in PCNA decrease silencing at telomeres and at the mating-type HMR locus (Zhang et al., 2000). Furthermore, mutations in the *Drosophila* PCNA gene mus209 suppress repression in the vicinity of heterochromatin (Henderson et al., 1994). The disruption of epigenetic silencing has been attributed to the inability of some of these mutants to associate with CAF-1. However, synergism of several PCNA mutants with CAF-1 mutants suggested that PCNA may participate in silencing through another factor.

During nucleosome assembly, histones H3 and H4 undergo transient acetylation before their deposition onto replicated DNA. In the process of chromatin maturation,

newly deposited acetylated histones are generally deacetylated by a mechanism that is poorly understood (Sobel et al., 1995).

In this study, we tested whether HDAC1 could be involved in this process. HDAC1 is a member of a growing family of proteins that currently consists of 16 isoforms divided into three classes (Fischer et al., 2002). HDACs have been found in multiprotein complexes involved in transcriptional regulation, cell cycle, differentiation, and DNA repair. HDAC1 and HDAC2 have been shown to associate with the NuRD nucleosome remodeling complex (Zhang et al., 1998) as well as with the mammalian transcription repressor Sin3 and to be targeted to promoter elements to cause transcriptional repression (Zhang et al., 1997). Recruitment of HDAC1 by retinoblastoma protein (Rb) to E2F1-responsive promoters supports its role in cell cycle regulation (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). In addition, HDAC1 and HDAC2 have been shown to associate with DNA methyltransferase 1 and play a role in transcriptional silencing (Fuks et al., 2000; Rountree et al., 2000), thereby establishing a link between two major epigenetic modifications: DNA methylation and histone deacetylation.

Similar to DNA replication, DNA repair machinery utilizes chromatin remodeling activities to make the DNA more accessible and to reestablish the proper chromatin structure after DNA repair. In accordance, Hus1 and Rad9, two human checkpoint proteins, were shown to form a complex with HDAC1 (Cai et al., 2000). In addition, another histone-modifying enzyme, histone acetyltransferase p300, has been shown to form a complex with PCNA and was proposed to play a role in DNA repair after UV-induced DNA damage (Hasan et al., 2001).

We therefore tested the hypothesis that similar to DNA methyltransferase 1 and CAF-1, HDAC1 associates with PCNA, which may serve as a recruiting factor for histone deacetylases to the sites of DNA replication and repair. We also tested the hypothesis that disruption of HDAC activity would disrupt cell cycle progression.

MATHERIALS AND METHODS

Cell Culture and Transfections-- A549 human non-small cell lung carcinoma cells (ATCC CCL 185) were grown in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. HEK293 human adenovirus type 5-transformed human embryonal kidney cells (ATCC CRL 1573) were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. MRHF male human foreskin fibroblasts (72-213A; BioWhittaker) were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum and 2 mM glutamine. For transient transfections, HEK293 cells were plated 18 h before transfection at a concentration of 5×10^5 cells/100-mm tissue culture dish. 6 μg of pcDNA3-HDAC1-F plasmid (a kind gift from Dr. T. Kouzarides) (Fuks et al., 2000) were transfected using the calcium-phosphate precipitation method. The medium was replaced 24 h after transfection, and the cells were harvested 48 h after transfection.

Purification of GST Fusion Proteins-- GST-HDAC1 fusion constructs were a kind gift from Dr. T. Kouzarides (Fuks et al., 2000). GST and GST fusion proteins were expressed in *Escherichia coli XA90*, induced with 0.1 mM isopropyl-1-thio--D-galactopyranoside for 4 h, and purified following the protocol from Amersham Biosciences, with modifications. To isolate fusion proteins from inclusion bodies, the bacterial pellet was lysed in STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) on ice, lysozyme was added to a final concentration of 0.1 mg/ml, and incubation was continued for an additional 15 min on ice. Triton X-100 was then added to a final concentration of 2%, and the samples were sonicated and centrifuged at 12,000 rpm. The supernatants were bound to glutathione-Sepharose beads, and the concentrations of the different fusion proteins were estimated by subjecting small portions of the samples to SDS-PAGE. Equal amounts of the fusion proteins were used in the binding assays, whereas glutathione-Sepharose beads were added to the binding mixtures to achieve equal amount of beads in the binding reactions.

GST Pull-down Experiments-- Equal amounts of GST fusion proteins bound to the glutathione-Sepharose beads were incubated in radioimmune precipitation buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with 2 mg of total cell extracts prepared from either A549 or HEK293 cells and pre-cleared with beads alone. The binding reaction was incubated by rocking overnight at 4 °C. The beads were washed four times with 1 ml of radioimmune precipitation buffer, subjected to SDS-PAGE, and analyzed by Western blotting. PCNA was transcribed/translated *in vitro* using the coupled transcription translation system (Promega) from pHPCNA15 vector (a kind gift from Dr. Edward K. L. Chan) (Huff et al., 1990). We performed pull-down assays with *in vitro* translated PCNA as described previously (Fuks et al., 2000), adding Complete Mini protease inhibitors (Roche Molecular Biochemicals) in all buffers.

Immunoprecipitations and Western Blot Analysis-- Total cell extract was prepared in radioimmune precipitation buffer containing Complete Mini protease inhibitors (Roche Molecular Biochemicals). For immunoprecipitations, 2 mg of cell extract were incubated with 10 μl of agarose-conjugated PCNA antibody (PC10; Santa Cruz Biotechnology) or agarose-conjugated mouse IgG by rocking overnight at 4 °C. Bound complexes were washed four times with 1 ml of PBS (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, and 150 mM NaCl) and resolved on SDS-polyacrylamide gels. After transferring to polyvinylidene difluoride membrane and blocking the nonspecific binding with 5% milk, HDAC1, PCNA, and FLAG-tagged proteins were detected using a 1:1000 dilution of HDAC1 antibody (H-51; Santa Cruz Biotechnology), a 1:1000 dilution of PCNA antibody (PC-10; Santa Cruz Biotechnology), and a 1:5000 dilution of FLAG antibody (M2; Sigma), respectively. Peroxidase-conjugated anti-rabbit IgG (A0545; Sigma) and anti-mouse IgG (115035-146; Jackson ImmunoResearch) were used at dilutions of 1:5000 and 1:20,000, respectively, followed by enhanced chemiluminescence detection (Amersham Biosciences).

Histone Deacetylase Assay-- 1 mg of HEK293 total cell extract was incubated with 10 μl of agarose-conjugated PCNA antibody or agarose-conjugated normal mouse IgG

overnight at 4 °C. The beads were washed four times with 1 ml of PBS and assayed for deacetylase activity using the HDAC Fluorescent Activity Assay/Drug Discovery Kit (AK-500; BIOMOL Research Laboratories). In short, beads were incubated with 100 μM acetylated substrate in 100 μl of assay buffer containing or lacking 1 μM trichostatin A. Incubation of the reaction at 37 °C for 30 min allowed deacetylation of the substrate, which sensitized it to treatment with the developer and produced a fluorophore detectable on a fluorometric reader (excitation at 360 nm and emission at 450 nm).

Immunofluorescence and Confocal Microscopy-- A549 cells were plated on coverslips and grown to 60-70% confluence. The cells were washed with PBS and fixed with 3% paraformaldehyde solution at room temperature for 20 min, followed by fixation in 100% methanol at -20 °C for 10 min. The cells were washed three times with PBS and stained for PCNA, followed by staining for HDAC1. All of the staining steps contained PBS and 0.1% Triton X-100. For PCNA staining, coverslips were blocked with 10% donkey serum for 20 min, incubated with a 1:100 dilution of anti-PCNA antibody (PC-10; Santa Cruz Biotechnology) in 5% serum for 1 h at room temperature, washed three times, incubated with a 1:500 dilution of rhodamine Red-X anti-mouse IgG (715-295-150; Jackson ImmunoResearch) in 5% serum for 45 min at room temperature, and washed three times. The coverslips were blocked again with 10% goat serum for 20 min, incubated with a 1:50 dilution of anti-HDAC1 antibody (H-51; Santa Cruz Biotechnology) in 1.5% serum for 1 h at room temperature, washed three times, incubated with a 1:200 dilution of fluorescein isothiocyanate-conjugated anti-rabbit IgG (sc-2012; Santa Cruz Biotechnology) in 1.5% serum for 45 min at room temperature, and washed three times. The coverslips were mounted onto slides using Immunomount (Shandon). The staining was analyzed using LSM 510 Laser Scanning Microscopy, version 2.5 (Zeiss). The fields were taken with a magnification of x 63. The inserts were zoomed three additional times.

Cell Cycle Analysis by FACS-- A549 cells were plated at a density of 5 x 10^5 cells/100-mm plate. The next day, cells were treated with 1 μM trichostatin A (Sigma)

for 6, 12, and 24 h. Control cells and treated cells were harvested, washed twice in PBS, and fixed in ice-cold 70% ethanol for 30 min. The fixed cells were stained for DNA with a 50 μg/ml solution of propidium iodide in PBS for 30 min at room temperature. The cells were analyzed for DNA content by FACS.

RESULTS

PCNA and HDAC1 Form a Complex in Vivo-- To test the hypothesis that PCNA and HDAC1 are physically associated in human cells, we first tested whether they reside in the same multiprotein complex by coimmunoprecipitation assays. Endogenous HDAC1 protein was coimmunoprecipitated by agarose-conjugated anti-PCNA antibody from two human cancer cell lines (A549 and HEK293) as well as from a non-transformed human fibroblast cell line (MRHF), suggesting that the interaction is not unique to the cancer cells (Fig. 1A). Control immunoprecipitation with an equal amount of agarose-conjugated normal mouse IgG failed to precipitate any HDAC1 (Fig. 1A). To further confirm this interaction, HEK293 cells were transiently transfected with FLAG-tagged HDAC1 (HDAC1-F). Anti-PCNA antibody, but not the control mouse IgG, coimmunoprecipitated HDAC1-F (Fig. 1*B*).

PCNA Interacts Directly with HDAC1 in Vitro-- To map the interaction domain of HDAC1, we expressed in bacteria and purified three different fragments of HDAC1 fused to GST (Fig. 2A). We used these fusion proteins to carry out GST pull-down assays from total extracts of A549 and HEK293 cells (Fig. 2, *B* and *C*). Western blot analysis using anti-PCNA antibody revealed that two of the GST fusion proteins (region 1 (amino acids 1-120) and region 2 (amino acids 150-242)), which span the catalytic domain of HDAC1, associate with endogenous PCNA. These regions were previously shown to interact with human Hus1 protein (Cai et al., 2000). Interestingly, it was proposed that Hus1, Rad1, and Rad9, three checkpoint Rad proteins, form a PCNA-like ring structure around the DNA during repair. In addition, region 2 of HDAC1 has been shown to bind DNA methyltransferase 1 and play a role in transcriptional repression (Fuks et al., 2000).

To test whether the interaction of PCNA and HDAC1 is direct, we translated PCNA *in vitro* and carried out GST pull-down assays with purified GST-HDAC1 fusion proteins (Fig. 2D). These experiments confirmed that PCNA interacts directly with region 1 and region 2 of HDAC1 but does not interact with GST alone or with region 3 (amino acids 332-482), which contains a LXCXE-like motif previously shown to interact with Rb (Magnaghi-Jaulin et al., 1998).

PCNA and HDAC1 Colocalize in A549 Cells in Vivo-- To confirm the association of PCNA and HDAC1 *in vivo,* we carried out colocalization studies in A549 cells (Fig. 3). We stained endogenous PCNA protein with anti-PCNA antibody, followed by a rhodamine-conjugated anti-mouse secondary antibody (*red*). The same slides were subsequently stained for endogenous HDAC1 protein with anti-HDAC1 antibody, followed by a fluorescein-conjugated anti-rabbit secondary antibody (*green*). Both proteins showed strong nuclear staining, and the merging of confocal images appeared mainly *yellow,* indicating that PCNA and HDAC1 colocalize *in vivo.*

PCNA Associates with Histone Deacetylase Activity-- To test whether PCNA associates with an active histone deacetylase, we immunoprecipitated endogenous PCNA from HEK293 cells using anti-PCNA or a control mouse IgG antibody (Fig. 4). The precipitated complexes were tested for their ability to deacetylate an acetylated histone substrate. We showed that PCNA associates with deacetylase activity and that this activity is completely abolished when the deacetylase inhibitor trichostatin A (TSA; 1 μM, final concentration) is included in the deacetylation reaction, suggesting that the histone deacetylase activity associated with PCNA is completely sensitive to TSA.

Trichostatin A Arrests A549 Cells at G2-M-- Because the histone deacetylase activity associated with PCNA is sensitive to TSA, we used TSA to study the functional consequences of inhibition of this activity for the progression of the cell cycle. We treated A549 cells with 1 μM trichostatin A. We followed the cell cycle profile by FACS analysis of control cells and cells treated with TSA for 6, 12, and 24 h (Fig. 5).

The normal cell cycle distribution was altered early, after only 6 h, causing the accumulation of cells in G2-M phase. These results are consistent with the hypothesis that the maturation of chromatin through deacetylation may be involved in the timing of the events that drive the cells from replication to cell division.

DISCUSSION

Efficient assembly of nucleosomes onto newly synthesized DNA is essential for maintaining proper genome function. Chromatin organization is influenced by variations introduced at the nucleosomal level (Rea et al., 2000). One such variation is the deacetylation of newly assembled nucleosomes, a modification involved in the formation of heterochromatin. The pattern of this modification has to be faithfully inherited during cell division. Studies in yeast (Ekwall et al., 1997; Grewal et al., 1998) and mammalian cells (Taddei et al., 2001) show that inhibition of deacetylation leads to severe defects in chromosome stability. However, the mechanism of histone deacetylation after DNA replication remains poorly understood.

Here, we present evidence that PCNA associates with histone deacetylase 1. Because PCNA is a resident of the DNA replication fork and plays important roles in recruiting proteins to the fork, this physical linkage between PCNA and HDAC1 can explain the deacetylation of histones immediately after DNA replication. We first show that HDAC1 and PCNA coimmunoprecipitate in two cancer cell lines (A549 and HEK293) as well as in a non-transformed cell line (MRHF) (Fig. 1). This indicates that this interaction is universal and is not confined to the cancer cells that might form aberrant complexes. Second, we utilized GST-HDAC1 fusion proteins expressed and purified from bacteria to pull down PCNA from A549 and HEK cells and show that regions 1 and 2, which span the catalytic domain of the HDAC1, both associate with PCNA (Fig. 2, *B* and *C*). These regions have previously been shown to interact with the human Hus1 protein involved in DNA repair (Cai et al., 2000). It has been proposed that Hus1, Rad1, and Rad9, three checkpoint Rad proteins, form a PCNA-like ring structure around the DNA during repair. Therefore, it is likely that this region of

HDAC1 recognizes a ring-like structure around DNA and is thus recruited to sites of DNA replication and repair. Third, region 2 of HDAC1 was shown to bind DNA methyltransferase 1 (Fuks et al., 2000), which in turn was shown to bind PCNA (Chuang et al., 1997), which raised the possibility that the interaction between HDAC1 and PCNA is indirect, through DNA methyltransferase 1. To exclude this possibility, we studied the interactions between purified GST-HDAC1 and *in vitro*-translated PCNA. The same two regions that interact with PCNA in nuclear extracts also interact with *in vitro*-translated PCNA (Fig. 2*D*), indicating that the interaction between PCNA and HDAC1 is direct and is not mediated by DNA methyltransferase 1. Fourth, to further confirm the association of HDAC1 and PCNA in living cells, we carried out colocalization studies. Double staining of A549 cells with antibodies against endogenous PCNA and HDAC1 indicated that both proteins localize to the nucleus. The merging of images obtained by confocal microscopy showed that the two proteins colocalize *in vivo* (Fig. 3).

Fifth, we demonstrate that PCNA recruits a functional histone deacetylase activity that is inhibited by TSA (Fig.4). However, because HDAC1 is a member of a large family of histone deacetylases, we do not exclude the possibility that other members of the family also bind to PCNA and contribute to the deacetylase activity in the replication fork. Sixth, to test whether deacetylase activity was necessary for the progression of the replication fork during DNA synthesis, we treated A549 cells with TSA and followed their cell cycle by FACS analysis (Fig. 5). If histone deacetylase activity or the presence of HDAC1 was essential for DNA replication *per se* or for the progress of the replication fork, then the replication fork should have stalled during the S phase of the cell cycle or at the G1-S boundary after TSA inhibition of HDAC1. In contrast to these predictions, we observed that the cells started to accumulate at the G2-M phase 6 h after TSA treatment (Fig. 5).

Whereas we cannot formally exclude the possibility that TSA has other effects that might have caused G2-M arrest, our data are consistent with the hypothesis that histone deacetylase activity is essential for the formation of proper chromatin structure

after the synthesis of DNA. A recent study has shown that after DNA replication, PCNA remains associated with DNA and serves as an imprinting factor for the chromatin assembly (Shibahara and Stillman, 1999). This provides a window of opportunity for PCNA to load HDAC1 and allow it to modulate chromatin, leading to its maturation.

Why do cells arrest at G2-M after TSA treatment? It is possible that the disorganized hyperacetylated chromatin interferes either directly with the exit from G2 or indirectly by failing to signal proper progression through the cell cycle to a putative G2-M checkpoint. The G2-M arrest might be induced by a cellular checkpoint that monitors the state of acetylation of the chromatin and guarantees that only cells bearing proper chromatin divide. Such a mechanism might have evolved to guarantee the integrity of the epigenome. This checkpoint might be triggered by the induction of specific genes that are especially sensitive to hyperacetylation of histones and induce G2-M cell arrest. Additional experiments are required to address these questions and define the mechanisms through which the cell coordinates the inheritance of the histone acetylation pattern with cell cycle checkpoints.

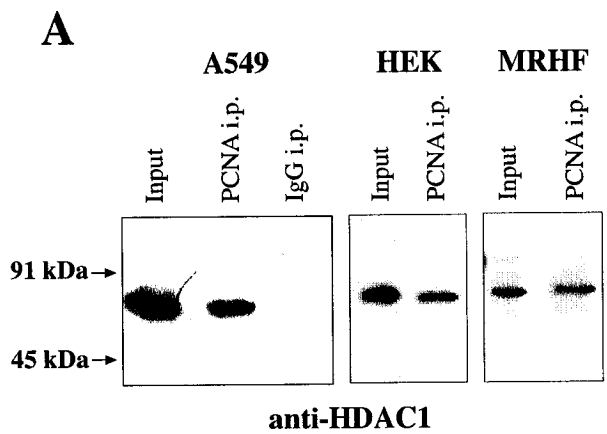
In summary, our data are consistent with a role for PCNA as a coordinator of DNA synthesis, epigenetic inheritance, and cell cycle control. The interaction of PCNA with HDAC1 as well as with DNA methyltransferase 1 and CAF-1 establishes its role in coupling two important processes, DNA replication and epigenetic inheritance.

ACKNOWLEDGEMENTS

We thank Dr. Tony Kouzarides for the kind gift of pcDNA3-HDAC1-F vector, GST-HDAC1 fusion constructs, and *E. coli XA90* and Francois Fuks for helpful suggestions regarding GST fusion protein purification. We also thank Dr. Edward K. L. Chan for the kind gift of pHPCNA15 vector.
This work was supported in part by the Canadian Institute of Health Research.

FIGURE LEGENDS

Fig. 1. PCNA and HDAC1 interact in vivo. Coimmunoprecipitations were performed with agarose-conjugated PCNA antibody or normal mouse IgG as described under "Materials and Methods." *A*, the complexes precipitated from A549, HEK293, and MRHF cells were analyzed by Western blots for the presence of HDAC1 using the anti-HDAC1 antibody. *B*, complexes precipitated from HEK293 cells transiently transfected with the HDAC1-FLAG construct were analyzed by Western blot using the anti-FLAG antibody.


A
A549
HEK
MRHF
Input
PCNA i.p.
IgG i.p.
Input
PCNA i.p.
Input
PCNA i.p.
91 kDa
45 kDa
anti-HDAC1

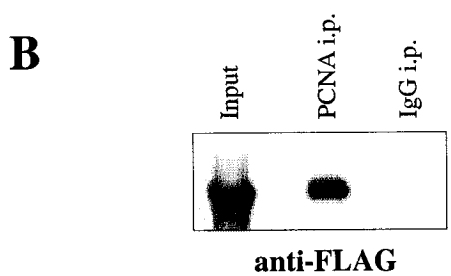

B
Input
PCNA i.p.
IgG i.p.
anti-FLAG

Fig. 2. Mapping of the PCNA interaction domain of HDAC1. *A*, schematic representation of the HDAC1 and the GST fusion proteins. *Gray* and ***black boxes*** indicate catalytic and L*X*C*X*E-like Rb-binding domains, respectively. ***B*** and ***C***, bacterially expressed and purified GST-HDAC1 fusion proteins bound to glutathione-Sepharose beads were incubated with total cell extracts from HEK293 cells (***B***) or A549 cells (*C*). Pull-down of endogenous PCNA was analyzed by Western blots using anti-PCNA antibody. *D*, GST-HDAC1 fusion proteins were used to pull down ***in*** *vitro*-translated and ^{35}S-labeled PCNA (*IVT PCNA*). The complexes were resolved on SDS-polyacrylamide gel and visualized by autoradiography.

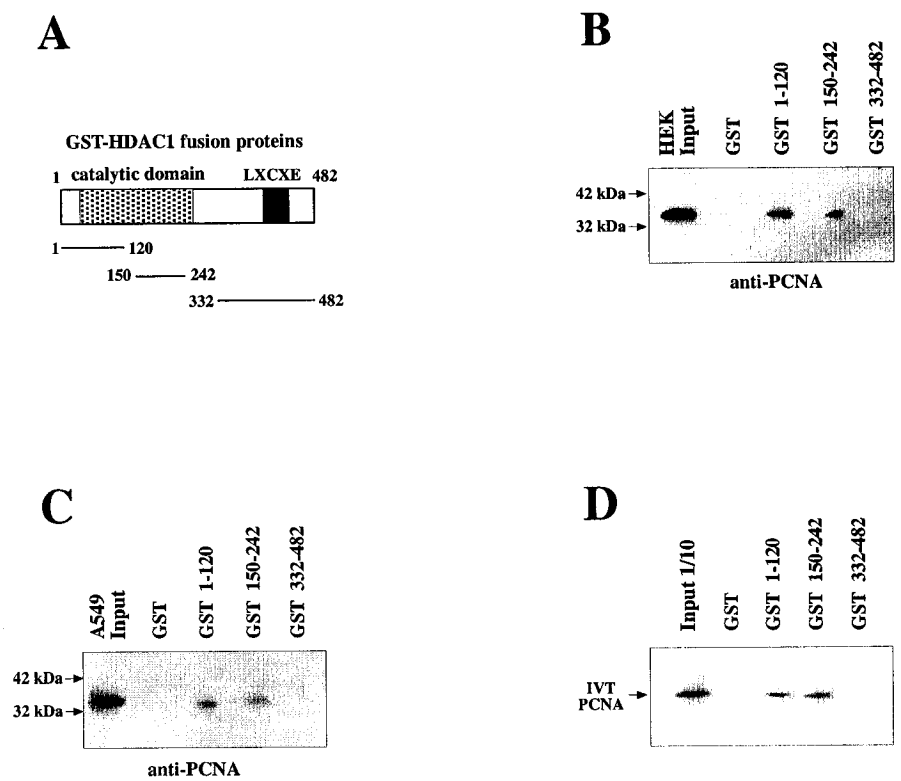

A
GST-HDAC1 fusion proteins
1 catalytic domain
LXCXE 482
1 120
150 242
332 482
B
HEK Input
GST
GST 1-120
GST 150-242
GST 332-482
42 kDa
32 kDa
anti-PCNA
C
A549 Input
GST
GST 1-120
GST 150-242
GST 332-482
42 kDa
32 kDa
anti-PCNA
D
Input 1/10
GST
GST 1-120
GST 150-242
GST 332-482
IVT PCNA

Fig. 3. Colocalization of PCNA and HDAC1 in A549 cells in vivo. *A*, A549 cells were grown on coverslips and stained with anti-PCNA antibody, followed by rhodamine-conjugated anti-mouse secondary antibody (*red*). *B*, the same cells were stained with anti-HDAC1 antibody, followed by fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (*green*). *C*, confocal merge of PCNA and HDAC1 staining shows colocalization (*yellow*) of PCNA and HDAC1 in the nuclei of A549 cells. The *insets* show a single cell from the field at a higher magnification.

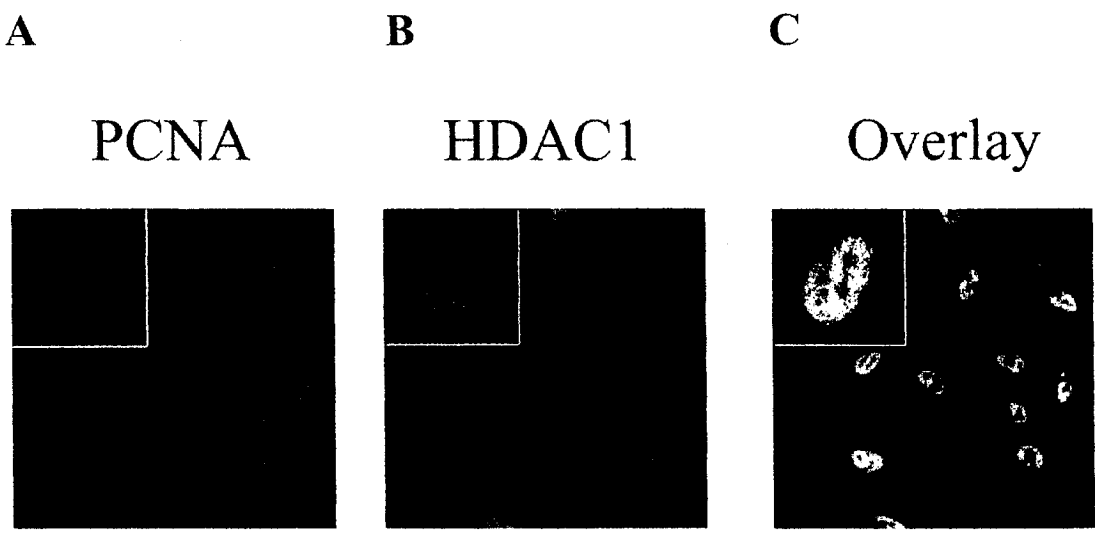

A
B
C
PCNA
HDAC1
Overlay

Fig. 4. PCNA associates with histone deacetylase activity. Histone deacetylase activity was precipitated from HEK293 cells using agarose-conjugated PCNA antibody or control mouse IgG as described under "Materials and Methods." The deacetylase activity associated with the precipitated complexes was assessed by their ability to remove an acetyl group from an acetylated substrate. Once the substrate becomes deacetylated, it reacts with the developer to produce a fluorophore detected on a fluorometric reader. In the presence of 1 μM TSA, the deacetylase activity is inhibited. The *error bars* represent S.D.s of triplicate experiments.

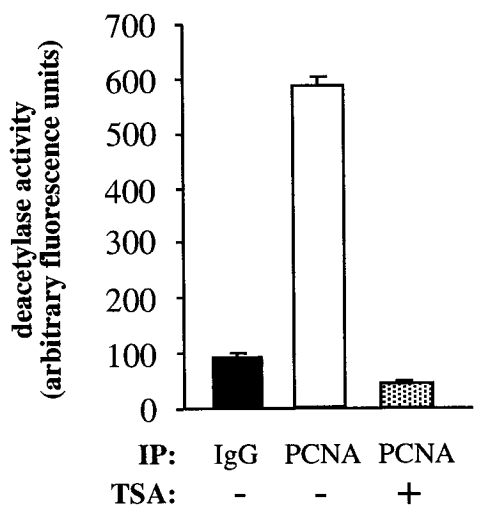
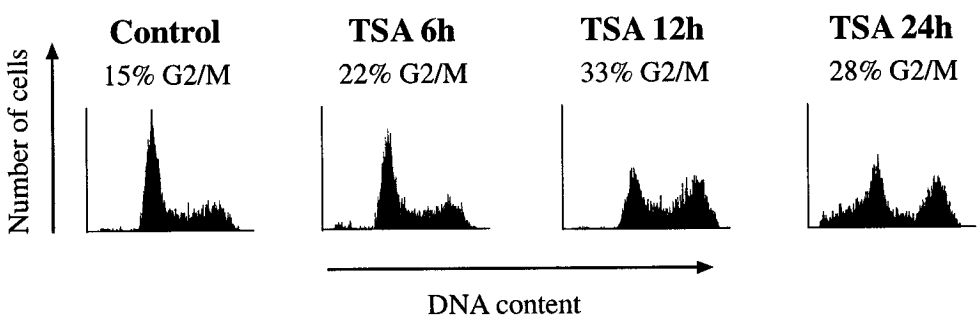

deacetylase activity
(arbitrary fluorescence units)
700
600
500
400
300
200
100
0
IP: IgG PCNA PCNA
TSA: - - +

Fig. 5. Trichostatin A arrests A549 cells at the G2-M. A549 cells were treated with 1 μM TSA for 6, 12, and 24 h. Cell cycle profile of control and treated cells was followed by FACS analysis. The percentage of cells in G2-M phase is shown.


Control
15% G2/M
TSA 6h
22% G2/M
TSA 12h
33% G2/M
TSA 24h
28% G2/M
Number of cells
DNA content

GENERAL DISCUSSION

Although DNMT1 biochemical activity is methylation of DNA, it is clear that it has many other roles in cellular regulation through its multiple protein-protein interactions. The general goal of this thesis was to understand the role that DNMT1 plays in cellular transformation since this is critical for proper targeting of the DNA methylation machinery in anticancer therapy. The specific aims of this thesis were therefore: 1. To determine the mechanism by which DNMT1 regulates gene expression 2. To define which of the functions of DNMT1 are critical for cellular transformation 3. To characterize the mechanism of the inheritance of chromatin states during DNA replication. The classical model of the role of DNMT1 in cellular transformation is that aberrant regulation of DNMT1 results in spurious methylation and inactivation of tumor suppressor genes, leading to uncontrolled growth. Thus, it is widely believed that it is the methyltransferase activity of DNMT1 that is critical for cellular transformation. The findings presented in this thesis challenge this model. Here we show that DNMT1 controls the expression of tumor suppressor genes and DNA replication by a mechanism which is independent of DNA methylation. The discovery of a novel role of DNMT1 in controlling gene expression, independent of its well-established methyltransferase activity, raises important questions regarding the use of different DNMT1 inhibitors in cancer therapy. The novel roles of DNMT1 in the regulation of the cell cycle and checkpoint control, and their potential mechanisms are also explored here. An additional mechanism for the inheritance of the chromatin states during DNA replication is proposed, which could explain some of the deficiencies of previously proposed models. Additionally, the antitumorigenic activity of HDAC inhibitors in cancer therapy is discussed in the view of these novel findings. The following discussion will summarize the results of this thesis and will attempt to explain how these novel findings might revise our current knowledge about the epigenome and its targeting in cancer therapy.

I. Targeting DNA methylation and its machinery in tumorigenesis

As previously discussed in the introduction, it is well established that cancer cells display aberrant changes in the methylation pattern: specific genes are hypermethylated whereas the genome is globally hypomethylated. The regional hypermethylation was shown to occur frequently in the promoters of tumor suppressor genes leading to their inactivation, which is believed to be crucial for the uncontrolled growth of cancer cells. In addition, the findings that DNMT1 protein levels are elevated in many cancers, and that its overexpression can lead to cellular transformation, have established DNMT1 as an important anticancer target. Two different inhibitors of DNMT1, the demethylating agent 5-aza-CdR and DNMT1 antisense oligonucleotides, are currently being tested in clinical trials. It is generally accepted that their mechanism of action involves demethylation of silenced tumor suppressors leading to their reactivation and cell cycle arrest. However, the data presented in this thesis unraveled a novel role of DNMT1 protein, independent of its methyltransferase activity, suggesting that these two drugs have different mechanisms of action because they target different functions of DNMT1. To determine whether DNMT1 tumorigenic actions are determined solely by its catalytic activity or by other functions of the protein, we tested whether inhibitors of DNMT1 that act by different mechanism will induce identical or different changes in gene expression. In chapter 1-3 it was demonstrated that treatment of cells with different inhibitors of DNMT1 (DNMT1 antisense oligonucleotide, a hairpin inhibitor of DNMT and 5-aza-CdR) produced different effects on cell growth and gene expression. The antisense oligonucleotide and hairpin inhibitor treatments, both of which reduced the amount of DNMT1 protein which is available for cellular functions, resulted in a rapid activation of unmethylated genes such as tumor suppressor *p21* and apoptosis inducer *BIK*. This suggests that the DNMT1 protein regulates the expression of these genes by a mechanism which does not involve DNA methylation. On the other hand, a demethylating agent 5-aza-CdR which inhibits the catalytic activity of DNMT1 during replication activated genes by demethylating them. The time course of action of 5-aza-CdR was different than the time course of gene activation caused by knock down of the DNMT1 protein. Whereas knockdown of DNMT1 causes a rapid increase in p21 and BIK, the DNA methylation inhibitor 5-aza-CdR causes a progressive increase

in gene activation. These differences in kinetics of gene induction most probably reflect differences in mechanism of action. While knock down of DNMT1 produces immediate effects on protein-protein interactions, demethylation of DNA requires DNA replication in the presence of 5-aza-CdR, resulting in progressive demethylation with every round of replication. However, it is important to note that some genes were activated by both antisense and 5-aza-CdR, suggesting that their functions overlap. In the case of 5-aza-CdR, although its main action is trapping DNMT1 protein during replication thus causing passive demethylation, it probably also reduces DNMT1 protein pool available for cellular functions, and therefore resembles the activity of antisense oligonucleotides. On the other hand, it was shown in chapter 2 and by other groups that DNMT1 antisense also leads to demethylation and activation of methylated tumor suppressors such as p16 (Fournel et al., 1999). However, the kinetics of this activation resembles that induced by 5-aza-CdR, and is different from the rapid induction observed with unmethylated genes. This is consistent with the mechanism involving demethylation, but it remains unclear whether this demethylation is passive or active. The data presented in chapter 2 demonstrates that DNMT1 knock down causes rapid replication arrest resulting in limited passive demethylation, suggesting that the observed demethylation of these tumor suppressors may be a result of active demethylation by MBD2b or other demethylases. Alternatively, the observed demethylation may be a result of residual replication in the absence of DNMT1. In order to test which of these two mechanisms is responsible for demethylation, future experiments will require simultaneous knock down of MBD2b/demethylase and DNMT1. If this would reduce observed demethylation it would mean that its mechanism involves active demethylation rather than being a consequence of passive demethylation in the absence of DNMT1.

II. DNA demethylation as a side effect in cancer therapy

The question of whether DNA demethylation is required for producing the antitumorigenic effects of DNMT1 inhibitors has very important implications on the

use of DNA methylation and DNMT1 inhibitors in cancer. If our data is correct, then antitumorigenesis could be accomplished by inhibition of DNMT1 DNA methylation independent actions. One might therefore avoid DNMT1 inhibitors which cause extensive demethylation. In the case of 5-aza-CdR, in addition to up-regulation of genes that are potentially antimitotic and proapoptotic, it was shown in chapter 2 that many cancer specific genes are also activated by this demethylating agent. As discussed in the introduction section, demethylation could lead to genomic instability and increased tumor invasion. In fact, there are multiple studies showing that treatment of cancer cells with 5-aza-CdR increases their metastatic potential through the activation of pro-metastatic genes (Frost et al., 1987; Guo et al., 2002; Habets et al., 1990; Sato et al., 2003). The data presented in chapter 2 suggests that knockdown of DNMT1 causes limited demethylation, thus this class of inhibitors might be a preferred approach. It would nevertheless be important to study the extent of demethylation after longer treatments with DNMT1 knock downs and to determine whether these might cause deleterious demethylation. Ongoing studies in our lab are directed at determining the effects of DNMT1 knockdown on metastatic potential of cancer cells.

Recent data suggests that MBD2b/demethylase is induced in some cancers and it was shown to be important for the anchorage-independent growth of cancer cells (Billard et al., 2002; Campbell et al., 2004; Hattori et al., 2001; Vilain et al., 1999). It was proposed that demethylation of genes involved in invasion, such as uPA, contributes to the metastatic potential of cancer cells (Guo et al., 2002). Therefore, it seems that DNA demethylation plays an important role in tumor progression by activating genes promoting metastasis. Therefore, it is tempting to speculate that simultaneous knock down of DNMT1 and MBD2b/demethylase would create a good drug combination in cancer therapy. The knockdown of DNMT1 produces a rapid cell cycle arrest, and the potentially dangerous demethylation produced with this treatment could be inhibited with the simultaneous knock down of MBD2b/demethylase.

III. DNMT1 as a multifunctional protein

Through the comparison of the effects of different DNMT1 inhibitors on cell growth and gene activation we were able to analyze different functions of this protein. An important difference in the mechanism of action of 5-aza-CdR and DNMT1 antisense was reflected in the kinetics of growth inhibition of cancer cells. In case of 5-aza-CdR, its activity of trapping DNMT1 onto DNA is dependent on its incorporation into DNA during replication, and therefore its effects on cell cycle arrest are limited. The reduced cell growth observed with 5-aza-CdR may be a result of the reduction of cellular pools of DNMT1, or at later time points, activation of demethylated tumor suppressors such as p16. On the other hand, knockdown of DNMT1 resulted in a dramatic reduction in cancer cell growth very early after the treatment, before any demethylation was observed. We propose that this rapid cell cycle arrest is a consequence of methylation-independent roles of DNMT1. It was previously demonstrated that DNMT1 knock down results in a rapid replication arrest through the inhibition of replication origin firing (Knox et al., 2000). This may be a consequence of inhibiting DNMT1 role in the regulation of the cell cycle.

There are a few possible ways how DNMT1 could be involved in the regulation of the cell cycle. As previously mentioned in the introduction, DNMT1 was shown to compete with the tumor suppressor p21 for binding to PCNA (Chuang et al., 1997). When p21 levels are high during Go/G1 phase, p21 competes out DNMT1 and forms an inhibitory complex with PCNA, therefore preventing inappropriate DNA methylation during this phase. As the cells enter into S phase, DNMT1 levels rise and it can now compete out p21 and enable PCNA to assemble at the replication fork. Therefore, DNMT1 seems to play a regulatory function in the initiation of replication by promoting assembly of the replication fork. Further support for the hypothesis that DNMT1 is involved in the cell cycle regulation comes from the recent finding that DNMT1 associates with Rb/E2F complex, which is critical for the progression from G1 to S phase of the cell cycle (Robertson et al., 2000a). During the G1 phase, Rb/E2F1 complex associates with HDAC and represses transcription from early S genes bearing E2F response elements (Ferreira et al., 2001). It is possible that as the cells enter into S phase, DNMT1 protein accumulates and associates with Rb/E2F to

repress genes with opposite function, such as tumor suppressor p14 (Robertson et al., 2000a). Thus, the presence of DNMT1 would promote progression into S phase by silencing tumor suppressors. The suppression of p14 by DNMT1 was shown to be histone deacetylase dependent (Robertson et al., 2000a). However, the data presented in chapter 3 proposes a novel role of DNMT1 in the regulation of gene expression, which is both methylation and histone modification independent. Similarly to its association with Rb/E2F1 complex, DNMT1 was previously shown to bind another transcription factor Sp1 (Song et al., 2001). Sp1 elements are found in genes that perform contradictory cellular roles, such as, thymidine kinase, which stimulates cell cycle, and p21, which causes cell cycle arrest (Kivinen et al., 1999; Li et al., 1998; Pagliuca et al., 2000). Since these two classes of genes are expressed at different phases of the cell cycle, there must be regulatory factors that switch their responsiveness to Sp1. The data presented in chapter 3 suggests that DNMT1 suppresses genes involved in cell cycle arrest though their Sp1 elements. Since this suppression does not involve either DNA methylation or histone modification, it is possible that DNMT1 directly interferes with the assembly of the transcriptional machinery at the regulated promoters. We propose that this is a novel role that DNMT1 plays in orchestrating the cell program toward DNA synthesis and S phase progression.

IV. Putative DNA methylation checkpoint

The rapid arrest of the cell cycle observed after the knock down of DNMT1 seems to be a result of inhibiting different DNMT1 functions. In addition to its effects on the replication fork assembly and the regulation of genes involved in the cell cycle, DNMT1 knock down was also shown to trigger an intra S phase arrest, and activation of genotoxic stress response genes (chapter 2). We propose that these effects are a consequence of the activation of a checkpoint similar to DNA damage checkpoints. This checkpoint has probably evolved to sense the presence of DNMT1 during replication in order to prevent buildup of DNA methylation errors as well as to

coordinate inheritance of genomic and epigenomic information. The intra-S-phase arrest and the activation of the stress response genes happens early after the knockdown of DNMT1, before any demethylation is observed, suggesting that it is the absence of DNMT1 protein and not the demethylation that triggers this checkpoint. In accordance, this protective mechanism is not induced by 5-aza-CdR which causes demethylation but does not reduce de novo synthesis of DNMT1 protein. The nature of this checkpoint is currently under investigation in our laboratory.

V. Chromatin inheritance during replication

Tight packaging of DNA into chromatin imposes a major obstacle to chromosomal events such as transcription, DNA replication, repair and recombination. Therefore, each of these events requires activity of numerous chromatin remodeling factors which open up chromatin and allow access to DNA. In addition, after DNA has been processed it needs to be repackaged into chromatin to retain its proper expression capacity. In accordance, both histone acetyltransferase p300 and a histone deacetylase HDAC1 have been proposed to play a role in DNA repair (Cai et al., 2000; Hasan and Hottiger, 2002). p300 has been suggested to participate in chromatin remodeling at DNA lesion sites to facilitate PCNA function in DNA repair synthesis. Similarly, HDAC1 was shown to be associated with DNA damage protein Hus1. It was suggested that Hus1 recruits HDAC1 to the region where DNA repair has been successfully completed to modify the local nucleosomal structure, which may have been changed during the repair process (Cai et al., 2000). Similarly to DNA repair, DNA replication also leads to major disruption of the chromatin structure in order to allow access of the replication machinery to DNA. After DNA replication, chromatin needs to be reconstituted by repackaging DNA into histones. As previously mentioned in the introduction, the newly deposited histones are acetylated and they undergo deacetylation as an important step in chromatin maturation (Annunziato and Seale, 1983; Sobel et al., 1995). In general, transcriptionally active euchromatin is rich in acetylated histones and it is replicated early in the S-phase, while the inactive heterochromatin is hypoacetylated and tends to be replicated later in S-phase (Sadoni et al., 1999). It was recently proposed that this deacetylation is carried out by

HDAC2, which is recruited to the replication foci through its interaction with DNMT1. Since this recruitment was limited to the replication foci of the late S-phase, it was proposed that HDAC2 is the enzyme responsible for the maturation of chromatin through histone deacetylation (Rountree et al., 2000). Since this model could not explain early S-phase deacetylation events which are probably necessary for the fine-tuning of different euchromatic regions, we hypothesized that there must be other HDACs responsible for chromatin maturation. We tested whether HDAC1 could associate with PCNA since HDAC1 was previously shown to interact with a PCNA-like ring structure formed by DNA damage proteins Hus1, Rad1 and Rad9. The data presented in chapter 4 demonstrates that HDAC1 binds PCNA directly, through a region outside of the DNMT1-binding domain. We also show that PCNA and HDAC1 co-localize in the cell nucleus. This data suggests an additional mechanism for the inheritance of the state of histone modifications through the direct interaction of PCNA and HDAC1. It was also recently discovered that PCNA mutations lead to disruption in inheritance of the silenced heterochromatin loci in yeast S. Cerevisiae (Zhang et al., 2000). Our data is consistent with this observation and suggests an evolutionary conserved role of PCNA in the epigenetic inheritance.

In addition to its roles in DNA repair and replication, PCNA has recently been demonstrated to play a role in transcriptional regulation as well. Recruitment of PCNA to gene promoters was shown to result in reduction of acetylation of associated histones, leading to gene suppression. Since PCNA was shown to bind to and inhibit the acetyltransferase activity of p300, it was proposed that its targeting to the promoters leads to indirect deacetylation through the inhibition of HAT activity at the targeted chromatin (Hong and Chakravarti, 2003). However, our data offers an alternative mechanism for PCNA transcriptional repression through direct recruitment of HDAC activity. Future experiments should test whether PCNA binding to the promoters increases binding of HDAC1, and whether PCNA mutants incapable of HDAC1 binding have a reduced transcriptional repressor potential. The hypothesis that PCNA and HDAC1 interaction may have other functions in addition to DNA replication is additionally supported by our immunostaining analysis showing that

they co-localize throughout the nucleus, and not exclusively in the distinct structures characteristic of replication foci.

We also show that HDAC1 activity is not essential for the progression of the replication fork, since treatment of cells with TSA allowed S-phase progression and caused arrest only later, at the G2/M phase. The G2/M arrest might be induced by a cellular checkpoint that monitors the state of acetylation of the chromatin and guarantees that only cells bearing proper chromatin divide. This is supported by previous reports showing that histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects (Cimini et al., 2003). The activation of this checkpoint may be a result of activation of genes sensitive to hyperacetylation of their promoter regions, such as p21 (Richon et al., 2000). Alternatively, check point proteins may exist that specifically recognize abnormally hyperacetylated regions, similarly to DNA damage sensing proteins.

Since TSA and other HDAC inhibitors are currently used in clinical trials as anticancer agents, the nature of this check point activation should be further investigated. The discovery of specific check point proteins could potentially establish novel anticancer targets. In addition, this activity of TSA may prove important in combination therapy. For example, HDAC inhibitors and DNMT inhibitors show synergistic effects on growth arrest in cancer cells. There are a number of examples where this synergism results in activation of tumor suppressors (Zhu and Otterson, 2003). However, our data raises the possibility that this synergism is also due to the activation of two independent check points, one arresting cells at the G1 and the other at the G2/M phase of the cell cycle.

VI. Conclusion

The main conclusion of this thesis is that the epigenome is a dynamic modulator of the otherwise static genome, and that it is a critical factor for the normal development and functioning of the organism. Both DNA methylation pattern and chromatin structure

are a result of action of multiple enzymes with opposing functions such as DNMTs and dMTase, HATs and HDACs etc. The plasticity of the gene expression is therefore achieved by modulation of any of these factors in response to physiological, environmental and pathological cues. Cancer is one example where pathological cues lead to alterations of the epigenome. Because of its reversibility, the epigenome has therefore emerged as an important target in anticancer therapy. A well-accepted mechanism of action of inhibitors of DNMTs and HDACs is believed to be through their direct effects on tumor suppressor gene expression. However, the data presented in this thesis unraveled novel roles of these enzymes and suggested an alternative mechanism of anticancer action of their inhibitors. In addition to their enzymatic activities DNMT1 and HDAC1 seem to have important roles in protein-protein interactions as well. The discovery that their inhibitors might trigger activation of particular check points may prove extremely important in the design of more specific and effective anticancer agents. Since the genome cannot function properly without the epigenome, it is easily imaginable that in addition to DNA damage checkpoints, epigenome checkpoints also exist to ensure its proper maintenance and inheritance. Therefore, our data suggests that these functions of DNMT1, and potentially HDAC1, should be targeted in anticancer therapy since this would result in cell cycle arrest without the dangerous loss of the epigenomic information resulting from the inhibition of the enzymatic activities of these proteins.

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McGill University Internal Radioisotope Permit

20040244

Permit Holder & Position	Building (Office)	Building (Lab)	Laboratory Classification		
MOSHE DR. SZYF, ASSOCIATE PROFESSOR	MCINTYRE BUILDING	MCINTYRE BUILDING	INTERMEDIATE	**PLEASE POST**	Date Issued 2003/09/01
Department	Room Number(s) / Telephone	Room Number(s) / Telephone			Expiry Date 2004/08/31
PHARMACOLOGY & THERAPEUTICS	1308 / 398-7107	1311 / 398-7107			

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Train.	Cond(s)	Class(es)	Radioisotope(s)
JOHANNE THEBERGE, TECHNICIAN	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
SNEZANA MILUTINOVIC, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
ANA CATALINA D'ALESSIO, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
IAN WEAVER, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
VERONICA BOVENZI, POST DOC	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
MOSHE SZYF, ASSOCIATE PROFESSOR	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
JING-NI OU, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
SHELLEY BROWN, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
JEROME TORRISANI, POST DOC	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
GULZHAKHAN SADVAKASSOVA, TECHNICIAN	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
SERGIY DYMOV, TECHNICIAN	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
AANAN MISRA, UNDERGRADUATE	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
ALEXANDER UNTERBERGER, GRAD STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
STEPHEN ANDREWS, GRADUATE STUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
KEISUKE SHIKIMI, GRADUATESTUDENT	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
SACHIN TENDULKAR, POST DOC	Y	2, 4	1	C-14, H-3, P-32, S-35, P-33
FABIEN DELAERE, SUMMER STUDENT	Y	2, 4	1	C-14, H-3, P-32, P-33, S-35
ANAGHA TENDULKAR, RESEARCH ASSISTANT	Y	2, 4	1	C-14, H-3, P-32, P-33, S-35
JEAN-PHILIPPE DEMERS, SUMMER STUDENT	Y	2, 4	1	C-14, H-3, P-32, P-33, S-35

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The permit must be posted with the CNSC safety poster in the permit holder's premises.
- Radioisotope handling shall be in accordance with the McGill Radiation Safety Policy Manual.
- The permit holder must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety prior to start of work.
- Radioactive work areas must be clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a functioning fume hood.
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their cloths are not contaminated.
- Purchase and disposal of radioisotopes must be kept electronically or documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Policy Manual and/or McGill WMP.
- Wipe tests must be performed and records be kept in a log book.
- The permit must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the RSO at 398-1538.
- The device(s) containing the sealed source(s) must have a radiation symbol and an identification label bearing the name and telephone number of the permit holder.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).
- Extremity dosimeter (i.e. ring or wrist badges) must be worn if 50 MBq or more of P-32, Sr-89, Sr-90 & Y-90 are used.
- Workers using I-125 or I-131 on open bench (5 MBq), in a fume hood (50 MBq) or a vented glove box

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored	Handled
H-3	< 100 MBq (2.7 mCi)	1309	1309-1311
P-32	< 400 MBq (11 mCi)	1309	1309-1311
C-14	< 100 MBq (2.7 mCi)	1309	1309-1311
S-35	< 400 MBq (11 mCi)	1309	1309-1311
P-33	< 100 MBq (2.7 mCi)	1309	1309-1311

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)				*Accessible Source(s)*			
Isotope	Activity	Stored	Handled	Isotope	Activity	Stored	Handled

Personnel Conditions

1. Must attend thyroid bioassays within 5 days of use if 50 MBq (1.35 mCi) of I-125 are manipulated in a fume hood.
2. Must wear a whole-body film badge, if gamma, x-ray or high energy beta emitters are used.
3. Must wear an extremity TLD dosimeter, if more than 50 MBz (1.35 mCi) of P-32, Sr-89, Sr-90 or Y-90 are used.
4. Classified as Radiation User.
5. Classified as Nuclear Energy Worker (NEW).
6. Does not work with any radioisotopes but may be indirectly exposed.

Workload Classes

1. Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2. Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3. Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4. Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5. Work with sealed sources.
6. Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vincelli
RSO & Occupational Hygienist
McGill Environmental Safety Office

Date

For: Dr. Louise Proulx, Chairperson
McGill Radiation Safety Committee
Vice Principal (Research)