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Identification of the DNA methylation machinery, its regulation and uses as potential anticancer therapeutics

By

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Abstract

DNA methylation is a vital component of several mammalian genome functions such as X-inactivation, allele and tissue specific gene expression, parental imprinting, and condensation of transcriptionally inactive chromatin. Aberrant post developmental fluctuations in the levels of DNA methylation and patterns of DNA methylation are commonly observed in transformed or cancerous cells. Proper control over the process of DNA methylation appears to be essential for proper cellular function, however the regulation of the expression of DNA MeTase has remained only loosely elucidated and is still debatable. Chapter 1 presents the complete genomic structure of the human *dnmt1* gene which provides an essential template to resolve the existing contradictions. Using this structure chapter 2 demonstrates that there are several different transcriptional control elements that influence the production of different *dnmt1* transcripts from several different points of initiation. Furthermore it demonstrates that each of the transcriptional control elements can respond individually to oncogenic and tumor suppressor pathways. Proper control over *dnmt1* expression in non transformed cells is achieved in a posttranscriptional manner. Chapter 3 of this thesis shows that the mechanism of the posttranscriptional control over *dnmt1* expression is mediated by a conserved stretch of sequence contained within the 3' untranslated region of its mRNA. Evidence is presented that a labile protein factor existing in the G_0-G_1 part of the cell cycle, which is rapidly cleared in S-phase, mediates the degradation of the dnmt1 mRNA. The pharmacological potential of specific inhibition of *dnmt1* expression in a whole organism tumor model is evaluated in Chapter 4. Inhibition of *dnmt1* expression, by systemic administration of a phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* mRNA, inhibits tumorigenesis with no overt toxicity.

The energetics of DNA demethylation suggested that it is an improbable event to occur in a cellular context, so alternative activities which repair DNA were rationalized to be the activities responsible for the observed demethylation

events. Chapters 5 and 6 of this thesis reveal a true or *bona fide* DNA demethylase (dMTase) activity which catalyzes the reverse reaction of DNA MeTase. Chapter 5 describes a cDNA which encodes *bona fide* DNA dMTase activity and chapter 6 purifies a DNA dMTase activity, characterizes the reaction and identifies the products of the reaction as methanol and nonmethylated cytosine. The DNA dMTase cDNA cloned in chapter 5 is shown to be over expressed in cancer cells and that DNA dMTase activity can only be isolated from tumor tissue. Inhibition of DNA dMTase inhibits the tumorigenicity of cells on semi-solid media, indicating that over expression of dMTase is also a critical component of tumorigenesis.

Taken together the results presented in this thesis demonstrate that the DNA methylation machinery is a target for the development of anticancer therapeutics. The data presented here provide the rationale that direct inhibition of DNA dMTase or inhibition at molecular points of regulation are targets to be potentially exploited for therapeutic use.

French language abstract

La méthylation de l'ADN est une composante vitale de plusieurs fonctions du génome mammifère comme l'inactivation en croisé, l'expression de gènes spécifiques à certains tissus ou allèles, l'empreinte parentale, et la condensation de la chromatine transcriptionellement inactive. Des fluctuations post-développementales abberantes dans les niveaux de méthylation de l'ADN sont couramment observées dans les cellules transformées ou cancéreuses. Le contrôle adéquat du procédé de méthylation de l'ADN semble être essentiel au fonctionnement cellulaire normal. Cependant, les mécanismes régulant l'expression de l'ADN méthyltransférase (DNA MeTase) demeurent vaguement élucidés et sont encore contestables. Le chapître 1 présente la structure génomique complète du gène dnmt1 humain, qui fournit un guide pour résoudre les contradictions existantes. En utilisant cette structure, le chapître 2 démontre qu'il existe plusieurs différents éléments de contrôle transcriptionel de dnmt1 qui influencent la production de différentes transcriptions de dnmt1 à partir de plusieurs régions génomiques uniques d'initiation. De plus, ce chapître démontre que chaque élément de contrôle transcriptionel peut répondre individuellement aux signaux oncogénique et suppresseur de tumeur. Le contrôle adéquat de l'expression de dnmt1 dans les cellules non-transformées est accompli de manière post-transcriptionelle. Le chapître 3 de cette thèse montre que le mécanisme de contrôle post-transcriptionel de dnmt1 est modéré par une étendue de séquence conservée localisée dans la région 3' non-traduite de son propre ARNm. L'évidence y est présenté qu'un facteur protéique labile existant dans la partie G0-G1 du cycle cellulaire, rapidement éliminé en phase S, supervise la dégradation de l'ARNm de dnmt1. Le potentiel pharmacologique de l'inhibition spécifique de l'expression de dnmt1 dans un modèle de tumeur implanté dans un organisme entier est évalué au quatrième chapître. L'inhibition de l'expression de dnmt1 par l'administration systémique d'un antisens phosphorothioate dirigé contre l'ARNm de dnmt1 inhibe la croissance tumorale sans toxicité évidente.

Les énergies requises pour la déméthylation de l'ADN suggère que cette réaction est improbable dans un contexte cellulaire, donc les activités alternatives qui réparent l'ADN furent raisonnées comme étant responsables des événements de déméthylation observés. Les chapîtres 5 et 6 démontrent que cette notion est incorrecte en révélant une véritable activité ADN déméthylase (DNA dMTase) qui catalyse la réaction inverse de la DNA MeTase. Le chapître 5 identifie un ADNc codant l'activité DNA dMTase et le chapître 6 montre la purification de cette activité, caractérise la réaction et identifie les produits de la réaction (méthanol et cytosine déméthylée). L'ADNc de DNA dMTase cloné au chapître 5 est surexprimé dans les cellules cancéreuses et son activité ne peut être isolée que dans les tumeurs. L'inhibition de DNA dMTase abolie la tumorogénécité des cellules en milieu semi-solide, indiquant que la surexpression de DNA dMTase est aussi une composante critique à la tumorogénèse. Ensemble, les résultats présentés dans cette thèse démontre que la machinerie de méthylation de l'ADN et les mécanismes qui la régulent sont des cibles pour le développement de thérapies anticancereuses.

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Manuscripts and Authorships**

"Candidates have the option of including as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These copies must be bound as an integral part of the thesis.

If this option is chosen, connecting texts, providing logical bridges between different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation". The thesis must include: a table of contents, a general abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final overall conclusion and summary, and a bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent: supervisors must attest to the examiners is made perfectly clear the responsibilities of the different authors of co-authored papers."

**Taken from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University.

Statement of Contributions

This thesis is written in manuscript format and is composed of six manuscripts. The contribution of each author is described below.

Chapter 1: Genomic structure of the human DNA methyltransferase gene
Shyam Ramchandani*, Pascal Bigey*, and Moshe Szyf

Figure 1a: Physical restriction enzyme map was put together by Shyam Ramchandani.
Phages ϕ tb, ϕ ftr1, ϕ utr were cloned by Shyam Ramchandani
Phages ϕ ftr2, ϕ p494, ϕ p4 were cloned by Pascal Bigey

Figure 1b: Exon intron map was constructed by Shyam Ramchandani

Figure 1c: Southern blots were all done by Shyam Ramchandani

Figure 2: Compiled by Pascal Bigey

Preparation of the text of the manuscript was done by Shyam Ramchandani and Dr. Moshe Szyf.

Chapter 2: Transcriptional regulation of the DNA Methyltransferase *dnmt1* gene
Pascal Bigey*, Shyam Ramchandani*, Johanne Theberge, and Moshe Szyf

All RACE-PCR clones were generated by Shyam Ramchandani (Summarized in figure 1b).

Sequence analysis of potential transcription factor sites was done by Shyam Ramchandani (figure 2).

RNase protection analysis shown in figure 3 was performed by Shyam Ramchandani.

All CAT assay constructs shown in figure 4a were generated by Shyam Ramchandani.
All CAT assay constructs shown in figure 4b,c and d were generated by Pascal Bigey.
All CAT assays were performed by Johanne Theberge.

Electromobility Shift assay shown in Figure 5 was performed by Pascal Bigey.

Figure 6 was designed and constructed by Dr. Moshe Szyf.

Preparation of the text of the manuscript was done by Dr. Moshe Szyf

Chapter 3: A novel RNA element mediates growth dependent posttranscriptional regulation of DNA methyltransferase expression
Shyam Ramchandani and Moshe Szyf

All experiments were performed by Shyam Ramchandani

Preparation of the text was done by Shyam Ramchandani and Dr. Moshe Szyf.

Chapter 4: Inhibition of tumorigenesis by a cytosine-DNA methyltransferase antisense oligodeoxynucleotide.
Shyam Ramchandani, A.Robert MacLeod, Marc Pinard, Eric von Hofe, and Moshe Szyf

All experiments in figure 1 were performed by A. Robert MacLeod.

Blood work reported in Table 1. was generated by the McGill animal facility

All experiments in Figures 2 and 3 were designed and performed by Shyam Ramchandani.

Marc Pinard assisted in tumor extractions and measurements, and generated the densitometry shown if figure 3c.

Dr. Eric von Hofe provided all oligonucleotides used in the studies.

The text of the manuscript was prepared by Shyam Ramchandani, Marc Pinard and Dr. Moshe Szyf.

Chapter 4: A mammalian protein with specific demethylase activity for mCpG DNA.
Sanjoy Bhattacharya*, Shyam Ramchandani*, Nadia Cervoni, and Moshe Szyf

Figure 1a: EST search was performed by Dr. Moshe Szyf

Figure 1b: Protein motif analysis was performed by Dr. Moshe Szyf and Shyam Ramchandani

Figure 1c: Northern blot analysis was performed by Shyam Ramchandani

The DNA dMTase cDNA reported was cloned by Shyam Ramchandani

Figure 2a: Construction of the vector shown was done by Shyam Ramchandani and *in vitro* translation experiment was done by Shyam Ramchandani

Figure 2b: This experiment was performed by Shyam Ramchandani and Sanjoy Bhattacharya

Figure 3a: Western Blot was performed by Johanne Theberge

Figure 3b: This experiment was performed by Shyam Ramchandani and Sanjoy Bhattacharya

Figure 3c: This experiment was performed by Sanjoy Bhattacharya

Figure 3d: This experiment was performed by Shyam Ramchandani

Figure 3e: This experiment was performed by Sanjoy Bhattacharya

Figure 4: All experiments presented were performed by Sanjoy Bhattacharya

All of the reported experiments were repeated several times, in all cases transfections and *in vitro* translations were performed by Shyam Ramchandani, all enzyme sample dialyses were performed by Sanjoy Bhattacharya.

The text of the manuscript was prepared by Dr. Moshe Szyf, Sanjoy Bhattacharya and Shyam Ramchandani

Chapter 6: DNA methylation is a reversible biological signal.
Shyam Ramchandani*, Sanjoy Bhattacharya*, Nadia Cervoni and Moshe Szyf

Figure 1: All purification steps were done several times by Sanjoy Bhattacharya and Shyam Ramchandani.

All concentrations and dialysis steps were done by Sanjoy Bhattacharya

Figure 2: Demethylation reactions for analysis by Gas Chromatography/ Mass Spectrometry were done by Sanjoy Bhattacharya and the McGill Medical Spectrometry Unit.
(Similar preliminary experiments using only Gas Chromatography were done by Sanjoy Bhattacharya and Shyam Ramchandani)

All kinetic parameters (Tables 1 & 2) were generated by Sanjoy Bhattacharya and Dr. Moshe Szyf.

Figure 3a: This experiment was performed by Sanjoy Bhattacharya and Shyam Ramchandani.

Figure 3b: Bisufite mapping shown in was done by Nadia Cervoni

Figure 3c&d: Demethylation experiments shown were done by Sanjoy Bhattacharya.

The text of the manuscript was prepared by Shyam Ramchandani and Dr. Moshe Szyf.

All of the work presented in this thesis was initiated from concepts of Dr. Moshe Szyf and performed under his competent, and constant supervision.

* authors made equal contributions

Contribution to original knowledge

In this thesis, I have:

1) cloned, mapped and constructed the first complete genomic structure of a mammalian DNA methyltransferase gene;

2) identified 3 previously unknown promoters of the human *dnmt1* gene and verified that the human *dnmt1* gene contains the same promoter as the previously described promoter of the mouse *dnmt1* gene;

3) demonstrated that the promoters identified for the human *dnmt1* gene respond differentially to oncogenic (jun) and tumor suppressor (Rb) signalling pathways;

4) demonstrated that posttranscriptional regulation of *dnmt1* mRNA requires a conserved 54 nucleotide sequence of the 3' untranslated region of its mRNA;

5) demonstrated that a cell growth cycle dependent factor(s) recognizes the the 3' untranslated region of *dnmt1* mRNA;

6) shown that a labile protein factor limited to G_0-G_1 of the cell cycle is responsible for destabilization of *dnmt1* mRNA;

7) demonstrated that inhibition of *dnmt1* expression, by treating tumor cells with a phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* , can result in demethylation of specific genes;

8) demonstrated that inhibition of *dnmt1* expression, by administration

of a phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* , inhibits tumorigenicity of cancer cells in culture;

9) demonstrated that inhibition of *dnmt1* expression, by systemic administration of a phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* , inhibits tumorigenicity of cancer cells *in vivo* ;

10) demonstrated that inhibition of *dnmt1* expression, by systemic administration of a phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* , can cause demethylation of specific genes and reactivation of their transcription;

11) shown that there is no apparent toxicity to the phosphorothioate modified antisense deoxyoligonucleotide directed against *dnmt1* ;

12) purified the first biological example of a true DNA demethylating activity;

13) cloned a cDNA that encodes DNA demethylating activity;

14) characterized that the DNA demethylase is specific for methylated cytosine residing in a CpG dinucleotide;

15) identified the products of the DNA demethylation reaction to be nonmethylated cytosine residing in a CpG dinucleotide and methanol;

16) demonstrated that DNA demethylase activity is restricted to transformed cells;

17) demonstrated that the cloned DNA demethylase cDNA is over

expressed in cancer cells;

18) demonstrated that inhibition of the cloned DNA demethylase inhibits the tumorigenicity of cancer cells in culture.

Table of contents

Introduction and literature review

Biological methylation is a ubiquitous reaction mediated by a class of enzymes known as methyltransferases. The reaction is carried out in a wide spectrum of organisms, including mammals, fungi, bacteria and plants, and results in the modification of both small molecules and macromolecules for different functional and regulatory purposes (Chiang, et al., 1996). The primary methyl donor is S-adenosylmethionine (AdoMet or SAM) for all of the different AdoMet- dependent methyltransferases (Schluckebier et al., 1995). The methyl group from AdoMet can be transfered to an amino group, hydroxyl group, or C5 atom of the cytosine ring of acceptor molecules by different methyltransferases. Methylation reactions can be divided into N-, O-, or C- methylation on the basis of the identity of the atom that is methylated and the individual methyltransferases are denoted as N-, O-, and C-methyltransferases, respectively.

A similar nomenclature is given to the known demethylation or dealkylation reactions and is based on the identity of the atom being demethylated/dealkylated, and are known as N-, O-, and S- demethylation/dealkylation reactions. The enzymes that carry out these reactions, to date, are all primarily microsomal P450 enzymes involved in the metabolism and clearance of endogenous and exogenous small molecules.

In the section to follow, biological methylation and demethylation reactions of molecules other than DNA will be briefly covered based on the substrates being methylated/demethylated. The focus of this thesis is the enzymatic machinery that determines how DNA methylation patterns are set up, maintained, changed and the regulation of these enzymes in the normal and transformed state of a cell. DNA methylation will be reviewed in a comprehensive fashion in the section that follows the other biological methylation/demethylation sections.

1. Enzymatic methylation of small molecules

1.1 Phenolic compounds

Phenylpropanoids and flavonoids, play important roles in plant growth and development, as well as plant interactions with the environment (Hahlbrock and Scheel, 1989; Dixon and Pavia, 1995). Many AdoMet-dependent O-methyltransferase cDNAs have been cloned, and divided into 4 groups according to the substrates that they can methylate (Ibrahim et al., 1997). A majority of the plant O-methyltransferase cDNA clones reported are involved in methylation of lignin precursors, which are of nodal importance in cellular differentiation and plant development (Ibrahim, 1997). The second major category of plant O-methyltransferases are involved in the methylation of flavonoid compounds. Flavonoid compounds are used as ultra-violet light protectants and as pigments that attract insects by plants. There are hundreds of different flavonoid compounds and range from mono- to polymethylated (Wollenweber et al., 1981).

1.2 Phospholipid

Phosphatidylcholine (PC), the most abundant phospholipid in eukaryotic cells, is synthesized from choline which is first phosphorylated, then cytidine is transfered to it which is subsequently traded for diacylglycerol to from PC. However, the liver also has the capacity to use phosphatidylethanolamine (PE) as a substrate and through three successive methylation reactions of its amino group produce PC (Vance et al., 1997). The enzyme that performs this reaction is known as phosphatidyl-N-monomethylethanolamine methyltransferase and genes from human, yeast and E. Coli have been identified that encode different forms of the enzyme (Kodaki, et al., 1991; Arondel, et al., 1993; Walkey et al., 1999). There is some evidence that phospholipid methylation might be involved in the modulation of some biological processes, such as calcium transport (Moore, et al., 1984) and, hepatocyte proliferation and liver cancer (Tessitore et al., 1999), however, its biological functions other than as an alternate route for the synthesis of phosphatidylcholine remains an open question (Walkey et al., 1997).

1.3 Catecholamine neurotransmitters

Catecholamine neurotransmitter biosynthesis and metabolism requires N- and O- methylation. The enzyme phenylethanolamine-N-methyl transferase methylates norepinephrine on its amino group to form epinephrine (Park *et al.*, 1982; Kaneda *et al.*, 1988). This enzyme is largely restricted to the adrenal medulla, shows poor substrate specificity and can transfer methyl groups to the nitrogen atom on a variety of β- hydroxylated amines (Yu, 1978; Elayan *et al.*, 1990). The enzyme catechol-O-methyltransferase (COMT) (Axelrod and Tomchick,1958) transfers a methyl group to the hydroxyl group at the C3 position of the catechol ring, which is a common structural feature of L-dopa, dopamine, norepinephrine and epinephrine. Catabolic degradation of catecholamines is important for terminating neurotransmitter action and regulating catecholamine functions, and is acheived by COMT in concert with monamine oxidase (Kopin, 1985). Recent studies suggest that O-methylation of the C3 position of the catechol ring can reduce the susceptibility of catecholamine to autoxidation (Bindali et al., 1992) which may play a significant physiological role in the brain's defense against oxidative stress (Miller et al. 1996).

2. Enzymatic methylation of macromolecules

In distinction from small molecule methylation, which is commonly associated with biosynthesis or degradation processes, macromolecule methylation is a post-synthesis process which functions to modulate and regulate the action of the macromolecule. Each macromolecule has a specific function that needs to be regulated properly *in vivo* , and methylation plays a role with differing degrees of importance within each individual organismal context.

2.1 Protein methylation

In eukaryotic cells, proteins can be methylated on the carboxyl groups or on the side-chain nitrogen of the amino acids lysine, arginine and histidine. Protein methylation is involved in cellular stress responses and aging / repair functions of proteins (Desrosiers and Tanguay, 1988; Ladino and O'Connor, 1992; Wang, et

al., 1992; Najbauer, et al., 1996). N-methylation of proteins has generally been regarded as a constitutive and irreversible post-translational modification (Najbauer and Aswad, 1990). The carboxymethylation of proteins sparked interest initially from its role in bacterial chemotaxis (Springer et al. 1979), however a similar role for carboxymethylation in mammals still remains as an open question (Hrycyna and Clarke, 1993).

Data from several recent studies has indicated that there may be reversible protein methylation in cellular signaling (Hrycyna and Clarke, 1993; Yamane and Fung, 1993; Philips, et al., 1993; Parish, et al., 1995). Several signal-transducing G-proteins, Ras, Rac, and Rho, contain the CaaX motif (C=cysteine, a=aliphatic amino acid and X=any amino acid motif). The cysteine residue in this motif (usually found in the C-terminals of G proteins) can be isoprenylated and then be methylated on the prenyl carboxyl group. This is the only modification that is known to be reversible in this pathway (Glomset, et al., 1990; Rando, 1996) and its role appears to be to enhance membrane association of the G-protein and coupling to potential effectors such as Phosphatidyl-inositol-3 kinase (PI3K). A unique class of protein carboxyl methyltransferases may be catalyzing the methylation of Ras and other signaling proteins (Clarke, et al., 1988).

The protein arginine N-methyltransferase (PRMT1) provides more evidence for an involvement of protein methylation in cell signaling pathways. PRMT1 is the first identified and most characterized enzyme of this family (Lin, et al., 1996). PRMT1 can asymmetrically or symmetrically dimethylate arginine residues located in specific sequence contexts. This family of arginine methyltransferases methylate many different RNA binding proteins involved in processing and transport of pre-mRNA to mRNA (Lin, et al., 1996; Najbauer, et al., 1993). Many of the proteins targeted for methylation are found in the nucleus and nucleolus, but the exact role or consequences of this methylation are still not clear. Recently, PRMT1 has been shown to bind the interferon receptor INFAR and possibly be a positive modulator

of the antiviral interferon signal mediated through INFAR1 receptor (Abramovich, et al., 1997). PRMT1 is also probably involved in the signaling triggered by NGF in PC12 cells (Lin, et al., 1996). However, the link between a specific methyltransferase and ligand-stimulated signal transduction pathways is still indirect and requires some rigorous genetic study in defined cellular models before precise mechanisms that are regulated by protein methylation can be identified.

2.2 RNA methylation

Methylation of RNA can occur at the 2'-OH group of the ribose or at adenosine and guanosine residues and depends on the identity of the RNA species being methylated. Mammalian ribosomal RNA (rRNA) is methylated primarily on ~80 % of 2'-OH groups, shortly after it is synthesized as ribosomal precursor RNA in nucleoli but before it is cleaved to mature rRNA (Maden, 1990). Methylation of rRNA at the 2'-OH group is thought to protect it from endogenous RNAses since their mechanism of action is thought to require a free 2'-OH group. Methylation of rRNA often affects the efficiency of its transport to cytoplasm, its interaction with cellular factors, and ribosomal assembly (Alix, 1986; Van Buul, et al., 1983; Maden, 1990; Lane *et al.*, 1995).

In higher eukaryotes, N6-methyladenosine (m6A) is the major methylated base on mRNAs (Tuck, 1992). Eukaryotic mRNA has a cap structure - m7G(5')ppp(5')N - at its 5' terminus, consisting of a 7-methylguanosine residue joined to the transcript's initial 5' nucleotide via a 5' to 5' triphosphate bridge. A cap can be 2'-OH methylated at the transcripts first nucleoside (cap-1), at its first two nucleosides (cap-2), or at neither (cap-0, predominantly in unicellular eukaryotes). If the first nucleoside is adenosine, it can also be N- methylated on its 6 position. The N-7 methyl group of the cap largely determines the translational efficiency *in vitro* (Both et al., 1975), possibly because it enhances the binding of eIF-4E (Sonenberg et al., 1975). In xenopus oocyte maturation, cap ribose methylation has been suggested to underly polyadenylation-dependent increases in some

maternal mRNA translation (Kuge et al., 1995; 1998)

3. Biological demethylation reactions

In higher eukaryotes, demethylation reactions are primarily carried out by cytochrome P450 enzymes and are classified as phase 1 reactions. These can be subdivided into three categories based on the identity of the atom being demethylated (or dealkylated); N-demethylation, which is an oxidative demethylation event, O-demethylation, and S-demethylation. Drugs such as morphine, benzphetamine, aminopurines, caffiene, theophylline, and sildenafil (Viagra) undergo N-demethylation. Codeine and *p*-nitroanisole are examples of drugs that are O-demethylated, and 6-methylthiopurine and methitural are drugs that are S-demethylated. These reactions are generally considered dealkylations and are not very selective so they may not represent truly specific demethylation reactions. Curiously, there is no sign of C-demethylation in the literature, unlike methylation reactions. This is due to the fact the C-demethylation requires the cleavage of a very stable and nonpolar bond between two carbon atoms and therefore it has always been assumed (although not documented) that this reaction is very unlikely to occur in biological systems.

4. DNA Methylation

The heritable genetic information in cellular organisms is coded for within the organism's DNA. DNA is made up of 4 different deoxynucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine), linked together by phosphodiester bonds to form a polymer consisting of a double stranded molecule consisting of 2 anti parallel chains with complementary nucleotide sequences (Watson and Crick, 1953). As organisms grow or multiply they need to faithfully pass on the primary sequence of their DNA, therefore the primary base sequence and composition cannot change, but as Hotchkiss revealed (Hotchkiss, 1948), there are other minor nucleotides in DNA. In bacteria and some lower

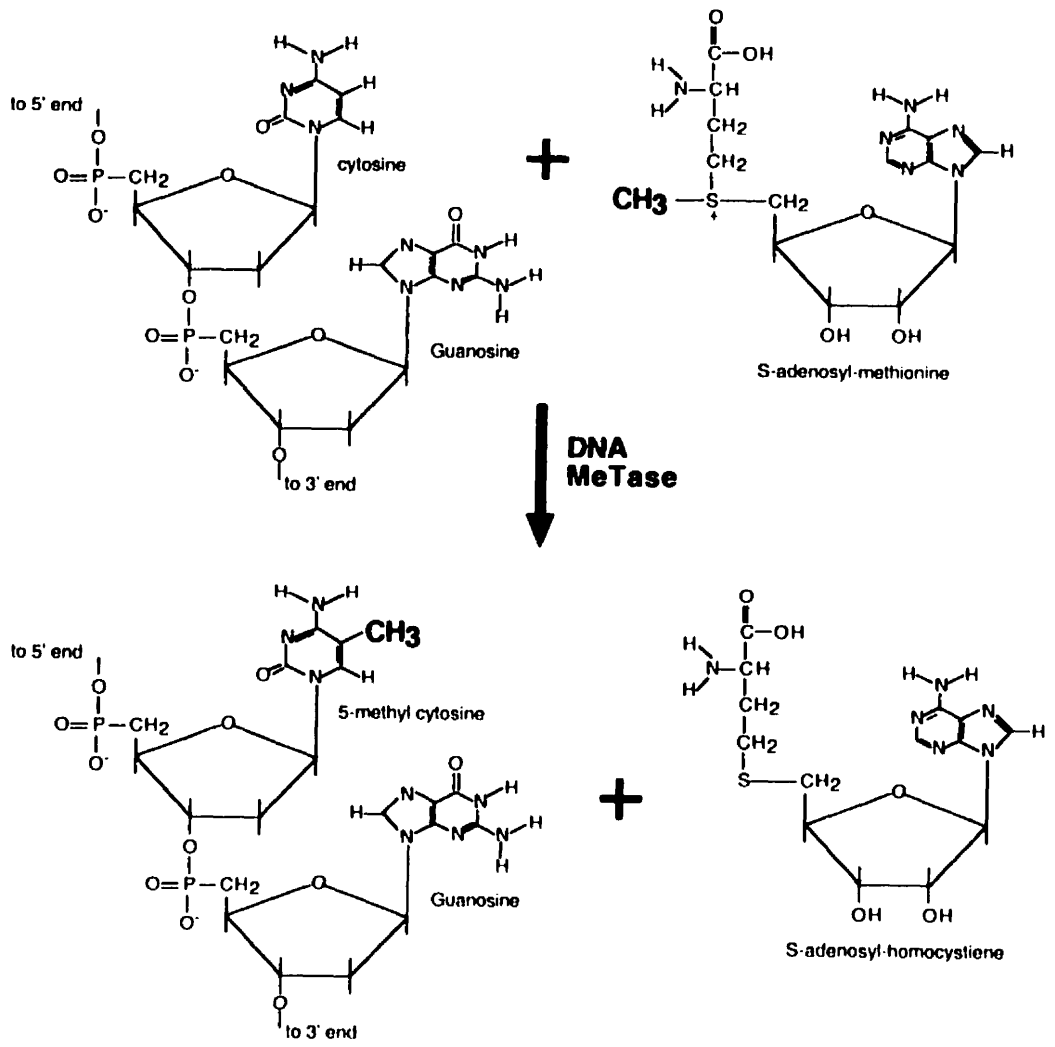
eukaryotes, 6-methyl-deoxyadenosine and 5-methyldeoxycytidine have been identified (Szyf and Razin, 1984), but in higher eukaryotes only 5-methyldeoxycytidine is found (Szyf and Razin, 1984). These methylated nucleotides do not alter the complementary nature of the DNA primary sequence. The methylation of DNA is sequence specific, meaning that not all deoxycytidines are substrates for methylation. In vertebrates, the vast majority of methylation occurs on the deoxycytidine residue in the dinucleotide sequence CG (Gruenbaum *et al.*, 1981b), although there have been published data showing that deoxycytidines in sequence contexts such as CC, CT and CA can be found that are methylated (Woodcock *et al.*, 1987, 1988; Toth *et al.*, 1990; Clark *et al.*, 1995). The occurrence of methylated deoxycytidine in the latter 3 sequence contexts is rare and their significance is poorly understood. DNA methylation occurs very shortly after DNA replication (Araujo *et al.*, 1998) and is catalyzed by the enzyme DNA methyltransferase (DNA MeTase) (Fig.1-1). DNA MeTase specifically recognizes deoxycytidine in its appropriate CG context (Gruenbaum *et al.*, 1982; Wu and Santi, 1985). Since DNA methylation is carried out by an autonomous enzymatic machinery, it provides a mechanism for epigenetic demarkation of the genome, and therefore a potential versatility to the genome without compromising its primary sequence structure.

5. DNA methylation patterns

The genetic information (DNA primary sequence) within all of the cells that constitute a multicellular organism is identical, however, the expression of functions encoded by the genome varies in each different cell type. An example is the phenomenon of X-inactivation. Two alleles of the same gene can be differentially expressed in the same cell. X-inactivation involves the inactivation of the allele of a gene that is located on the inactive X-chromosome, while the other allele of the same gene that is located on the active X-chromosome is active (Migeon *et al.*, 1994) (Fig. 1-2). Allele specific expression is also seen in the phenomenon of

Figure 1-1: The 5-methyl cytosine DNA methylation reaction

Chemical structures of DNA containing a CG dinucleotide and S-adenosyl-methionine. DNA methyltransferase (DNA MeTase) catalyzes the transfer a methyl group from S-adenosyl-methionine onto the 5th position of the cytosine ring. This is the reaction that the mammalian DNA MeTase catalyzes.


to 5' end
cytosine
Guanosine
to 3' end
S-adenosyl-methionine
DNA
MeTase
5-methyl cytosine
Guanosine
to 3' end
S-adenosyl-homocystiene

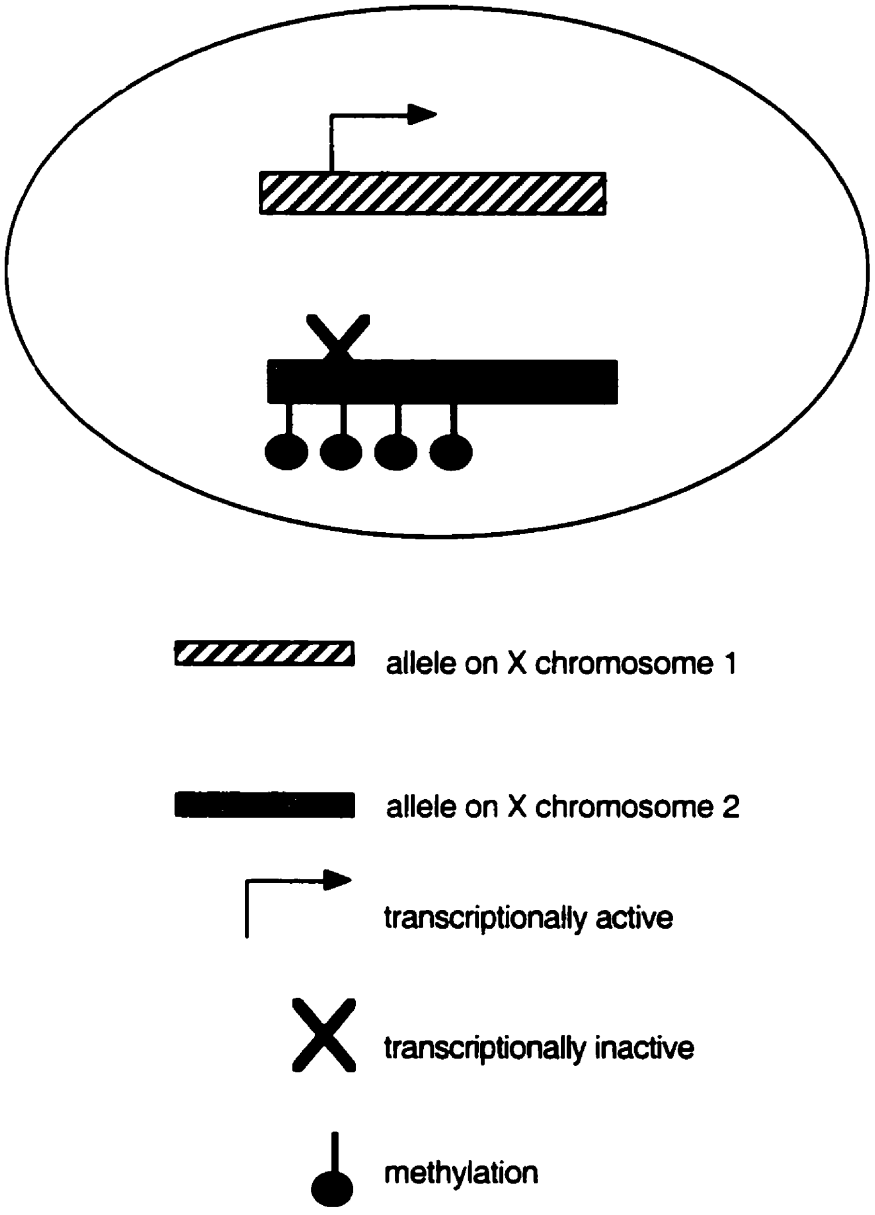


Figure 1-2 X-inactivation.

Inactivation of one of a pair of identical alleles residing on the X-chromosomes by DNA methylation.

Parental imprinting. This term defines the observation that, in some cases, an allele of a gene that is inherited from one parent is active, whereas the other allele inherited from the other parent is inactive (Sapienza *et al.*, 1987; Peterson and Sapienza, 1993).

A fundamental question arises which asks, "How can the exact same (identical) genetic information encode so many diverse functions in different cells and tissues?". The fact that DNA contains modifications that are not encoded in the primary sequence, but added covalently to the DNA using an autonomous enzymatic machinery, raises the possibility that DNA methylation is a candidate to encode the additional level of information that controls the differential expression of the genome or, DNA methylation represents "epigenetic information" (Holliday and Pugh, 1975; Holliday, 1990; Razin and Riggs, 1980). The obvious hypothesis that became essential to test was thatdifferent tissues within the same organism have different levels of 5-methyl-deoxycytidine. Many studies were conducted (Ehrlich *et al.*, 1982; Gama-Sosa *et al.*, 1983a; Gruenbaum *et al.*, 1981b; Razin *et al.*, 1984), but all concluded that, even though some differences exist between the total level of deoxycytidine methylation in different tissues (low levels in placenta and high levels in brain and thymus), these differences *per se* cannot explain the differentiation state of these tissues. Using a modification of the "nearest neighbour" assay (Gruenbaum *et al.*, 1981b), which allows for the evaluation of the state of methylation of the deoxycytidine residue in a specific dinucleotide context, it was demonstrated that 70-80 % of deoxycytidines residing in the sequence context CG were methylated in most tissues and cell lines analysed (Razin *et al.*, 1984).

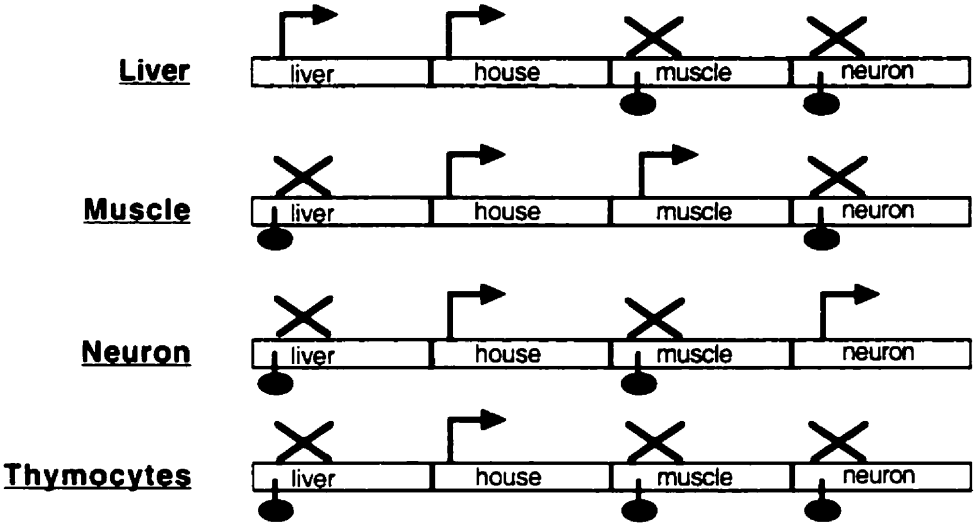
From these studies it became apparent that it is critical to answer the question, "How are these non-methylated deoxycytidines distributed?". The development of Southern blotting techniques to visualize patterns of restriction enzyme cleavage of specific genes, combined with the advent of a methylation-sensitive (Hpa II) and insensitive (Msp I) restriction enzyme pair that recognise a subset of CG sequences (CCGG) (Waalwijk and Flavel, 1978a,b; Singer *et al.*,

1979), unveiled that nonmethylated deoxycytidines are non randomly distributed (Waalwijk and Flavel, 1978b). A large number of genes were analyzed in a similar fashion in the following 15 years and the results are generalized as follows, "genes are nonmethylated at certain CG sites in tissues where they are expressed, but they are methylated in non expressing tissues." (Szyf, 1996; reviewed in Yisraeli and Szyf, 1984) (Fig. 1-3). More sensitive techniques such as ligation mediated polymerase chain reaction coupled to genomic sequencing (Pfiefer *et al.*, 1989, 1990ab) and bisulfite mapping (Frommer *et al.*, 1992; Clark *et al.*, 1994), which allow one to analyze the the state of methylation of every deoxycytidine within a specific gene, have verified the generalization. The results of the experiments described above have led to the development of the fundamental concept that DNA methylation encodes its information by forming specific patterns of methylation. Since the default state of deoxycytidine residing in the sequence CG is methylated and since nonmethylated CGs are located 5' of expressed genes or throughout the complete sequence of an expressed gene (Yisraeli and Szyf, 1984), it has been proposed that hypomethylation of specific genes marks them for expression (Razin and Riggs, 1980) (Fig. 1-3). The pattern of distribution of nonmethylated deoxycytidines is what may encode epigenetic information.

The fact that genes that are expressed are nonmethylated suggests that methylation of a gene will repress its expression. Many studies have established that the state of expression of a gene correlates with the hypomethylated nature of the regulatory sequences controlling a gene's expression (Yisraeli and Szyf, 1984) (Fig. 1-3). These correlations hold true for X-inactivated genes (Gartler and Riggs, 1883; Pfeifer *et al.*, 1989, 1990a,b; Migeon *et al.*, 1994) (Fig. 1-2). Analysis of the CG island (a region of DNA that has an unusually high density of CG dinucleotides) 5' to the phosphogycerate kinase gene (a gene located on the X-chromosome) shows that the copy of the gene located on the inactive X-chromomsome is heavily methylated while the copy located on the active X-chromosome is not methylated (Pfeifer *et al.*, 1990a). This correlation has been shown to hold as it pertains to parentally imprinted genes (Sapienza *et al.*, 1987,

Figure 1-3: Patterns of methylation and gene expression.

A hypothetical pictorial representation of DNA from different cell types from the same organism. The methylation of genes correlates inversely to their state of transcriptional activity. Bold and underlined text indicates the tissue of origin of the DNA. Note that housekeeping genes or genes that are required by all cell types are generally transcriptionally active and generally found to be hypomethylated.



liver — liver specific gene

house — housekeeping gene

muscle — muscle specific gene

neuron — neuron specific gene

transcriptionally active

transcriptionally inactive

methylation

Swain *et al.*, 1987) and some genetically transmitted genomic abnormalities such as fragile-X syndrome, where the amplification of a trinucleotide repeat is correlated with the inactivation of the gene and its hypermethylation (Sutcliffe *et al.*, 1992; Knight *et al.*, 1993). Similar correlations have been demonstrated for retroviral gene expression and integrated adenoviral gene expression (Sutter and Doerfler, 1980; Harbers *et al.*, 1981; Kruczek and Doerfler, 1983; Simon *et al.*, 1983). The differences in methylation patterns of a gene that is active and inactive respectively, can explain why it is expressed in some tissues while not in others, and can even explain how two identical copies of the same DNA sequence can be differentially expressed within the same cell, even though they are exposed to the same environment containing the same transcription factors. The correlation between methylation and inactive genes is strong but it still does not reveal whether methylating a gene will render it inactive or whether an inactive gene becomes methylated. If methylation is a consequence of the state of gene expression, then a change in the methylation pattern should not alter the pattern of gene expression. On the other hand, if the pattern of methylation can control gene expression, altering the state of methylation of a gene should alter its expression. This would mean that the pattern of methylation is determined by factors that are independent of gene expression, and that methylation patterns encode epigenetic information.

6. In vitro methylated genes are inactive when exogenously introduced into mammalian cells.

In order to test whether the state of methylation determines the expression status of a gene, one can introduce an *in vitro* methylated cloned gene or the same gene unmethylated into a cell line and monitor its expression. Many experiments using the described paradigm have been performed on several different genes and the results demonstrate that *in vitro* methylated genes are repressed in mammalian cells. The questions that arise from this are, "Is a specific site required to be methylated to mediate the repression of gene expression or does the methylation

have to be regional, nonspecific and only in close proximity to the gene in order to repress its expression?" and "Does methylation directly inhibit the transcription process or does it in turn attract methylation specific factors that bind and inhibit gene expression?". In some cases, such as the E2A adenovirus promoter (Langner *et al.*, 1984) and the proenkaphalin gene (Comb and Goodman, 1990), the methylation of specific sites, which are mainly transcription factor binding sites (AP-2 for the proenkephalin gene) is sufficient to repress transcription. In other examples, site specificity of methylation is not required for repression of gene expression, methylation of different sites within the coding sequence (Yisraili *et al.*, 1988) or of associated sequences, or even of neighbouring plasmid sequences can mediate repression of expression of several different genes (Bryans *et al.*, 1992; Komura *et al.*, 1995). These observations support both possibilities, in some cases methylation must be site specific and interferes with the binding of a transcription factor (Comb and Goodman, 1990; Prendergast *et al.*, 1991) while in other cases the methylation is not site specific and involves creating an inactive chromatin structure around the gene (Kass *et al.*, 1993, Jones *et al,*. 1998; Nan *et al.*, 1998). Both of these mechanisms have been supported by molecular and biochemical data and will be discussed later.

7. Inhibition of DNA methylation induces expression of silenced genes

Data accumulated from experiments where genes were *in vitro* methylated and subsequently introduced into mammalian cells is slightly problematic since it is not obvious that exogenously introduced genes behave like endogenous genes. In order to demonstrate that DNA methylation has a role in controlling the expression of endogenous genes one must inhibit the methylation of endogenous genes and monitor the effects on the expression of endogenous genes. The cytidine analogue 5-aza-cytidine (5-azaC), can be transformed into a triphosphate by cellular enzymes and then incorporated into newly synthesized strands of DNA

during DNA replication. Once it has been incorporated into DNA it can covalently bind DNA MeTase and inhibit DNA methylation (Wu and Santi,1985), which results in DNA hypomethylation. Treatment of cells with 5-azaC can induce the expression of many silent genes (Constantinides, 1977; reviewed by Jones, 1985) and induce the differentiation of Friend erythroleukemia cells (Creusot *et al.*, 1982) and fibroblast lines such as NIH 3T3 and 10 T 1/2, which differentiate into muscle, fat, and oesteoclast cells (Taylor and Jones, 1979). Induction of the *myoD* gene by its demethylation using 5-aza-C as a demethylating agent in 10 T 1/2 fibroblasts is sufficient to convert them into myoblasts (Lassar *et al.*, 1986; Davis *et al.*, 1987).

Inferences of the role of DNA methylation based on experiments using 5-azaC must be reserved and confirmed by alternate means due to the nature of the agent. It is a nucleoside analogue that can effect other DNA metabolizing enzymes and may interfere with other DNA binding functions that are essential for maintaining the state of gene expression. 5-azaC has been shown to induce new developmental phenotypes in *Asperfillus* , whose DNA does not contain any 5-methyl-cytosine (Tamame *et al.*, 1983). Another report suggests that 5-azaC can act by a mechanism that involves trapping of the DNA MeTase enzyme by its covalent binding to the incorporated 5-azaC residue in the DNA, rather than inhibition of DNA methylation (Jutterman *et al.*,1994). 5-azaC is mutagenic in bacteria (Call *et al.*,1986; La *et al.*, 1988), yeast, which do not contain 5-methyl-cytosine in their genome (Zimmerman and Scheel, 1984) and mammalian cells (Amacher and Turner, 1987). 5-azaC induces DNA damage (Covey *et al.*, 1986) at fragile sites in chromosomes and chromosomal breakage (Lavia *et al.*, 1985; Snyder and Lachmann, 1989; Djalali *et al.*, 1990) in *Drosophila* which do not bear any 5-methylcytosine in their genome (Katz, 1985; Osgood and Seward, 1989). It is teratogenic in mouse (Takeuchi and Takeuchi, 1985; Matsuda, 1990) and rat (Rosen *et al.*, 1990). The toxic side effects of 5-azaC are consistent with those of other nucleoside analogues, which is exemplified by the rapid anti viral effects it elicits, which do not seem to be mediated by an inhibition of DNA methylation

(Bouchard *et al.*, 1990). To rule out the pleiotropic nature of the action of 5-azaC, new approaches to inhibition of DNA MeTase were investigated. Partial inhibition of DNA MeTase by using an antisense approach, was achieved in 10 T 1/2 cells, and demonstrated that inhibition of DNA MeTase resulted in conversion of the cells to a myogenic phenotype (Szyf *et al.*,1992) as 5-azaC did previously (Taylor and Jones, 1979). These results support the hypothesis that DNA methylation controls the state of differentiation in somatic cells.

8. Mechanisms of action of DNA methylation patterns

8.1 Blocking binding sites of transcription factors

Gene expression is heavily influenced by *cis*-acting sequences (Gillies *et al.*, 1983). One can simply envisage that the recognition sequence of a transcription factor could become disguised by its methylation. This simple concept has been validated by the demonstration that certain transcription factors are inhibited by methylation in their recognition sequence. For example AP-2 binding to the adenoviral E2A promoter (Langer *et al.*, 1984; Herman and Doerfler, 1991), the interaction of transcription factors to the tyrosine aminotransferase promoter (Becker *et al.*, 1987), the binding of the cAMP-dependent activator CREB to its canonical site (Comb and Goodman, 1990), and Myc/Max interaction to its cognate sequence (Prendergast and Ziff, 1991; Prendergast *et al.*, 1991), are inhibited by methylation in their specific binding sequences. The vast majority of transcription factors do not contain the CG dinucleotide in there binding site so their ability to bind to their cognate site is not directly effected by DNA methylation and so it stands to reason that this mechanism alone cannot explain the wide spread correlation between methylation and repression of gene expression.

8.2 Chromatin structure

Chromatin structure has been determined to be a major contributor in

determining the potential for gene activity. Also, the combination of data from numerous different studies demonstrates that, gene specific methylation patterns correlate inversely with state of gene activity (reviewed in Yeivin and Razin, 1993), that exogenous demethylation of methylated genes results in their activation and that *in vitro* methylation of genes and promoters causes repression of gene expression activity (reviewed in Razin and Cedar, 1991). Only recently has there been a conclusive molecular link demonstrated between methylated DNA and its specific precipitation of condensed or inactive chromatin structure even though it has been known for over two decades that regions of heterochromatin in the mammalian genome are associated with high levels of DNA methylation (Razin and Cedar, 1977). Experiments that introduced *in vitro* methylated genes into eukaryotic cells show that DNA methylation results in the formation of inactive chromatin (Keshet *et al.*, 1986), and that the repression of gene expression exerted by methylation is only observed after the inactive chromatin structure is formed on the newly introduced DNA (Buschhausen *et al.*, 1987). Methylation *per se* can repress gene activity, but this repression can be alleviated by a strong transcriptional activator like GAL4-VP16, but this strong activator cannot overcome repression once chromatin structure was formed on the methylated gene (Kass *et al.*, 1997). These facts point to the conclusion that silencing of gene expression by methylation requires the assembly of a chromatin structure that masks the DNA from the transcription initiation complex, which begs the question, "How does methylated DNA signal the formation of inactive chromatin?".

Deacetylation of the core histones H3 and H4 at lysine residues by multi protein complexes mediate transcriptional repression by forming a tightly bound, DNAse 1 insensitive chromatin structure (Wolffe,1997). In mammalian cells E-box related signals mediated through Mad:Max heterodimer, or by heterodimers of unliganded hormone receptors like the thyroid hormone receptor and retenoic acid receptor (TR:RXR) attract the mSin3A corepressor, which is associated with at least 8 different polypeptides, including the histone deacetylases HDAC1 and HDAC2 (Pazin and Kadonaga, 1997). These deacetylases remove acetyl

groups from specific lysine residues on H3 and H4 histones, revealing positively charged lysine residues. The positively charged lysines on H3 and H4 are thought to interact more tightly to the negatively charged DNA than their acetylated forms, which restricts nucleosome mobility on the DNA and creates a closed or inactive chromatin structure which leaves DNA with this structure inaccessible to the transcription initiation complex (Ura *et al.*, 1997).

Recently, it has been shown that DNA methylation can mediate the formation of a multi protein complex that represses transcription and can induce changes in histone acetylation. The methylated DNA binding protein MeCP2, contains a domain responsible for binding methylated DNA (MBD), and a transcriptional repressor domain (TRD) (Nan *et al.*, 1997). The TRD of MeCP2 directly interacts with the corepressor mSin3A and immunoprecipitation of MeCP2 coprecipitates mSin3A, HDAC1 and HDAC2 (Nan *et al.*, 1998), which suggests that methylated DNA attracts a complex that can remodel chromatin to an inactive structure via histone H3 and H4 deacetylation. In support of this theory, trichostatin A (a histone deacetylase inhibitor) can relieve the transcriptional repression mediated by MeCP2 (Nan *et al.*, 1998; Jones *et al.*, 1998; Eden *et al.*, 1998). The results of these experiments clearly demonstrate that methylation of genes induces transcriptional repression through binding to MeCP2, which in turn anchors a repression multi protein complex which includes the corepressor protein mSin3A, and the histone deacetylases HDAC1 and HDAC2 (see model in Razin, 1998).

9. The enzymatic DNA methylation machinery: DNA methyltransferase

The enzyme that catalyzes the bulk of the DNA methylation seen in vertebrate cells is encoded for by the gene *dnmt1* and is known as the DNA methyltransferase (DNA MeTase). It is a 190 kDa protein that is composed of a number of interesting motifs and consists of a catalytic domain that is homologous to other 5-cytosine DNA MeTases (Kumar *et al.*, 1994). It has a zinc finger DNA binding domain (Bestor *et al.*, 1988) and a nuclear localization domain within another domain that targets and mediates the association of the enzyme to the DNA

replication machinery (Leonhardt *et al.*, 1992) through an interaction with the proliferating cell nuclear antigen (PCNA) (Chuang *et al.*, 1997).

Until recently, there was only one DNA MeTase demonstrated in vertebrate cells. Other candidate genes *dnmt2*, *dnmt3a*, and *dnmt3b* have been found but appear to be minor contributors to the methylation status of the genome, and based on homozygous deletion experiments it is clear that the major and essential contributor to the genome's methylation is *dnmt1* (Li *et al.*, 1992). Furthermore, forced expression of human *dnmt1* in NIH-3T3 cells results in an increase in total genomic methylation (Wu *et al.*, 1993) and inhibition of *dnmt1* by expression of a portion of the *dnmt1* cDNA in the antisense orientation leads to a reduction in the level of methylation of the genome (Szyf *et al.*, 1992; MacLeod and Szyf, 1995). The *dnmt1* gene product can catalyze the methylation of DNA molecules that are methylated on one strand (maintenance methylation), as well as nonmethylated DNA (*de novo* methylation) (Tollefsbo and Hutchinson, 1995), but displays a preference for the hemi-methylated substrate (Gruenbaum *et al.*, 1982).

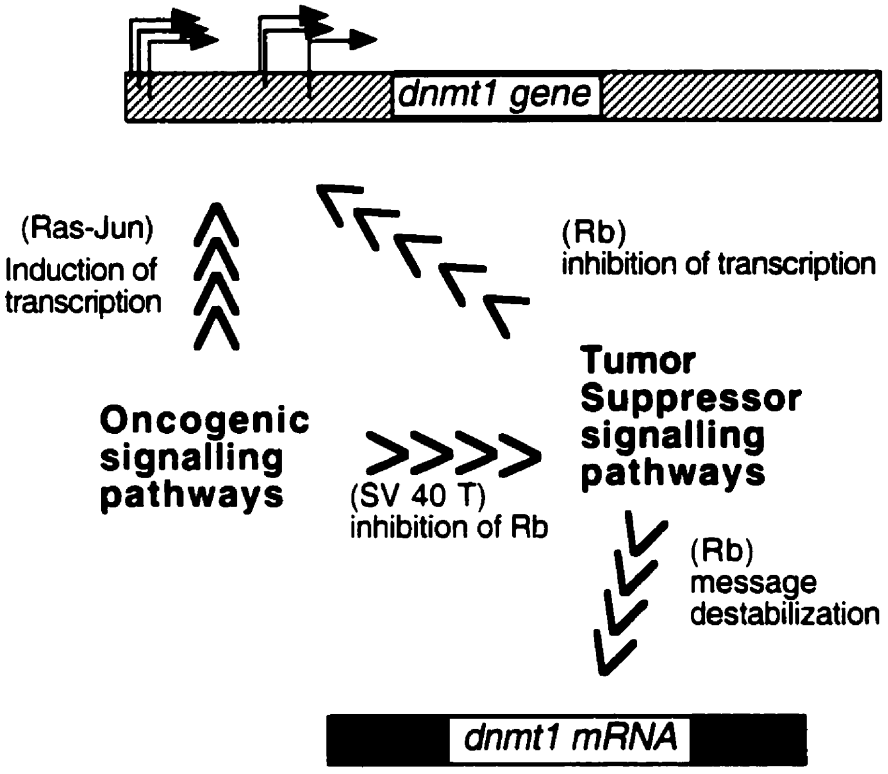
If the level of DNA MeTase activity can determine DNA methylation patterns, then DNA MeTase gene expression should be tightly regulated. There should be signals for altering the level of DNA MeTase activity when a programmed change in DNA methylation occurs. Many studies have established that DNA MeTase activity is regulated with the cell cycle, and that it is mainly expressed in growing cells and induced at the G_1-S boundary (Szyf *et al.*, 1985; Singer-Sam *et al.*, 1990b; Szyf *et al.*, 1991; el-Deiry *et al.*, 1991). The nuclear localization of the enzyme is restricted to S-phase of the cell cycle (Leonhardt *et al.*, 1992). During development the levels of DNA MeTase are also tightly regulated (Monk *et al.*, 1991; Carlson *et al.*, 1992), as exemplified by the regulated levels of DNA MeTase activity in spermatogenesis (Trasler *et al.*, 1992; Benoit and Trasler, 1994).

DNA MeTase gene expression is regulated at a number of different levels in order to be able to respond to different types of signals, such as maintaining limiting levels of expression under normal conditions, and responding to needs for

increased DNA methylation under other circumstances (Fig. 1-4). Preliminary analysis of the internal promoter region of the mouse *dnmt1* has revealed much information on the regulatory events controlling *dnmt1* expression and the potential cross talk between DNA methylation and other signalling pathways (Rouleau *et al.*, 1992, 1995). This regulatory region contains no TATA, CG rich Sp-1 elements or any previously identified initiator sequences. It also contains a GT-repeat sequence that is a potential repressor sequence and a cluster of AP-1 binding sites (Rouleau, *et al.*, 1992). These AP-1 sequences were demonstrated to be responsible for induction of the promoter activity (Rouleau *et al.*, 1995). The Ras oncogenic signalling pathway induces the formation of the AP-1 transactivation complex which is composed of Jun homodimers or Fos:Jun heterodimers (Angel and Karin, 1991; Binetruy *et al.*, 1991; Lowy and Willumsen, 1993). This was the first demonstration that DNA MeTase can be regulated by major oncogenic signalling cascades which provided a rationale for the upregulation of DNA MeTase seen in transformed cells. The Y1 adrenocortical cell line contains a 40 fold amplification of the *ras* gene and as a result is a tumorigenic line (Schwab *et al.*, 1983). Using this cell line as a model, it has been demonstrated that intervention at several different levels of the Ras signalling cascade will cause an inhibition of this cell line's tumorigenic potential, and that *dnmt1* expression is also inhibited, along with concomitant DNA hypomethylation of the genome (MacLeod *et al.*, 1995). Furthermore, inhibition of *dnmt1* by an antisense strategy produced an inhibition of the transformed state of the Y1 cell line (MacLeod and Szyf, 1995). The cell cycle regulation of the DNA MeTase (*dnmt1*) is controlled mainly at the post-transcriptional level (Szyf *et al.*, 1991). This mode of regulation is dependent upon the Rb tumor suppressor pathway and is inhibited in SV40 large T-antigen mediated cellular transformation (Slack *et al.*, 1999). The possibility that DNA MeTase activity, and therefore DNA methylation patterns, can be influenced by extracellular and intracellular signals raises the possibility that methylation patterns have an element of plasticity even in mature somatic tissue. This notion is the basis for revisiting the idea that DNA methylation patterns are static guardians of

Figure 1-4: Regulation of DNA methyltransferase gene expression.

A picture where multiple signalling pathways can interact at multiple levels of the regulation of gene expression. Both oncogenic and tumor suppressor signalling pathways can effect the expression of the *dnmt1* gene at different points of regulation. At the transcriptional level the Ras-Jun signalling pathway can induce transcription from the *dnmt1* gene. The tumor suppressor protein Rb can inhibit transcription from the *dnmt1* gene (see chapter 2). Cellular transformation through expression of the SV 40 large T antigen induces the relative stability of *dnmt1* mRNA posttranscriptionally, in an Rb dependent manner, suggesting that Rb, either directly or indirectly, can signal the decay of *dnmt1* mRNA.


dnmt1 gene
(Ras-Jun)
Induction of
transcription
(Rb)
inhibition of transcription
Oncogenic
signalling
pathways
(SV 40 T)
inhibition of Rb
Tumor
Suppressor
signalling
pathways
(Rb)
message
destabilization
dnmt1 mRNA

epigenetic information, but they may be dynamic regulators of the genome and are consequently influenced by both extra and intracellular signals.

10. DNA methylation patterns and cancer

Much evidence which has been accumulated over the last 15 years has established that the initiation and progression of cancer involves a change in the expression of the genetic program. This process involves the aberrant activation of growth promoting genes (oncogenes) (Weinberg, 1985), and the inactivation of genes that inhibit aberrant growth, know as tumor suppressor genes (Hinds and Weinberg, 1994). Many different proteins can become oncoproteins (protoncogenes) when they are aberrantly activated, such as receptors (Schlessinger and Ullrich, 1992), intracellular kinases (Schlessinger and Ullrich, 1992) and signalling molecules (G-proteins) (Lowy and Willumsen, 1993), as well as transcription factors (Fos, Myc, Jun) (Herschman, 1991), and translation factors (eIF-4E) (Lazaris-Karatzas*et al.*, 1990). The loss of function of tumor suppressor genes was demonstrated to be an essential component in the progression towards cancer. Examples of these genes are retinoblastoma protein (Rb) (Levine, 1993; Riley *et al.*,1994), Wilm's tumor protein (WT) (Hastie, 1994), Neurofibromatosis-1 protein (Cawthon *et al.*, 1990), p16 and p21 (Elledge and Harper, 1994). Evidence suggests that most tumor suppressors function in controlling the progression of the cell cycle. If DNA methylation encodes epigenetic control of gene expression, then it may be likely that this process is involved in the process of carcinogenesis. Many of the changes that result in the activation of oncogenes or inactivation of tumor suppressor genes involve stable alterations of the DNA that codes for them, such as mutations and rearrangements, but it seems reasonable that epigenetic alterations, such as DNA methyiation, may initiate changes in gene expression that lead to cancer also.

10.1 Hypermethylation

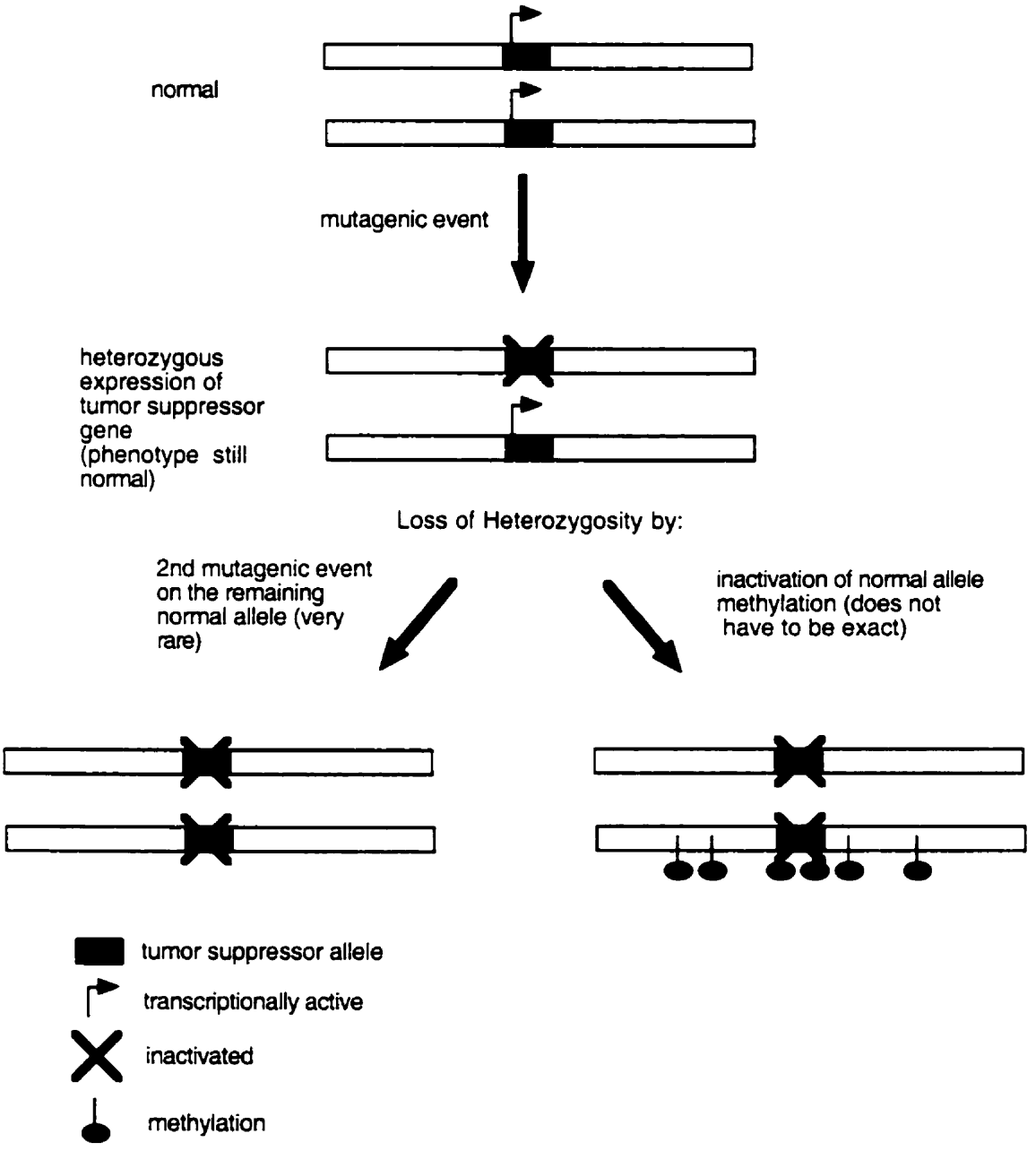
The realization that inhibition of gene expression could be a mechanism that can cause cancer surfaced with acceptance of the fact that tumor suppressors are an

important component of oncogenesis. Since inactivation of tumor suppressor activity requires the genetic alteration of both alleles, which is a highly improbable event by conventional mutagenesis, an alternative possibility is that loss of heterozygocity of the remaining wildtype allele by hypermethylation results in its inactivation (Scrable *et al.*, 1989) (Fig. 1-5). The loss of tumor suppressor activity with no apparent change in the primary DNA structure in several tumors has been demonstrated and shown to be associated with the hypermethylation of the tumor suppressor gene, examples of which are Rb (Ohtani-Fujita *et al.*, 1993), WTs (Royer-Pokora and Schnieder, 1992), p16 (Merlo *et al.*, 1995), p15 (Herman *et al.*, 1996), VHL (Herman *et al.*, 1994), and E-cadherin (Yoshira *et al.*, 1995).

The hypermethylation of DNA sequences seen in cancer can be subdivided by the identity of the DNA sequence into three categories; 1) tumor suppressor genes, 2) CpG island sequences, and 3) tissue specific genes. Regional, site specific hypermethylation has been demonstrated to occur in cancer in several different tumor types (deBustros *et al.*, 1988) and has led to the suggestion that this process is associated with the progression of cancer (Baylin *et al.*, 1991). For example, hypermethylation of the calcitonin gene marks the progression of chronic myelogenous leukemia (Nelkin *et al.*, 1991). Distinct regional hypermethylation occurs on chromosome 17p in colon and lung cancer DNA (Makos *et al.*, 1992). In both of the previous tumor types hypermethylation of similar regions results in the loss of heterozygocity, which suggests that residing within this region of the chromosome are genes whoseinactivation is critical for the progression of cancer (Makos *et al.*, 1992). *HIC-1* (Hypermethylated In Cancer) is a putative tumor suppressor gene and was cloned by the analysis of the hypermethylated region on chromosome 17p13.3. *HIC-1* is expressed ubiquitously in normal tissues, but is under expressed in many different tumor cell types, where it is also found to be hypermethylated (Makos-Wales *et al.*, 1995). Therefore it appears that hypermethylation in cancer cells demonstrates an element of specificity by being targeted to specific chromosomal loci and specific DNA sequences.

Figure 1-5: Tumor suppressor gene inactivation: Mutagenesis vs Methylation.

A diploid genome from 1 cell is depicted as two identical horizontal, unfilled, bars. In a normal, or wild type cell, there is expression of the tumor suppressor gene from both alleles. After a mutagenic event has inactivated one of the two alleles of the tumor suppressor gene there is still proper expression from the unaffected allele resulting in a heterozygous genotype but the phenotype still remains wildtype. In activation of the second allele, or loss of heterozygocity can occur through another mutagenic event, which is an extremely rare event, or by methylation of the allele, which does not have to be an exact site specific event.


normal
mutagenic event
heterozygous expression of tumor suppressor gene (phenotype still normal)
Loss of Heterozygosity by:
2nd mutagenic event on the remaining normal allele (very rare)
inactivation of normal allele methylation (does not have to be exact)
tumor suppressor allele
transcriptionally active
inactivated
methylation

CG island containing sequences, which are normally found at the 5' end of housekeeping genes and are hypomethylated in normal somatic tissue (Bird *et al.*, 1985), are one class of sequences that appear to be targeted for hypermethylation in cancer cells (Antequera *et al.*, 1990). Hypermethylation of the *myoD* CG island has been shown to occur in transformed cell lines (Jones *et al.*, 1990a,b), and proceeds in a progressive fashion during the process of cellular transformation (Rideout *et al.*, 1994). CG island sequences on chromosomes 11p and 3p have also been shown to be hypermethylated in human lung tumors (Baylin *et al.*, 1986; de Bustro *et al.*, 1988). Oncogene induced transformation of human bronchial epithelial cells has been shown to be associated with hypermethylation of CG island sequences (Vertino *et al.*, 1993). Hypermethylation of the CG islands located proximal to the estrogen receptor gene is associated with the resistance of breast cancer tumors to hormone therapy (Ottaviano *et al.*, 1994).

Tissue specific genes also undergo hypermethylation and inactivation in cancerous cells. This loss of expression of tissue specific genes is thought to produce the "dedifferentiated" nature of transformed cells in comparison to their non transformed parental cells. An example of this is the adrenal-specific 21-hydroxylase gene (C21). C21 is expressed in the adrenal cortex and the gene is found in a relatively hypomethylated state, however, C21 becomes hypermethylated and inactivated in the adrenocortical carcinoma cell line Y1 (Szyf *et al.*, 1989, 1990a) An exogenous C21 gene, when introduced into the Y1 cell line, becomes *de novo* methylated, which suggests that the cancer cell maintains the ability to specifically recognize the C21 gene and target it for *de novo* methylation (Szyf *et al.*, 1989).

A candidate mechanism for the hypermethylation of DNA in cancer cells is the loss of regulation and therefore hyperactivation of the DNA MeTase enzyme, which is observed in many cancer cells (Kautiainen and Jones, 1986; el Deiry *et al.*, 1991). Therefore it is essential to understand the mechanisms involved in the regulation of the DNA MeTase expression and how they are deregulated in the

transformed state, in order to develop specific cellular targets for the design of anticancer therapeutics. The first half of this thesis is dedicated to this issue.

10.2 Hypomethylation

A series of studies have shown that the genomes of tested cancer cells are widely hypomethylated when either the general level of methylated cytosines is assayed (Gamma-Sosa *et al.*, 1983a,b; Feinburg *et al.*, 1988), or when specific genes are analysed using methylation sensitive restriction endonucleases and Southern blotting (Feinburg and Vogelstein, 1983; Goelz *et al.*, 1985; Wahlfors *et al.*, 1993). The design of the first studies dissecting the correlation between cancer and DNA methylation was influenced by the consensus that activation of proto-oncogenes leads to cancer. However, DNA hypomethylation is not restricted to oncogenes, and genes that are not involved in cancer progression, such as β-globin, are also hypomethylated (Ribieras, *et al.*, 1994). Therefore, with respect to cancer, the state of DNA methylation is not a direct reflection of the state of activity of oncogenes, nor is demethylation a site specific mechanism for activation of oncogenes. The possibility still exists that the general DNA hypomethylation observed in cancer cells plays a causal role in oncogenesis, by also hypomethylating and activating oncogenes repressed by methylation, as part of a process that is not site specific, however as of yet, there is no clear example of a methylation repressed proto-oncogene that is activated by hypomethylation. It may be the case that this general hypomethylation is induced as a compensentory mechanism in response to the hyperactivation of DNA MeTase.

The DNA methylation pattern is established by sequential demethylation at the preimplantation stage and waves of *de novo* methylation and site specific demethylation events (Monk *et al.*, 1987; Brandeis *et al.*, 1993a; Kafri *et al.*, 1993; Chapman *et al.*, 1984). Originally, it was suggested that demethylation could be accomplished by a passive process which relies on the inhibition of DNA methylation during DNA replication (Razin and Riggs, 1980). However, a number

of observations have demonstrated that an active process of demethylation, which does not involve DNA replication, occurs in mammalian cells, such as demethylation of the *vitellogenin II* promoter in response to estrogen in hormone responsive tissue from chicken (Wilks *et al.*, 1984), and the demethylation of the Epstein-Barr virus genome upon induction of a lytic cycle in producer lines, before the initiation of DNA replication (Szyf *et al.*, 1985). Also, it has been shown that an exogenous α-actin gene is actively demethylated at specific sites in C2C12 cells (Paroush *et al.*, 1990) and that CG islands are specifically and actively demethylated in mouse embryonal cell lines (Frank *et al.*, 1991). It appears that DNA demethylation can be directed to specific DNA sequences (Paroush *et al.*, 1990; Lichtenstein *et al.*, 1994), similar to the process of *de novo* methylation (Szyf *et al.*, 1989, 1990b). *In vitro* methylated sequences introduced into mouse zygotes during development undergo active demethylation at the preimplantation stage (Kafri *et al.*, 1992,1993). In addition to site specific demethylation, there is evidence that genome wide demethylation occurs during cellular differentiation (Razin *et al.*, 1984, 1985; Szyf *et al.*, 1985; Razin *et al.*, 1986). Since DNA demethylation can be genome wide the possibility exists that, in a fashion similar to DNA methylation, DNA demethylation may be controlled by the availability of a general DNA demethylase activity (Szyf, 1994; Szyf *et al.*, 1995).

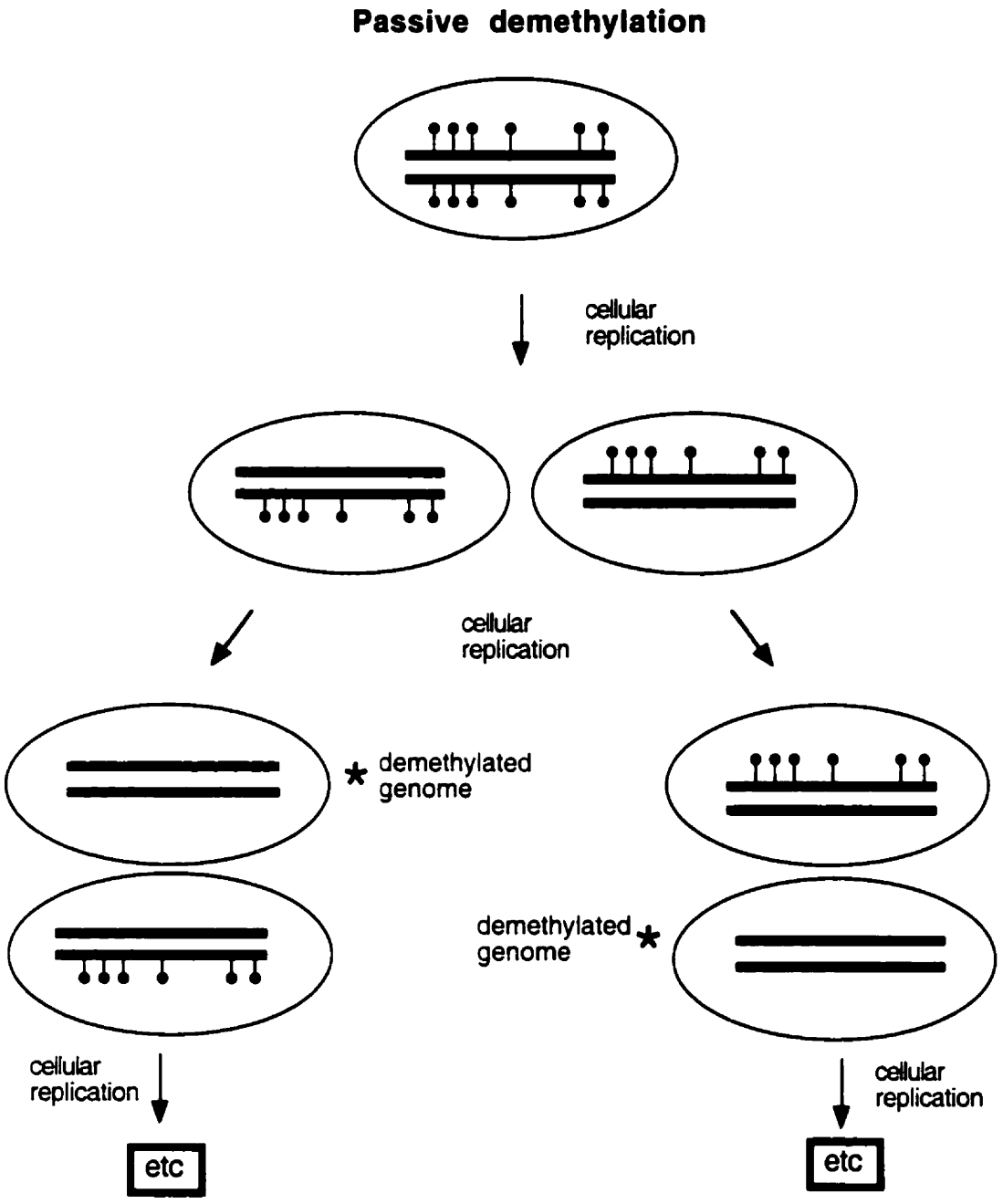
Until recently there has been no identification of an activity that truly removes methyl groups from DNA. The second half of this thesis describes the identification of this activity (Ramchandani *et al.*, 1999) and the cloning of a cDNA which encodes true demethylating activity (Bhattacharya *et al.*, 1999). There has been a consensus in the field that true DNA demethylation is energetically unlikely, (although this notion is not documented) so alternative mechanisms and activities were identified and reasoned as the activities responsible for DNA demethylation (Fig. 1-6a,b).

In 1982, a DNA demethylating activity was crudely described in murine erythroleukemia cell nuclei (Gjerset and Martin, 1982), but the work was apparently not followed up. Later, it was suggested that the DNA demethylation seen in

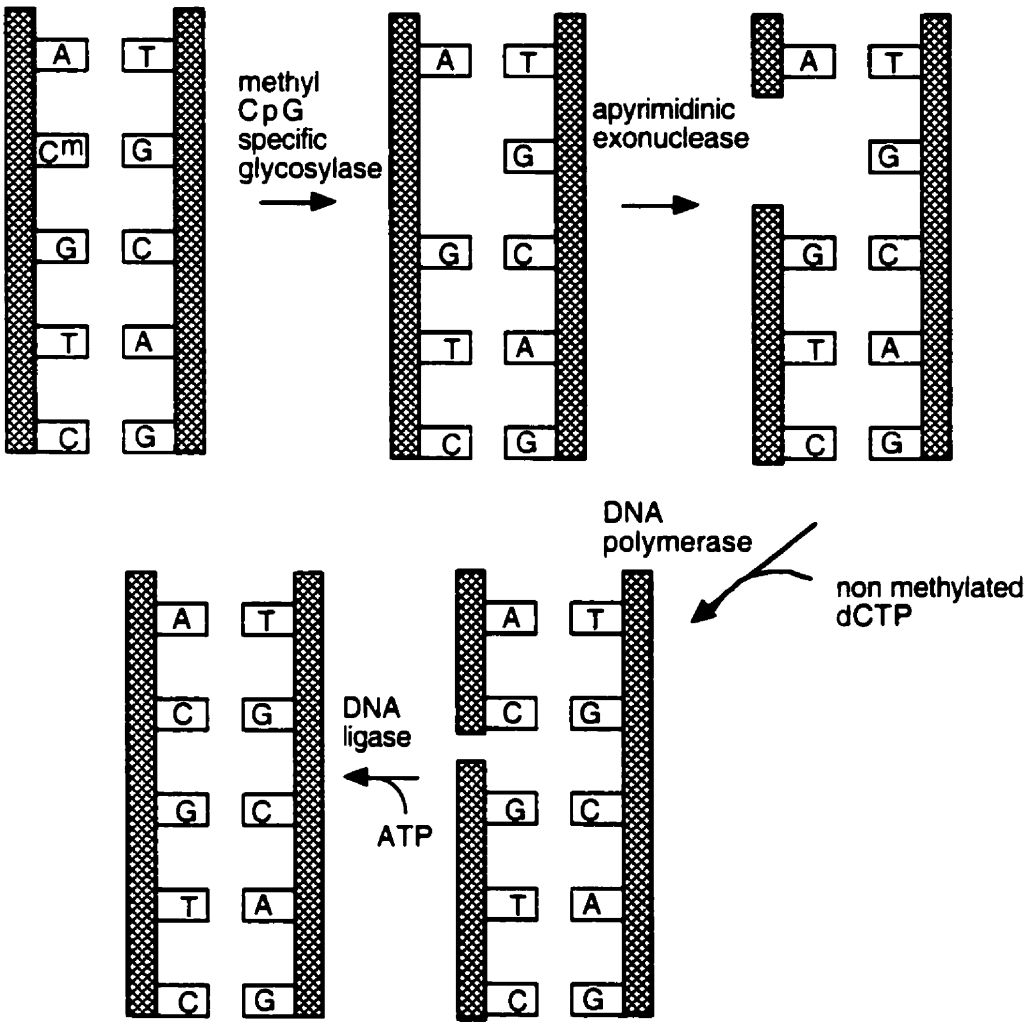
Figure 1-6: Proposed mechanisms of DNA demethylation.

a. Passive demethylation. A nucleus from one cell is shown containing a double stranded methylated genome. After 1 cell division in the absence of DNA methyltransferase activity 2 offspring cells are generated that are now only methylated on a single strand. When these 2 cells replicate to become 4, the cells derived from the parent containing DNA replicated from the nonmethylated strand are now completely demethylated (delineated by *). When these cells go through further rounds of replication they will continue producing demethylated progeny. Thick horizontal lines represent one strand of DNA. Filled "lollipop" structures represent DNA methylation.

b. Excision and Replacement. Double stranded anti parallel DNA, properly base paired, containing 1 methylated cytosine (C^m) in a CpG dinucleotide is shown. A methylCpG specific glycosylase recognizes the methylated CpG dinucleotide and excises the methylated cytosine base, resulting in an apyrimidic site. The apyrimidinic site is a signal for apyrimidinic exonuclease activity to create a single stranded break at the apyrimidinic site. DNA polymerase then fills in the missing nucleotide with a non methylated cytosine (C). The single stranded break is then filled in by a DNA ligase activity. Vertical crosshatched bars represent the phosphodiester backbone of DNA. The rectangles extending inwards from the phosphodiester back bone represent bases and are labelled A (adenosine), T (thymidine), G (Guanosine), C (cytosine) or C^m (5-methyl cytosine).


Passive demethylation
cellular replication
cellular replication
demethylated genome
demethylated genome
cellular replication
cellular replication
etc
etc

Excision and Replacement



differentiating Friend erythroleukemia cells occurs by the removal of the methylated cytosine base and its replacement with a nonmethylated cytosine base, termed as "excision and replacement" (Razin *et al.*, 1986). An enzymatic glycosylase activity that can remove 5-methyl-cytosine bases from DNA was identified in nuclear extracts from Hela cells (Vairapandi and Duker, 1993), but it was not clear whether this activity was specific to methylated cytosines rather than a general pyrimidine glycosylase. Another proposed mechanism for DNA demethylation is a 5-methyl-cytosine directed endonuclease activity found in chicken embryos (Jost, 1993) and mouse 10T1/2 cells (Jost and Jost, 1994) which can initiate the the excision and replacement of methylated cytosines in hemimethylated DNA in the presence of deoxynucleotide triphosphates and DNA polymerase. Purification of this activity and identification of its components revealed that it contains a combination of 5-methylcytosine glycosylase and apyrimidine-endonuclease (Fig. 1-6b). This activity was still not a complete explanation to the demethylation events described because of its preference for hemi-methylated substrates, which is suggested to be a "mismatch repair" activity, required for removal of methyl groups that are aberrantly introduced into the nascent stand of DNA during DNA replication (Jost *et al.*, 1995), which suggests that it is a "maintenance" activity and not a "*de novo*" activity.

Since extensive demethylation occurs in early development (Razin *et al.*, 1984; Monk *et al.*, 1987; Kafri *et al.*, 1993) a possible source for a demethylation activity could be an embryonal cell line. Demethylation of an exogenously methylated plasmid occurs in P19 cells, which is independent of DNA replication and is induced 10-100 fold when the activated Ras oncogene is expressed (Szyf *et al.*, 1995). These observations provide the initial basis for a search for a true or *bona fide* DNA demethylase activity which removes methyl groups from methylated DNA. First, global demethylation by base or nucleotide excision is potentially dangerous for the cell. The human diploid genome contains approximately 10^8 CG dinucleotides, and it has been estimated that 60 to 80% percent of those are methylated. This means that during early embryonic

development, 120 million 5-methylcytosines bases are removed in one cell resulting in the same number of apyrimidinic sites. Removal of such a large number of these bases and subsequent cleavage of the apyrimidinic sites created by that process would cause large amounts of single-strand breaks which might overwhelm downstream repair enzymes and be a possible source for DNA damage (Chu and Mayne, 1996), and therefore be very unlikely to happen at the preimplantation stage. An enzyme that just removes the methyl group while maintaining the integrity of the DNA strand would be much more advantageous and less prone to mistakes. Where could one look for a DNA demethylase enzyme? Combining the observations that oncogenic signals can activate DNA demethylase activity and the wide hypomethylation observed in tumor cells, leads to the hypothesis that transformed cells may be a good source for a *bona fide* DNA demethylase activity.

Rationale

Clearly, a large body of evidence has established that aberrations of DNA methylation patterns occur in tumor cells. The seemingly contradictory observations of the hyperactivation of DNA MeTase activity concurrently with a genome wide hypomethylation in tumor tissues implies that one has to establish the identity of the machinery responsible for these aberrations before these observations can be understood. Much data has accumulated demonstrating that the expression of *dnmt1* is up regulated in cancer cells, although the level of this induction is still controversial, and that limiting its expression may have therapeutic potential. In chapter 1 this thesis delineates the complete genomic structure of the human *dnmt1* gene as a starting point to identify the sequences responsible for its transcriptional regulation, and as a template for future structure function studies of the enzyme. Chapter 2 determines the regions of the gene that impart transcriptional control over human *dnmt1* expression, and reveals the potential oncogenic and tumor suppressor cascades that influence its expression. Chapter 3 describes a posttranscriptional mechanism for the cell cycle control of *dnmt1* expression that is

directed to the 3' untranslated region of the *dnmt1* mRNA. Chapter 4 tests the potential of the inhibition of *dnmt1* expression *in vivo* as a legitimate pharmacological target for anti-cancer therapeutic targeting, by the use of an antisense oligonucleotide directed against mouse *dnmt1*. Chapter 5 describes the first demonstration of an enzymatic activity that can remove methyl groups from methylated DNA and the cloning of a cDNA that encodes a DNA demethylation activity (Bhattacharya *et al.*, 1999). Chapter 6 characterizes the demethylation reaction with respect to substrate specificity and the products of the reaction (Ramchandani *et al.*, 1999). Taken together this work identifies major components of the machinery responsible for DNA methylation, sheds new light how the process is regulated and misregulated in cancer, provides the basis for the initiation of clinical trials to begin with antisense deoxyoligonucleotides directed against *dnmt1* , and identifies a new and unprecedented enzyme which has the potential to be a target for the development of anticancer compounds.

Chapter 1

Genomic Structure of the human DNA methyltransferase gene.

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We determined the genomic structure of the gene encoding human DNA methyltransferase (DNA MeTase). Six overlapping human genomic DNA clones which include all of the known cDNA sequence were isolated. Analysis of these clones demonstrates that the human DNA MeTase gene consists of at least 40 exons and 39 introns spanning a distance of 60 kilobases. Elucidation of the chromosomal organization of the human DNA MeTase gene provides the template for future structure function analysis of the properties of mammalian DNA MeTase.

Key words: cloning/DNA methylation/exon-intron boundaries.

In mammalian cells the transfer of a methyl group from S-adenosyl-methionine (AdoMet) to the 5 position on cytosine residues located in a CG dinucleotide context is catalysed by the DNA methyltransferase enzyme (DNA MeTase). Methylation of DNA provides an important mechanism of epigenetic control over genome function (Razin and Riggs, 1980; Razin and Cedar, 1991). The genome contained within any mammalian cell is 60 to 80 percent methylated at its CG dinucleotides but the distribution of the unmethylated CGs is distinct from cell to cell (Yisraeli and Szyf, 1984) and correlates with tissue specific gene expression (Monk, 1995). Allele specific gene repression marked by DNA methylation is observed in X inactivation (Beard et al.,1995; Carrel et al., 1996a; Carrel et al., 1996b; Luo et al., 1993) and parental imprinting (Lalande,1996; Efstratiadis, 1994). During development, patterns of methylation are set by the processes of *de novo* methylation and demethylation (Shemer et al., 1991; Brandeis et al., 1993) and are maintained in somatic cells (Paroush et al., 1990; Szyf et al., 1984). The results of inhibition of DNA MeTase, by 5 Aza-deoxycytosine (Laird et al., 1995), antisense (MacLeod and Szyf, 1995), or targeted disruption of the DNA MeTase gene by homologous recombination (Li et al., 1992), indicate that methylation patterns are critical for embryogenesis and maintenance of the identity of cells.

It has been suggested that one possible mechanism for controlling DNA methylation patterns is by regulating DNA MeTase levels (Szyf et al 1984; Szyf 1991). Several observations have shown that aberrant methylation patterns arise in cancer cells (Feinberg and Vogelstein, 1983). Both global hypomethylation (Feinberg and Vogelstein, 1983) as well as hypermethylation of specific CG sites in the DNA of cancer cells relative to normal cells has been documented (de Bustros et al., 1988; Nelkin et al., 1991). One possible explanation for hypermethylation of DNA is that aberrant regulation of DNA MeTase in cancer cells leads to an increase in DNA MeTase activity which in turn results in some hypermethylation (Szyf,1994). In accordance with this hypothesis, several reports have shown increased DNA MeTase activity (Kautiainen and Jones, 1986) and

mRNA (el-Deiry et al., 1991) in cancer cells. However, a recent report suggests that the DNA MeTase activity in cancer cells in vivo is not significantly different than the activity found in normal dividing cells (Lee et al., 1996). Recent evidence suggests that DNA MeTase is regulated by the Ras-Jun nodal cellular signalling pathways that is known to be involved in oncogenesis (Szyf,1994; Rouleau et al., 1992; Rouleau et al.,1995; MacLeod et al., 1995; Yang et al., 1997). Thus there is a possible molecular link between regulation of DNA MeTase and oncogenic pathways.

Several lines of evidence are consistent with the hypothesis that activation of DNA MeTase might play a causal role in cellular transformation. First, ectopic expression of DNA MeTase in NIH 3T3 cells leads to hypermethylation and cellular transformation (Wu et al., 1993). Second, expression of a 5' segment of the cDNA from mouse DNA MeTase in the antisense orientation reverses tumorigenesis of Y1 adrenocortical carcinoma cells (MacLeod and Szyf, 1995). Third, Laird et al., have shown that the DNA MeTase inhibitor 5-azadeoxycytidine suppresses intestinal neoplasia in *Min* mice bearing the mouse homologue of the human mutant APC gene (Laird et al., 1995). Fourth, Inhibition of DNA MeTase expression by *in vivo* administration of an antisense oligodeoxyribonucleotide against DNA MeTase suppresses the growth of Y1 tumors in syngeneic mice (Ramchandani et al., 1997). These results have also led to the proposal that DNA MeTase is a potential therapeutic target in anticancer therapy (Szyf, 1996)

Information regarding the chromosomal organization of the human DNA MeTase gene is critical for a comprehensive analysis of the mechanisms that underlie the regulation of DNA MeTase expression in oncogenic progression and developmental processes. For example, analysis of potential alternative splicing products requires a knowledge of the exon-intron structure. Many regulatory functions such as enhancers (Jenuwein et al., 1997) and promoters (Lamas et al., 1996) reside in intronic genomic regions. A complete genomic structure will also

allow for maximizing the number of possible antisense oligodeoxyribonucleotides available for potential anticancer therapeutic agents.

In order to obtain overlapping DNA fragments spanning the entire human DNA MeTase gene several cDNA fragments spanning the known human DNA MeTase cDNA (Yen et al., 1992; Yoder et al., 1996) were generated via RT-PCR (Fig. 1A) and used as probes to screen human genomic DNA libraries from fetal placenta and lung tissue. Six phage containing unique and overlapping DNA fragments were isolated, subcloned and exon sequenced to identify exon-intron boundaries of the known human DNA MeTase cDNA (Fig. 1B, Figure 2 for intron-exon boundaries). Intron sizes were calculated by DNA sequencing (for introns less than 150 bp) and/or by amplifying the introns by PCR using sequence from 5' and 3' flanking exons as primers (for introns up to 2 kb). For larger introns (>2kb), the distance between the exons was estimated by restriction enzyme analysis of phage insert and mapping the exons to the different restriction fragments using exon specific oligonucleotide probes (see physical map in figure 1A). The physical map obtained by restriction enzyme analysis of phage DNA (Fig. 1A) was verified by a restriction enzyme-Southern blot analysis of human genomic DNA prepared from human lung carcinoma cell line A549 and bladder carcinoma T 24 (Fig. 1C) and hybridization to cDNA probes encoding the different domains of the human DNA MeTase (see Fig. 1A for physical map). The fragments predicted by the physical map of the different phages (Fig. 1A) were visualized with the cDNA probes in the Southern blots of genomic DNA (Fig. 1C, arrows indicate the restriction enzyme fragments, and their sizes, visualized with each cDNA probe).

The results of the cloning, sequencing and mapping experiments demonstrate that the 5.2 kilobase cDNA for the human DNA MeTase, as described by Yen et al.,1992, and recently updated by Yoder et al., 1996, is organised as 40 exons and 39 introns, with completely conserved splice acceptor

and donor sites (Figure 2), on 60 kilobases of chromosome 19p13.2-13.3 (Fig. 1B and Yen et al.,1992). This gene can therefore be classified as a "large gene" similar to Rb (70 kb) (Friend et al., 1987) and apolipoprotein B (79.5 kb) (Linton et al., 1993).

Several correlations regarding structure function and exon organization have been previously proposed (Gilbert, 1978; Gilbert, 1985). In accordance with the exon theory of genes (Gilbert, 1978; Gilbert, 1985), the functional domains of the DNA MeTase appear to be grouped together as a number of small exons and introns separated from neighbouring domains by large introns (Fig.1B). First, exons 6-8 code for the nuclear localization signal and exist within an isolated cluster that contains exons 2-8 and flanked by the large introns 1 and 8 (12 and 11 kilobases respectively). Second, the region described to be critical for targeting of the enzyme to replication foci (FTR) (Leonhardt et al., 1992) is coded for by exons 13-20. These exons are organized into two distinct chromosomal regions, exons 13-16 make up the first region, and exons 17-20 make up the second, and are separated by the large intron 16 (6000 bases). It is tempting to speculate that each region consists of an individual structural motif such that when combined together can target the enzyme to replication foci. Third, the region responsible for zinc binding is coded for by exon 22, and in its genomic organization, along with exon 23, is isolated by the large flanking introns 21 and 23. Fourth, the catalytic domain of the enzyme is coded for by exons 30-39. The catalytic domain of all of the known CpG methyltransferases share 10 conserved motifs of which 1,4,6,8,9, and 10 appear to be essential for catalytic activity (Kumar et al.,1994). Conserved motif 1 is entirely contained within exon 31 and codes for the AdoMet binding peptide. Conserved motif 4 is entirely contained within exon 32 and contains the Pro-Cys motif that catalyzes methyl transfer. Fifth, two postulated translation initiation codons exist (Fig.1B) and the genomic organization of the exons in which they reside suggests that they form distinctly different structural motifs. Based on the exon theory of genes the genomic structure of the human DNA MeTase gene

is consistent with the hypothesis that the enzyme is made up of numerous functional domains that have come together to form the mammalian DNA MeTase enzyme.

To date most antisense compounds have been targeted to mRNA sequence (Akhtar et al., 1997). However, a study describing the correcting of aberrant splicing in the human beta-globin gene by an antisense oligonucleotide has demonstrated that antisense oligonucleotides can gain access to the nucleus and influence splicing (Sierakowska et al., 1996). Targeting intron-exon boundaries is ideal because they only exist in nuclear pre-mRNA (Smith etal., 1995). The attack occurs on the RNA species before it can be translated and may actually prevent its transport to the cytoplasm. The antisense oligonucleotide would hybridise to the target intron-exon boundary by Watson and Crick hybridisation and effectively mask the splice junction. There is confidence that this approach can be successfully exploited for DNA MeTase because the gene offers 78 unique intron-exon junctions (Fig. 2) for antisense oligonucleotide development. Genomic structure information becomes a vital tool for molecular medicine since it has already been demonstrated that *in vivo* treatment with an antisense oligonucleotide against DNA MeTase is effective at inhibition of tumorigenesis (Ramchandani et al.,1997).

Elucidation of the chromosomal organization of the human DNA MeTase gene is consistent with the concept that the mammalian DNA MeTase is a complex protein consisting of several distinct functional domains. This information provides the template for future structure function analysis of the properties of the mammalian DNA MeTase enzyme.

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Canada University-Industry postdoctoral fellowship.

Figure 1: Cloning and Physical mapping of the human DNA MeTase gene

(A) Restriction map and phage clones of the human DNA MeTase gene. Human genomic DNA libraries from lung and placenta, in Lambda FIX II (Stratagene), were screened with several RT-PCR generated (mRNA source Hela and A549 cells) cDNA probes spanning the entire known human cDNA sequence (Yen et al., 1992 and Yoder et al., 1996). The cDNA probes used for screening are indicated by arrows under the lines representing the genomic fragments contained in the phages (the name of each phage is indicated above the line) identified by each of the probes. The numbers under the arrow indicate the 5' and 3' ends of the cDNA sequences included in each of the probes. The cDNA is numbered as in Yoder et al., 1996. Genomic inserts were isolated from the phages by NotI digestion and sub-cloned into NotI linearized pBluescript SK+. Sub-clones were digested with restriction endonucleases (X = Xba 1, B = Bam H1, H = Hind III) Southern blotted and hybridized to exon specific ^{32}P labelled oligodeoxyribonucleotides or cDNA probes to produce a scale restriction map of the human DNA MeTase gene.

(B) Exon-intron structure of the human DNA MeTase gene

Sub-clones from A were exon sequenced to determine exon-intron boundaries. Intron sizes were determined by either DNA sequencing (for introns less than 150 bp), PCR using 5' and 3' flanking exon sequences as primer sources (for introns less than 2Kb) and restriction enzyme-Southern blot analysis using the enzymes indicated in A (data not shown) using oligonucleotides for each specific exon to verify the restriction enzyme fragment that the exon is positioned in. The positions of exons determined by PCR analysis were verified by Southern blot analysis. The following primers were used to map by PCR the relevant intron boundaries and sizes: exons 4 to 5 : sense : 5'-aaacgggaaccaagcaagaa ; antisense : 5'-tgagatgtgatggtggttt ; exons 5 to 6 : sense : 5'-ctgaaccttcacctagcccc ; antisense : gatggactcatccgatttgg ; exons 6 to 7 : sense : 5'-ccctgccaaacggaaacctc ; antisense : 5'-gttctctggatgtaactcta ; exons 7 to 8 : sense : agacgtagagttacatccag ; antisense : 5'-

gctctttcaggttcttctgc ; exons 9 to 10 : sense : 5'-aagaaaagagactccgaagt ; antisense : tttctcgtctccatcttcgt ; exons 10 to 11 : sense : 5'-gtcagcccttaggagctgtt ; antisense : 5'-ggaaacagctatgaccatg (M13 reverse primer) ; exons 11 to 12 : sense : 5'-gatgagaagaagcacagaag ; antisense : 5'-tcatcctcgtctttttcatcagaa ; exons 12 to 13 : sense : 5'-ttctgatgaaaaagacgaggatga ; antisense : 5'-cattaccatctgctttggat ; exons 13 to 14 : sense : 5'-aggagaagagacgcaaaacg ; antisense : 5'-agttcatgactgttttggcg ; exons 17 to 18 : sense : 5'-gtactgtaagcacggtcacc ; antisense : 5'-aggtgctgaagccgatgagg ; exons 18 to 19 : sense : 5'-tggatcactggctttgatgg ; antisense : 5'-ctcgatcttgttgatcaggt ; exons 21 to 22 : sense : 5'-aggcgagcccaggcgaggcg ; antisense : 5'-cgctcttggcaagcctgcttg ; exons 22 to 23 : sense : 5'-gtgtcagcagcctgagtgtg ; antisense : 5'-ctccgacccaagagatgcga ; exons 23 to 24 : sense : gtcccaatatggccatgaag ; antisense : 5'-gctagatacagcggttttgagg ; exons 24 to 25 : sense : 5'-cgtcaagactgatgggaagaagagt ; antisense : 5'-ctccatggcccagttttcgg ; exons 25 to 26 : sense : 5'-gtcacggcgctgtgggagga ; antisense : 5'-ttgaacttgttgtcctctgt ; exons 26 to 27 : sense : 5'-gacctacttctaccagctgt ; antisense : 5'-ttgaacgtgaaggcctcagg ; exons 27 to 28 : sense : 5'-ctctactactcagccaccaa ; antisense : 5'-tagaacttgttgacccgga ; exons 28 to 29 : sense : 5'-tgagactgacatcaaaatcc ; antisense : 5'-cgaggaagtagaagcggtg ; exons 29 to 30 : sense : 5'-cgagtgcgtccaggtgtact ; antisense : 5'-cttccctttgtttccagggc ; exons 31 to 32 : sense : 5'-gaagggcaagcccaagtccc ; antisense : 5'-agccatgaccagcttcagca ; exons 32 to 33 : sense : 5'-tgctgaagctggtcatggct ; antisense : 5'-cctgcagcacgccgaaggtg ; exons 33 to 34 : sense : 5'-tccttcaagcgctccatggt ; antisense : 5'-tagtctgggccacgccgtac ; exons 34 to 35 : sense : 5'-ccggtcagtacggcgtggcc ; antisense : 5'-agatctccagtgccgaggct ; exons 35 to 36 : sense : 5'-tgagctcgggtcctttccgg ; antisense : 5'-tccacgcaggagcagacccc ; exons 36 to 37 : sense : 5'-tcagacggcaccatggccag ; antisense : 5'-cttgcccatgggctcggggt ; exons 37 to 38 : sense : 5'-ctctatggaaggctcgagtg ; antisense : 5'-cggtgcttgtccaggatgtt ; exons 38 to 39 : sense : 5'-ctgacacctaccggctcttc ; antisense : 5'-ggcactctctcgggctttgg ; exons 39 to 40 : sense : 5'-ggagatcaagctttgtatgt ; antisense : 5'-gtccttagcagcttcctcct. The following introns were determined by sequencing : exons 2 to 3 ; 3 to 4 ; 14 to

15 ; 15 to 16 ; 19 to 20 ; 20 to 21 ; 30 to 31.
The following introns were determined by restriction mapping : exons 1 to 2 (using the following oligonucleotides as probes : exon 1 : 5'-cgcctgcggacatcgtcgggcagc ; T3 : 5'-aattaaccctcactaaaggg ; T7 : 5'-gtaatacgactcactatagggc) ; 8 to 9 (using the following oligonucleotides as probes : exon 8 : 5'-gctctttcaggttcttctgc ; exon 9 : 5'-aagaaaagagactccgaagt) ; 16 to 17 (using the following oligonucleotides as probes : exon 16 : 5'-tgagccacagatgctgacaaa ; exon 17 : 5'-gtactgtaagcacggtcacc).

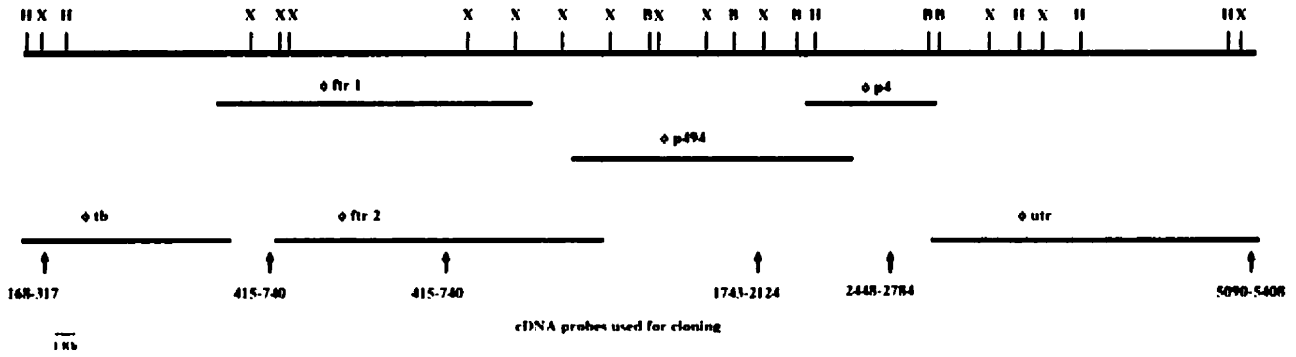
Exons are depicted as vertical bars and numbered above, introns as thick horizontal bars. Regions containing exons coding for specific function domains are depicted, NLS = nuclear localisation signal, FTR = replication foci targeting region, Zn = zinc binding domain, AdoMet Binding = S-adenosyl-methionine binding motif, Pro-Cys = proline-cysteine catalytic motif, Catalytic Domain = region conserved in all CpG methyltransferases. Exonal location of proposed initiation codons = ATG.

(C) Southern blot of human genomic DNA
Genomic DNA was prepared as described previously (Sambrook et al. 1989) from human lung carcinoma A549 cells (ATCC: CCL-185) and human bladder carcinoma cells: T24 (ATCC: HTB-4) and digested with restriction endonucleases Xba 1 (X), Bam H1 (B), or Hind III (H), electrophoresed on a 1.5% agarose gel and Southern blotted. The fragments encoding the different segments of the human DNA MeTase mRNA were visualized by hybridization to the following cDNA probes: 1. A probe bearing the first exon. 2. A probe bearing exons 3-5 (starting spanning nucleotides 415-740 of the known cDNA) 3. A probe bearing exons 7-20 4. A probe spanning exons 30-40. The cDNA probes are indicated under the map of the exon-intron structure, the dashed lines delineate the boundaries of exons spanned by each of the probes. The fragments visualized by each of the restriction enzymes are indicated by different shaded arrows. The size of the visualized fragments is indicated next to the arrows. The size of the

fragments visualized by each of the probes corresponds to the size predicted by the restriction enzyme analysis of the genomic phages.

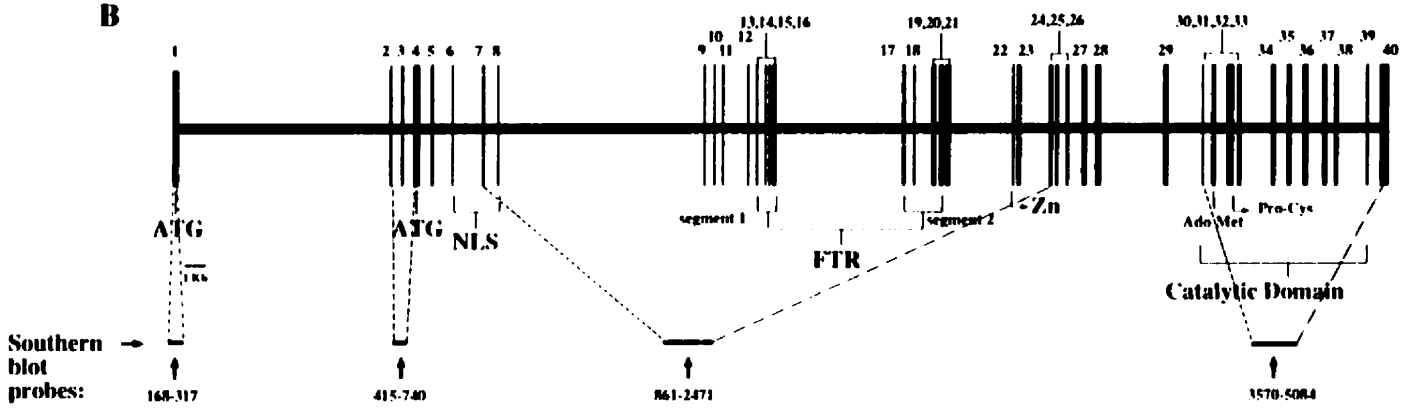
Physical map and cloned genomic inserts of the human DNA MeTase gene

A



Exon-intron structure of the human DNA MeTase gene

B



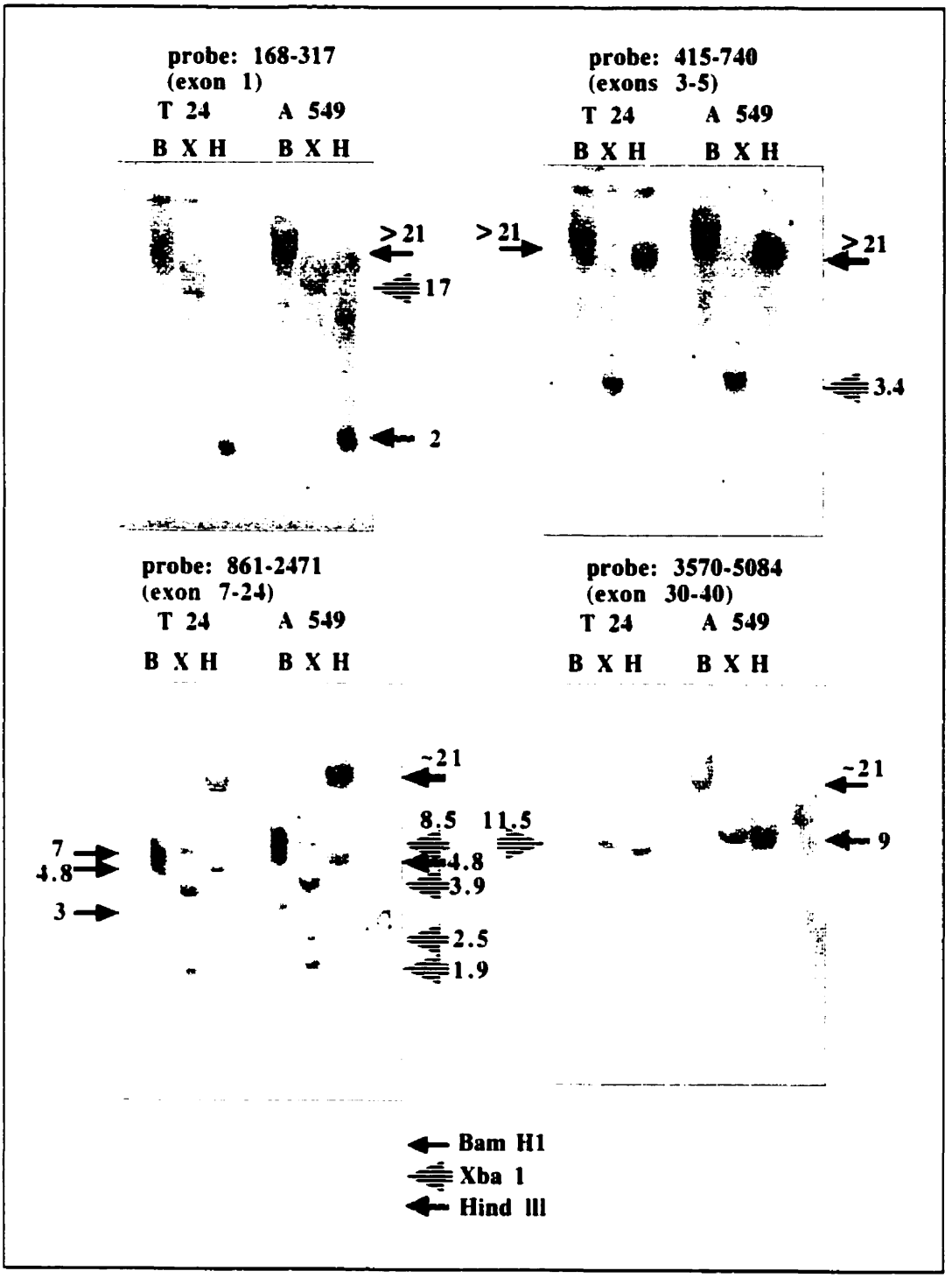
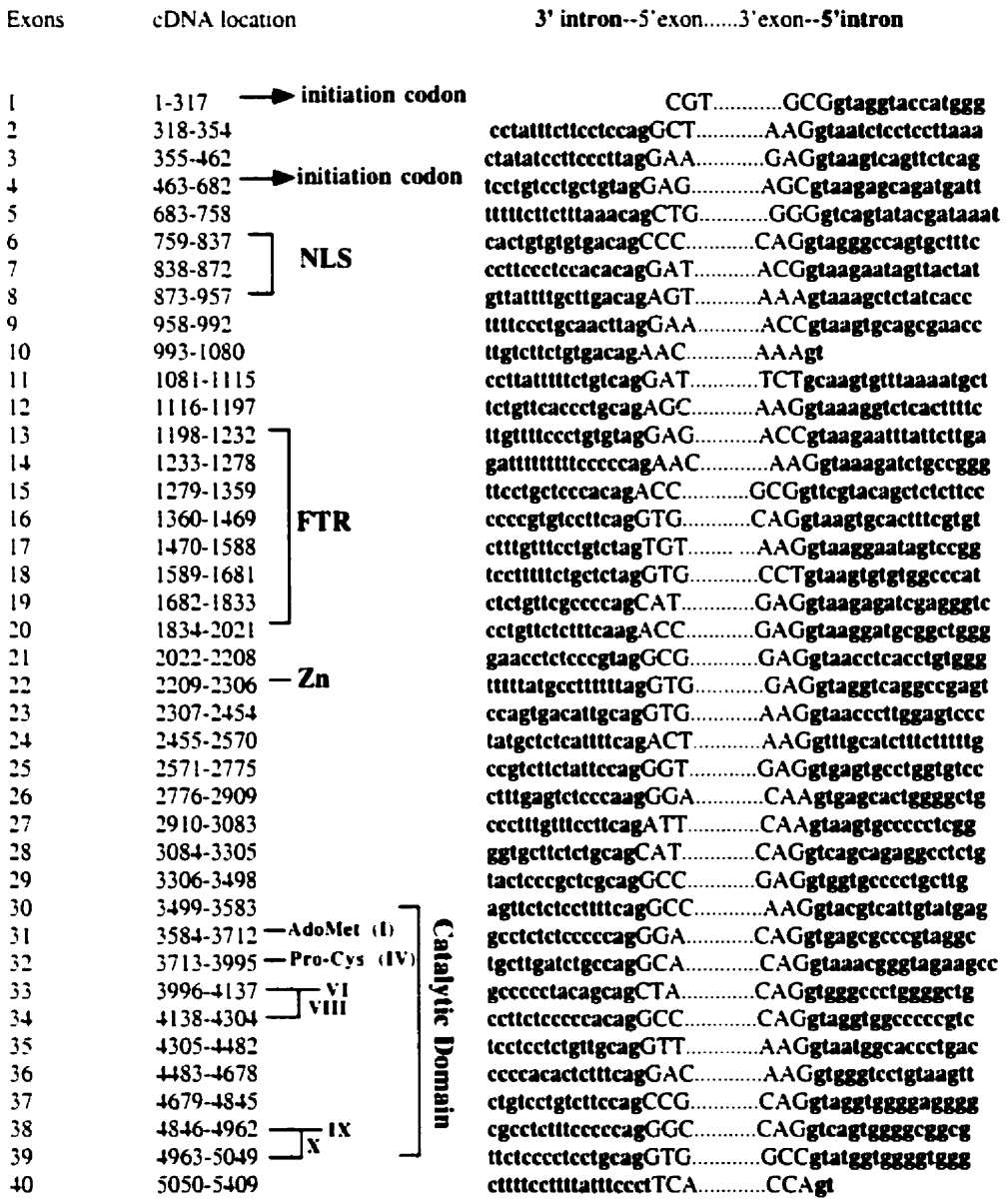

probe: 168-317
(exon 1)
T 24
A 549
B X H B X H
>21
17
2
probe: 415-740
(exons 3-5)
T 24
A 549
B X H B X H
>21
>21
3.4
probe: 861-2471
(exon 7-24)
T 24
A 549
B X H B X H
~21
8.5
11.5
7
4.8
3
4.8
3.9
2.5
1.9
probe: 3570-5084
(exon 30-40)
T 24
A 549
B X H B X H
~21
9
Bam H1
Xba 1
Hind III

Figure 2: Exon-Intron boundaries of the human DNA MeTase gene

Exonic organization of the sequences corresponding to the known human mRNA (Yen et al.,1992: Yoder et al., 1996). The intron-exon boundaries were determined by exon sequencing of the genomic fragments described in figure 1A. The intronic sequences flanking the boundaries are presented. Conserved splice acceptor (3' intron) and donor (5' intron) sites are depicted in bold. NLS = nuclear localisation signal (cDNA location : 817-874), FTR = replication foci targeting sequence (cDNA location : 1195-1938) (Leonhardt et al., 1992), Zn = zinc binding domain (cDNA location : 2194-2310), AdoMet = S-adenosyl-methionine binding motif (cDNA location : 3670-3687), Pro-Cys = proline-cysteine catalytic motif (cDNA location : 3910-3915 in domain IV), domain VI (cDNA location : 4003-4065), domain VIII (cDNA location : 4123-4197), domain IX (cDNA location : 4863-4935), domain X (cDNA location : 4948-5022)

Catalytic Domain = region conserved in cytosine- 5 methyltransferases (cDNA location : 3649-5083) (Kumar et al., 1994). Numbering of the nucleotides of the human DNA MeTase cDNA is as in (Yoder et al., 1996).

Figure 2: **Exon-Intron boundaries of the human DNA MeTase gene**



The resolution of the genomic structure of the human *dnmt1* gene in chapter 1 provides the foundation for an analysis of how transcriptional expression of *dnmt1* is controlled. Initial studies by our lab (Rouleau *et al.*, 1992) uncovered a unique class of transcriptional control element or promoter sequence which controls initiation of transcription of the mouse *dnmt1* gene. Furthermore, it was demonstrated that this promoter is responsive to the Ras-Jun signalling pathway (Rouleau *et al.*, 1995). However, later studies (Yoder *et al.*, 1996) identified new cDNA sequence which was located further upstream to the promoter sequence identified. This study concluded that there was only one protein isoform of DNA MeTase produced by the *dnmt1* gene, in which the translation initiation codon utilized is 5' to the previously identified promoter sequence. These data strongly suggested that the promoter described (Rouleau *et al.*, 1992) may be a cryptic transcriptional element that is not utilized *in vivo*. A later study (Gaudet, *et al.*, 1998) demonstrates that the short isoform of DNA MeTase (which initiates after the promoter described by Rouleau *et al.*, 1992) can functionally rescue embryonic stem cells that are *dnmt1* null. Taken together, all of the above observations seem to create a very convoluted picture of the *dnmt1* transcriptional initiation and control. It is quite possible that all of the observations are components of a multi-leveled transcriptional control of *dnmt1*. The following manuscript begins to dissect all of the possible regions where *dnmt1* transcription initiates and identifies some of the molecular players involved in their control.

Chapter 2

Transcriptional regulation of the DNA Methyltransferase *dnmt1* gene

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ABSTRACT

DNA methylation is an important component of the epigenetic control of genome functions. Understanding how the expression of the DNA Methyltransferase *dnmt1* gene is regulated is critical for deciphering how DNA methylation is coordinated with other critical biological processes. In this paper we delineate the transcriptional regulatory region of the human *dnmt1* gene using a combination of RACE, RNase protection analysis and CAT assays. We identified one major and three minor transcription initiation sites *in vivo* (P1-P4) which are regulated by independent enhancers and promoter sequences. The minimal promoter elements of P1, 2 and 4 are mapped within 256 bp upstream of the respective transcription initiation sites. P1 is nested within a CG rich area similar to other housekeeping genes whereas P2-P4 are found in CG poor areas. This bipartite organization of the transcription regulatory region of *dnmt1* is reflected in the differential responsiveness of these promoters to the protooncogene c-Jun. Three Jun dependent enhancers are located downstream to P1 and upstream to P2-P4, thus providing a molecular explanation for the responsiveness of this gene to oncogenic signals which are mediated by the Ras-Jun oncogenic signaling pathway.

INTRODUCTION

Mammalian DNA is modified by methylation of 60-80% of the cytosines residing in the dinucleotide sequence CpG (Razin and Szyf, 1984). DNA methylation is now being recognized as an important epigenetic control over different genome functions including differential gene expression (Razin and Cedar, 1991), allele specific expression in parental imprinting (Sapienza *et al.*, 1987) and X inactivation (Zucotti *et al.*, 1995). DNA methylation may serve as a signaling mechanism since vertebrate DNA is differentially methylated at CpG dinucleotide sequences forming site- gene- and tissue specific- patterns of methylation (Razin and Szyf, 1984). Methylation of CpG dinucleotides can inhibit gene expression by either directly interfering with the interaction of transcription factors with their cognate recognition sequences (Becker *et al.*, 1987) or by attracting the binding of methylated-DNA binding proteins that repress gene expression (Nan *et al.*, 1997). It is clear that understanding the mechanisms responsible for generating DNA methylation patterns is critical for elucidating the processes through which genomic expression programs are generated.

The enzyme catalyzing the modification of DNA by methylation is DNA methyltransferase (Adams *et al.*, 1979). One *dnmt1* cDNA has been cloned and extensively characterized to date in human and murine cells (Bestor *et al.*, 1988; Yen *et al.*, 1992). The 5' end of a murine and human *dnmt1* RNA have recently been characterized and it has been suggested that this is the only DNMT1 isoform expressed in somatic cells (Tucker *et al.*, 1996; Yoder *et al.*, 1996). However, splice variants have recently been characterized in germ cells (Mertineit et al., 1998) and somatic cells (Deng and Szyf, 1998).

It stands to reason that regulation of *dnmt1* expression plays an important role in coordinating DNMT1 activity with different developmental and cellular programs (Szyf, 1994; Szyf, 1998). In accordance with this hypothesis, it has been

previously shown that *dnmt1* is post transcriptionally regulated with the state of cell growth (Szyf *et al.*, 1991), that *dnmt1* expression is induced by the Ras-AP-1 signaling pathway (MacLeod et al., 1995) and that an AP-1 regulatory element regulates the expression of the murine *dnmt1* gene (Rouleau et al., 1995). We have therefore suggested that induction of expression of *dnmt1* is a downstream event in oncogenic programs, such as the Ras AP-1 signaling pathway and that it might play a causal role in cellular transformation (MacLeod and Szyf, 1995; Ramchandani *et al.*, 1997). A promoter and enhancer has been identified previously in the mouse, upstream to exons 3 and 4 (Rouleau *et al.*, 1992) but it is clear that transcription initiates upstream to the previously identified promoter as well (Tucker et al., 1996; Yoder et al., 1996). The transcriptional regulatory regions of this initiation site are unknown and it is not yet clear whether alternative transcription initiation sites are utilized *in vivo*.

The fact that the regulatory regions of *dnmt1* have been unresolved to date has limited our understanding of how its expression is coordinated with other important biological and pathological processes. This paper identifies the transcription initiation sites of *dnmt1* and the genetic elements regulating them.

RESULTS

Multiple *dnmt1* initiation sites and 5' splice variants determined by a RACE-PCR analysis of placenta and hippocampus mRNA. To identify elements regulating the expression of *dnmt1* one has to identify transcription initiation sites utilized *in vivo*. Previous studies on the mouse *dnmt1* using a combination of RNase protection and primer extension analyses have identified putative initiation sites at exons 3 and 4 and an AP-1 regulated promoter located upstream to these sites was demonstrated by CAT assays (Rouleau *et al.*, 1992; 1995). To delineate the initiation sites of *dnmt1 in vivo*, we performed a RACE analysis on human hippocampus and placenta mRNA. This technique allows one

to identify the 5' ends of mRNA transcribed in a given cell or tissue (Frohman et al., 1989). Double stranded cDNA prepared from both tissues and ligated at their ends to a Marathon adaptor were amplified with either a 3' antisense oligonucleotide which is complementary to a 27 bp sequence from exon 21 (oligo 145) or a 20 bp oligo from exon 6 and the Marathon anchor primer as described in Materials and Methods.

To precisely delineate the initiation sites obtained by our RACE analysis, the RACE amplification products were subcloned into pCR 2.1 vectors and subjected to dideoxy chain termination sequencing. The results of this analysis summarised in Fig. 1 show four distinct clusters of 5' ends of *dnmt1* mRNA (indicated by letters a-h in Fig. 1 and 2) are expressed *in vivo* at previously defined exons 1, 2, 3 and 4. The precise 5' boundaries of the RACE products are indicated in the sequence of the 5' region of *dnmt1* presented in Fig. 2. Our analysis also revealed 4 alternatively spliced new exons which are in frame with the rest of the coding sequence of *dnmt1*. Three of these alternatively spliced sites initiate at exon 1 (unpublished data).

RNase protection analysis of the 5' ends of *dnmt1*.

The fact that both the hippocampus and placenta RACE analyses reveal transcripts initiated at the 5' exon corresponding to the full known transcript (Fig. 1) suggests that the Marathon libraries used in this assay bear full length cDNAs. However, it is possible that the downstream 5' boundaries identified in this study reflect broken RNAs or incomplete primer extension by the reverse transcriptase. To verify that the 5' boundaries identified in the RACE analysis result from transcription initiation *in vivo* , we have resorted to an independent method for identifying 5' boundaries of RNA, RNase protection assay (Kreig and Melton, 1987). We analyzed RNA samples from placenta and two other cell lines (A549 and T 24) to exclude the possibility that a protected fragment represents broken RNA. It is highly improbable that two different preparations of RNA that are

randomly broken will have the same 5' boundaries. We first used a ^{32}P labelled riboprobe which is complementary to the first previously described 4 exons of *dnmt1* (Yoder et al., 1996). To ensure complete synthesis of the riboprobe, the synthesized probe was separated on a sequencing gel and the full size probe was eluted. mRNA prepared from two cancer lines (a bladder carcinoma line T-24 and a human lung carcinoma A549) and human placenta was hybridized with the labelled riboprobe, subjected to RNase treatment and the protected fragments were size fractionated on a sequencing gel. If *dnmt1* mRNA initiates exclusively at the first exon as has previously been suggested, the full probe should have been protected with no additional fragments to be observed. However, if *dnmt1* is initiated also at proximal sites, shorter fragments corresponding to the shorter messages including those identified in the RACE analysis would be protected. The size of the protected fragments will correspond to the distance between the initiation site and the start point of the RNA probe and do not correspond to the size expected from protection of the splice products (Fig. 3).

As observed in Fig. 3, a number of protected fragments are identified by this analysis. Three fragments which correspond to messages initiated in exon 1 are observed in both placenta and the cancer cell lines (corresponding to the RACE products a,b,c and e; Fig. 1 and 2), however there is a different relative use of these sites in the different tissues. Fragments corresponding to initiations upstream to exon 2 (corresponding to the RACE product e Fig. 1 and 2) and in exon 3 (corresponding to RACE product g as well as a 5' boundary not identified by RACE indicated as * in Fig. 3) are identified in the cancer cells.

To determine whether additional initiation sites are utilized in exon 4 we utilized a riboprobe encoding exons 3, 4 and 5. If transcription is exclusively initiated upstream to exon 3, the entire riboprobe should be protected (345 nt). Initiation downstream within exon 3 and 4 as predicted from the RACE analysis (Fig. 1 and 2 g,h) will result in shorter fragments as indicated in the scheme in Fig. 3. Both

placenta and cancer cells express *dnmt1* mRNA initiated within exon 3 (340 nt indicated by ** in Fig. 3 and 315 nt corresponding to RACE product g) and at exon 4 (258 nt corresponding to RACE product h in Fig. 1 and 2). Thus, the RNase protection analysis is consistent with the initiation clusters identified by the RACE analysis. Initiations at exon 1 are the most abundant as indicated by both the RACE and RNase protection analyses.

Nucleotide sequence of 5' region of the human *dnmt1*.

To determine whether the 5' region of *dnmt1* bears multiple promoters and regulatory regions as suggested by the previous analysis, we subcloned and sequenced the genomic regions flanking previously defined exons 1 to 4 from our previously described genomic *dnmt1* phages π TB and π FTR (Ramchandani *et al.*, 1998). An analysis of the sequences residing upstream to the transcription initiation sites identified by the RACE analysis reveals the absence of canonical TATAA or CAAT sequences which are characteristic of many housekeeping genes (Seiser et al., 1989). A common trait of TATAA-less housekeeping gene promoters is that they are nested in a CG rich area (Bird, 1986). Whereas the upstream transcription initiation region in exon 1 is CG rich and includes a large number of CG sites, the downstream initiation sites are nested within a relatively CG poor area which is consistent with different modalities of regulation of the upstream and downstream clusters of initiation sites as discussed below (Fig. 2). This is similar to the situation described for the mouse homologue (Rouleau *et al.*, 1992).

A computer analysis of the 5' sequences of putative recognition sequence for known transcription factors reveals multiple AP-1 recognition sites (Binetruy et al., 1991), an E2F recognition site (Bagchi et al., 1991) as well as multiple TCF binding sites (consistent with regulation by the APC-beta catenin-tcf pathway which has recently been implicated in tumor suppression) (Korinek et al., 1997). Whereas, identification of putative recognition sites does not obviously prove that they are

functional, it raises the possibility that *dnmt1* is regulated by central cellular signaling pathways (Szyf, 1998). This has to be verified experimentally once the regulatory regions of *dnmt1* are delineated.

Identification of the regulatory regions controlling the initiation sites of *dnmt1*. Verification of the four transcription initiation clusters identified above requires the demonstration of independent active promoter and enhancer sequences in their proximity using standard heterologous reporter expression assays. P19 cells were used because they bear a low background of AP-1(de Groot et al., 1990) and Rb (Slack et al., 1993) activity. Since expression of murine *dnmt1* has been shown to be induced by activation of the Ras-Jun signaling pathway (MacLeod et al., 1995; Rouleau et al., 1995) and to be regulated with the cell cycle (Szyf et al., 1991), we tested whether the independent promoter elements are responsive to either ectopic c-Jun or the tumor suppressor retinoblastoma. To verify that the CAT activities observed reflect true differences in promoter strength and not irregular differences in the quality of the plasmid preparation or transfection efficiency, two independent plasmids and three independent transfections were performed per construct. The AP-1 transactivation activity of the c-Jun expression vector was verified by testing its ability to trans activate a murine pMetCAT vector bearing consensus AP-1 sites and the lack of transactivation of a construct that does not bear AP-1 sites (data not shown). As indicated in Fig. 4D c-Jun does not activate the promoter elements in the antisense orientation suggesting that Jun acts on the promoter and not on cryptic elements in the plasmid vector.

As demonstrated in Fig. 4A, a promoter element resides within 250 bp upstream to the first transcription initiation cluster identified *in vivo* (P1) since an XbaI fragment bearing 0.256 kb upstream to the first exon (pMet-P1-DHX-CAT) can direct transcription of a promoter less CAT in the sense but not the antisense orientation. No additional elements that are required for expression of P1 are present in the 1.2

kb upstream to the P1 promoter (pMet-P1-CAT) since inclusion of this sequence does not enhance but rather represses the activity of the promoter. This 1.2 kb upstream fragment can not induce reporter activity by itself (pMet-P1-DXH-CAT). An enhancer resides in the region flanking the XbaI site (250 bp upstream to exon 1) which is expressed in Hela cells but not in P19 cells. This is indicated by the ability of the 2kb HIII fragment to enhance the transcription of an enhancer less vector bearing the TK promoter (pMet-P1-PrCAT) in both the sense and antisense orientations. This activity is lost once either the upstream 1.2 kb (pMet-P1-DXH-PrCAT) or 0.256 downstream fragments (pMet-P1-DHX-PrCAT) are removed. Adding 10 kb of downstream sequence does not affect the P1 promoter activity suggesting that all the elements required for P1 activity (data not shown) are contained within the minimal promoter 256 bp upstream of the first transcription initiation site.

The P1 promoter is not responsive to ectopic expression of c-Jun but is repressed by coexpression of Rb. This is consistent with the presence of an E2F recognition sequence downstream to the promoter (see physical map). The effect of Rb is more enhanced when the 1.2 kb sequence upstream to the XbaI site is present (pMet-P1-Cat). Since this fragment has a repressor effect in the absence of Rb in P19 cells, it is possible that Rb can increase a repressor interacting with this region.

A Jun dependent promoter activity resides within 160 bp upstream of the second transcription initiation cluster P2 identified *in vivo* (Fig. 4B). This is indicated by the ability of the XbaI-NcoI fragment spanning 160 bp upstream and 40 bp downstream to the second initiation cluster (pMet-P2-CAT) to direct CAT activity when inserted into a promoter less CAT vector in the sense but not antisense orientation only in the presence of ectopic c-Jun. A Jun dependent enhancer activity resides in the same region as demonstrated by its ability to enhance expression of CAT in both orientations in a Jun dependent manner when inserted into a vector bearing the TK promoter (pMet-P2-PrCAT, Fig. 4B). A single AP-1 recognition sequence is found downstream to exon 2 which can explain the

regulation of P2 by Jun. No other promoter or enhancer elements reside in the 2.4 kb upstream to the XbaI site (data not shown).

Both a promoter and enhancer activity which are inducible by Jun are found in the 1.2 kb of sequence upstream to the third initiation cluster observed *in vivo* , P3 as judged by its ability to direct CAT activity from a promoter less CAT construct in the sense orientation (pMet-P3-CAT), and to enhance the TK promoter activity in both sense and antisense orientations (pMet-P3-PrCAT) (Fig. 4C). The promoter activity is induced by Jun and repressed by Rb. The enhancer activity is enhanced by Jun in both orientations and is repressed by Rb only in the sense orientation.

A fourth promoter resides within 170 bp upstream of the fourth cluster of transcription initiation identified *in vivo* P4 (Fig. 4D) (pMet-P4-CAT). This promoter is also induced by c-Jun. The 263 bp fragment bearing P4 also contains a bidirectional enhancer (pMet-P4-PrCAT) which is dependent on expression of ectopic c-Jun.

In summary, in accordance with the hypothesis that four independent transcription initiation sites of *dnmt1* are found *in vivo* (Fig. 1,2,3), we have identified 4 promoter elements upstream to each of the sites identified *in vivo*. Three promoters were delineated within less than 256 bp from the transcription initiation site. The three downstream promoters are either dependent or inducible by c-Jun which is consistent with previous reports in the murine system that *dnmt1* is regulated by c-Jun (MacLeod *et al.*, 1995; Rouleau *et al.*, 1995). Three downstream enhancer elements which are inducible by c-Jun were also identified. Since these elements are bidirectional, they can act in concert to activate the entire *dnmt1* locus. These multiple elements regulated by c-Jun are consistent with a strong association of the level of expression of *dnmt1* with the intensity of signaling by the Ras-AP-1 signaling pathway (Szyf, 1994).

Consistent with the hypothesis that *dnmt1* is transactivated by c-Jun, we show that

double stranded oligonucleotides bearing either the sequence included in the putative AP-1 recognition sites upstream to P2 and the AP-1 site upstream to P3 form a specific DNA-protein complex that comigrates in an EMSA assay with the complex formed with a consensus AP-1 sequence (Fig. 5). The DNA-protein complexes formed with the putative AP-1 sequences upstream of P2 and P3 are competed out by an AP-1 consensus oligonucleotide but not by a mismatched AP-1 sequence (Fig. 5).

DISCUSSION

The complex DNA methylation patterns observed in vertebrate cells and the stringent requirement for its maintenance through multiple rounds of replication begs one to speculate that a variety of isoforms of *dnmt1* exist and that their expression is tightly regulated with the differentiation and physiological state of a vertebrate cell. Two translation initiation ATG sites that are in frame with the rest of the coding sequence of the *dnmt1* have been previously identified in the human cDNA. The first is located in exon 1 and the other located in exon 4 (Bestor *et al.*, 1988; Yoder *et al.*, 1996). A previous report has mapped a transcriptional regulatory sequence that is regulated by the Ras-Jun signaling pathway upstream to exon 3 in the mouse homologue (Rouleau *et al.*, 1992). Thus, one possible mechanism of differential expression of *dnmt1* isoforms is by alternative use of transcription initiation sites upstream to the different translation initiation codons (Fig. 6).

This manuscript dissects the basic modes of regulation of *dnmt1* gene. Three lines of evidence point towards the conclusion that *dnmt1* transcription is initiated from at least four independent transcription initiation sites. First, RACE-PCR analysis defines four distinct 5' boundaries of *dnmt1* transcripts *in vivo* (Fig. 1). Second, RNase protection assays identify the corresponding protected fragments (Fig.3). Third, promoter and enhancer elements were identified in proximity to (within 256 bp to P1,2 and 4) the transcription initiation sites identified *in vivo*.

The putative transcription initiation sites are differentially utilized *in vivo*. The most upstream initiation sites upstream to exon 1 (P1) are the most abundant in all the tissues studied here as determined by both RACE (26 out of 36 sequenced clones) and RNase protection analysis. This is consistent with previous published data (Tucker *et al.*, 1996; Yoder *et al.*, 1996). This differential use of the putative transcription initiations sites is reflected in the bipartite organization of the genomic regions controlling their expression and their mode of regulation by c-Jun. The downstream and upstream transcription initiation regions are separated by ~ 14 kb of genomic sequence. Whereas the 5' transcription initiation site is nested within a CG rich area (Fig. 6) similar to many other housekeeping promoters, the downstream sites are located within CG poor areas as previously described in the mouse homologue (Rouleau *et al.*, 1992). No consensus TATAA sequences were identified upstream to either P1 or P2-P4. Thus the 5' region of *dnmt1* bears a typical TATAA less CG rich promoter and atypical TATAA less CG poor promoters 14 kb downstream. P1 has a high basal activity and is not induced by ectopic expression of c-Jun (Fig. 4) but is repressed by the tumor suppressor Rb. P2-P4 have low basal activity and are significantly induced by c-Jun (Fig. 4).

The differential response of the *dnmt1* promoters to c-Jun provides the cell with a potential mechanism to fine tune the relative rate of firing of the different promoters and thus the relative representation of the short and long *dnmt1* isoforms in response to a nodal signaling pathway.

In addition to the 4 promoters, the *dnmt1* 5' bears 3 enhancers that are either exquisitely dependent on ectopic expression of c-Jun or are strongly induced by it. Since enhancers act bidirectionally, they might act in combination to induce transcription from all *dnmt1* promoters. These multiple AP-1 response elements can explain the hyperresponsiveness of *dnmt1* to induction of the Ras-AP-1 signaling pathway in mouse (MacLeod *et al.*, 1995; Rouleau *et al.*, 1995) and human cells (Yang *et al.*, 1997) and the hyperactivation of *dnmt1* during

tumorigenesis (el-Deiry *et al.*, 1991).

Our analysis reveals that the *dnmt1* gene bears the capacity to respond to two nodal cellular regulatory programs signaling through c-Jun and Rb. The fact that the *dnmt1* gene is structured this way and that this framework of genomic regulatory elements is conserved in the mouse, rat (unpublished data) and human points towards the significance of coordinating expression of *dnmt1* with other cell cycle events (Szyf 1998). Accumulating evidence strongly suggests that multiple mechanisms are in place to tune expression of *dnmt1* to other cell cycle events such as mRNA stability (Szyf et al., 1991) and protein-protein interactions (Chuang *et al.*, 1997). The fact that *dnmt1* bears the capacity to respond to both protooncogene and tumor suppressor pathways is consistent with the hypothesis that *dnmt1* plays an important role in oncogenic programs triggered by either inhibition of tumor suppressors or activation of protooncogenes.

In summary, our study has delineated the transcriptional start sites of *dnmt1*, the regulatory elements controlling its expression and their capacity to respond to different cellular signals. The identification of these elements will make it possible to establish the molecular basis of the coordination of *dnmt1* expression with other critical biological processes.

MATERIALS AND METHODS.

Cell culture and RNase protection

Human non small cell lung carcinoma cell line A549 (ATCC CCL 185), human bladder carcinoma cell line T24 (ATCC HTB 4), Human cervical carcinoma cell line HeLa (ATCC CCL2) and mouse embryonal carcinoma cell line P19 (ATCC CRL 1825) were cultured in Dulbecco's modified Eagle's medium (with low glucose for A549 and high glucose for the other cell lines) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics. Total

cellular RNA was prepared by extraction with guanidine thiocyanate (Chirgwin et al., 1979). Human mRNA samples were purchased from Clontech.

Two probes were used for RNase protection bearing either sequences encoding bases 168 to 611 (exons 1 to 4) or bases 396 to 740 (exons 3 to 5) in the antisense orientation (see Fig. 2 for map). The antisense RNA probe was transcribed using T7 RNA polymerase and [a-^{32}P]UTP and was hybridized with either T24 RNA, A549 RNA, human hippocampus mRNA or human placenta mRNA at 50°C overnight. The hybridized RNA was treated with RNases A and T1 as described (Kreig and Melton, 1987), and the protected fragments were resolved on a 8 M urea sequencing gel next to a century ladder (Ambion).

RACE-PCR analysis of 5' ends of human brain *dnmt1* mRNA.

Human hippocampus and placenta cDNA libraries with a Marathon cDNA adaptor ligated to its ends were obtained from Clontech. The 5' ends of *dnmt1* mRNA were determined by RACE PCR (Frohman et al., 1989) amplification using as the 5' oligo the AP1 (adaptor primer Clontech) adaptor and as a 3' amplimer either oligo 145 (ATCGAAGATCTGGTAGACCAGCTTGGT) (Fig. 1) encoding a sequence from exon 21 or oligo 161 (TTTCCAAGTCTTTGAGCCTG) encoding a sequence from exon 6 (Fig. 1). 10 pg of marathon ready cDNA were amplified using KlenTaq (Clontech) and the following parameters: 94°C 0.5 min., 55°C 0.5 min., 68°C 6 min. for 40 cycles which were followed by 6 min. incubation at 68°C. The amplification products were resolved on an agarose gel. An aliquot of the amplification reaction was ligated to pCR2.1 and the resulting colonies were sequenced to determine the sequence of the 5' end of the human *dnmt1*. The sequence was aligned to the genomic sequence of dnmt1 shown in Fig. 2. The 5' of the RACE products is indicated by an arrow in Fig. 2.

Genomic cloning and construction of plasmids

All plasmids were constructed using genomic subclones of *dnmt1* pTB and pFTR1 that were previously described (Ramchandani *et al.*, 1998). pTB (20 kb genomic insert) was digested with either *Hind III* or with *Xba I*. A 2 kb *Hind III* fragment containing exon 1, a 12 kb *Xba I* fragment containing exon 1 and a 10 kb *Hind III* fragment containing exon 1b were isolated and inserted into a Hind *III*-digested pBluescript SK- plasmid or a *Xba I*-digested pBluescript SK- plasmid. The 2 kb *Hind III* insert was fully sequenced by the dideoxy chain termination method using a T7 DNA sequencing kit (Pharmacia). Fusion CAT constructs containing different fragments from the 5'-flanking *dnmt1* gene were then generated. The following CAT vectors were used: pOCAT bears the bacterial chloramphenicol acetyl transferase gene with no eukaryotic promoter or enhancer, pCAT promoter bears the Herpes virus thymidine kinase promoter but no enhancer activity and is used to determine enhancer activity (CAT constructs were purchased from Promega). pMet-P1-CAT sense and antisense were obtained by ligating the 2 kb end-blunted *Hind III* fragment into a *Xba I*-digested and end-blunted pOCAT. pMet-P1-PrCAT at either sense or antisense were obtained by ligating the 2 kb *Hind III* fragment respectively in a *Hind III*-digested pCAT Promoter (Promega) and a *Hind III*-digested pCAT Enhancer (Promega).

The 2 kb *Hind III* fragment described above was digested with *Xba I*, a 1.2 kb *Hind III-Xba I* fragment (ΔHX) and a 0.8 kb *Xba I-Hind III* fragment containing exon 1 (ΔXH) were isolated and end-blunted. pMet-P1-ΔHX-CAT (in either orientation) and pMet-P1-ΔHX-PrCAT (in either orientation) were generated by inserting the 0.8 kb *Xba I-Hind III* fragment into an end-blunted *Xba I*-digested site of pOCAT and pCAT Promoter. pMet-P1-ΔXH-CAT (in either orientation), and pMet-P1-ΔXH-PrCAT (in either orientation)were generated by inserting the 1.2 kb *Hind III-*

Xba I into the same vectors.

pFTR1 (20 kb) (Ramchandani *et al.*, 1998) was digested with *Xba I*. The reaction was run on a gel, and all 5 fragments were purified and inserted into a Xba *I*-digested pBluescript SK plasmid. A 1.4 kb *Xba I* fragment containing exons 2 and 3 and a 0.3 kb *Xba I* fragment containing part of exon 4 were identified by DNA sequencing. pMet-P4-CAT (in either orientation) and pMet-P4-PrCAT (in either orientation) were generated by ligating the 0.3 kb *Xba I* fragment into the appropriate *Xba I*-digested plasmid.

The 1.4 kb *Xba I* fragment was digested with *Nco I*, resulting in a 0.3 kb *Xba I-Nco I* fragment containing exon 2 and a 1.1 kb *Nco I-Xba I* fragment containing exon 3. pMet-P2-CAT (in either orientation) and pMet-P2-PrCAT (in either orientation) were generated by ligating the end-blunted 0.3 kb *Xba I-Nco I* fragment containing exon 2 into the appropriate end-blunted *Xba I*-digested plasmid. pMet-P3-CAT (in either orientation) and pMet-P3-PrCAT (in either orientation) were generated by ligating the end-blunted 1.1 kb *Nco I-Xba I* fragment containing exon 3 into the appropriate end-blunted *Xba I*-digested CAT vector. The orientations of all these constructs were determined by DNA sequencing.

CAT assays

P19 cells or Hela cells were plated at a density of 10^5/well in a six-well tissue culture dish (Falcon) 24 hours prior to transfection. 15 µg of plasmid DNA were incorporated into a calcium phosphate precipitate (Rouleau et al., 1992). For cotransfection assays, 15 µg of the CAT construct and 7.5 µg of either SK as a control, RSV-cJun (Binetruy *et al.*, 1991), pLRbRNL (ATCC R81 65003). Two hundred µl of the precipitate were applied per well containing 2 ml of the

appropriate medium. The medium was replaced after 24 hours and cells were harvested for CAT assays 48 hours post-transfection. Cells were dislodged by scraping in phosphate buffered saline, and extracts were prepared in 100 µl of 100 mM Tris-HCl (pH 8) by five freeze-thaw cycles. CAT assays were performed on 50 µl of extract (~ 10 µg of protein) using [^{3}H]acetyl coenzyme A (0.5 µCi/assay, 3.8 Ci/mmol) essentially as described (Rouleau et al., 1992). All transfection assays were performed in triplicate and were repeated using two different plasmid preparations and two different cultures of either P19 or HeLa cells.

Electro mobility shift assay

The following oligonucleotide duplexes containing putative AP-1 binding sites from promoters 1-3 were used : AP-1 consensus: 5'-CGCTTGATGAGTCAGCCGGAA (sense), 5'-TTCCGGCTGACTCATCAAGCG (antisense) ; 1a : 5'-GCTGGGATGACAGGCGTG (sense), 5'-CACGCCTGTCATCCCAGC (position indicated in Fig.3) (antisense) ; 2a : 5'-GAAGAATGAGTTATTCGGG (sense), 5'-CCCGAATAACTCATTCTTC (antisense) ; 2b : 5'-ACAAGGTGACTCATGCCTG (sense), 5'-CAGGCATGAGTCACCTTGA (antisense) ; 3b : 5'-TGCCATTCACTCATTGTG (sense), 5'-CACAATGAGTGAATGGCA (antisense). The sense oligonucleotide was radiolabelled in a mixture containing 5 µM of oligonucleotide, 50 µCi of (g-^{32}P)-ATP (3000 Ci/mmol, Amersham), and 10 units of T4 polynucleotide kinase (Boehringer-Mannheim) in a final volume of 50 µl and the labelled oligonucleotide was then purified on a Sephadex G25 microcolumn (Pharmacia). The antisense strand was then added (1µg) and the duplex was annealed following 5 min. of

boiling by gradual cooling of the reaction mixture to room temperature.

The DNA binding reactions contained 20 mM Hepes pH = 7, 12% glycerol, 1mM EDTA, 4 mM DTT, 0.1% NP 40, 3 mM $MgCl_2$, 5 µg of A549 nuclear extract prepared as previously described (28), 5 µg BSA, 2 µg poly dI-dC, and 10 ng of labelled duplex in a 10 µl final volume.

The mixtures were incubated for 20 minutes at room temperature, following which a 300-fold excess of competitor was added and the reaction was incubated for 20 additional minutes at room temperature. The mixtures were then analyzed by electrophoresis on a 4% non-denaturing polyacrylamide gel at 4°C for 3 hours at 220 volts. Gels were dried and exposed for autoradiography.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institute of Cancer (Canada) and a contract with MethylGene Inc. Montreal.

Figure 1: RACE-PCR determination of initiation sites of *dnmt1*.

A. The genomic organization of *dnmt1* is shown in the first line. Boxes indicate exons and the arrows under the line indicate the position of the 3' amplimers used in the RACE analysis. The 5' ends of *dnmt1* mRNA in either human hippocampus or placenta Marathon cDNA library were determined by PCR amplification using as the 5' oligo the AP1 (adaptor primer Clontech) adaptor and as a 3' amplimer either oligos 161 encoding a sequence from exon 21 or 145 (Fig. 2) encoding a sequence from exon 21. 10 pg of marathon ready cDNA were amplified using KlenTaq (Clontech). **B**. The RACE-PCR products were subcloned and sequenced. The new start sites identified are shown as horizontal arrows and are indicated by letters a-h. The first arrow corresponds to the published boundary of exon 1. Grey arrows indicate initiations identified by RNase protection shown in Fig. 3 that were not identified in the cloned RACE products.

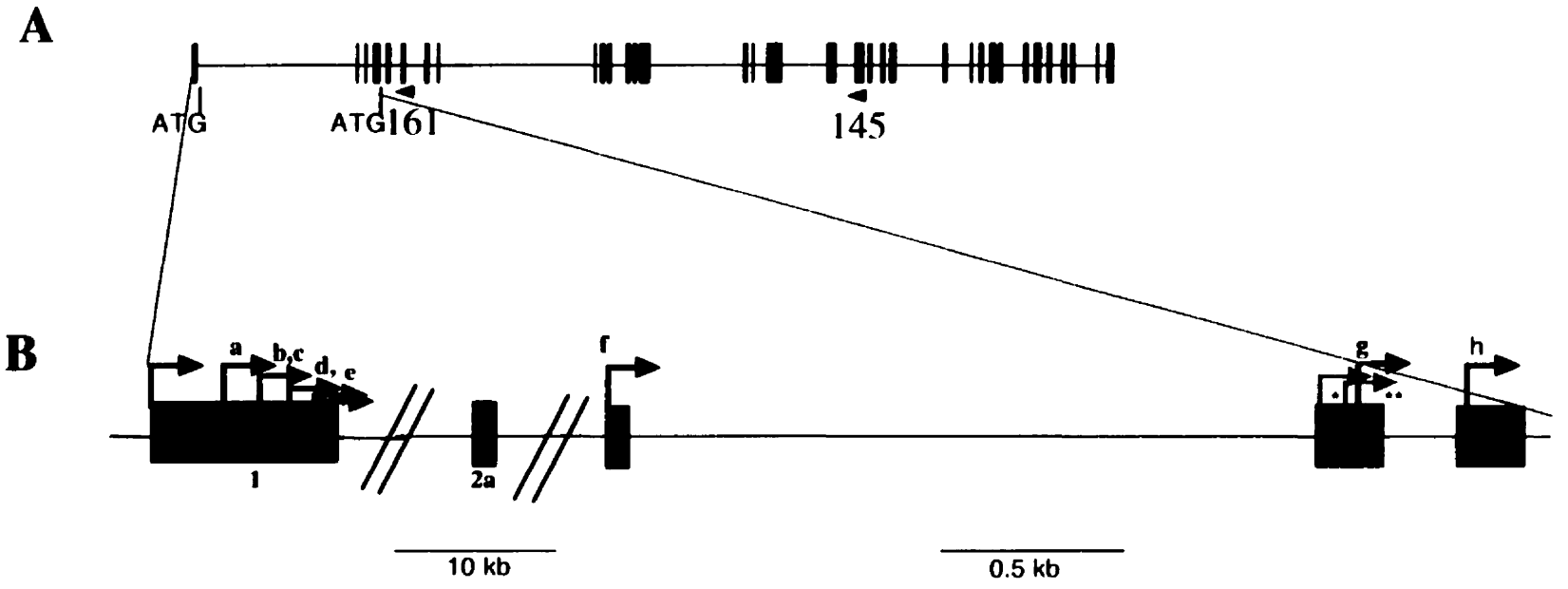
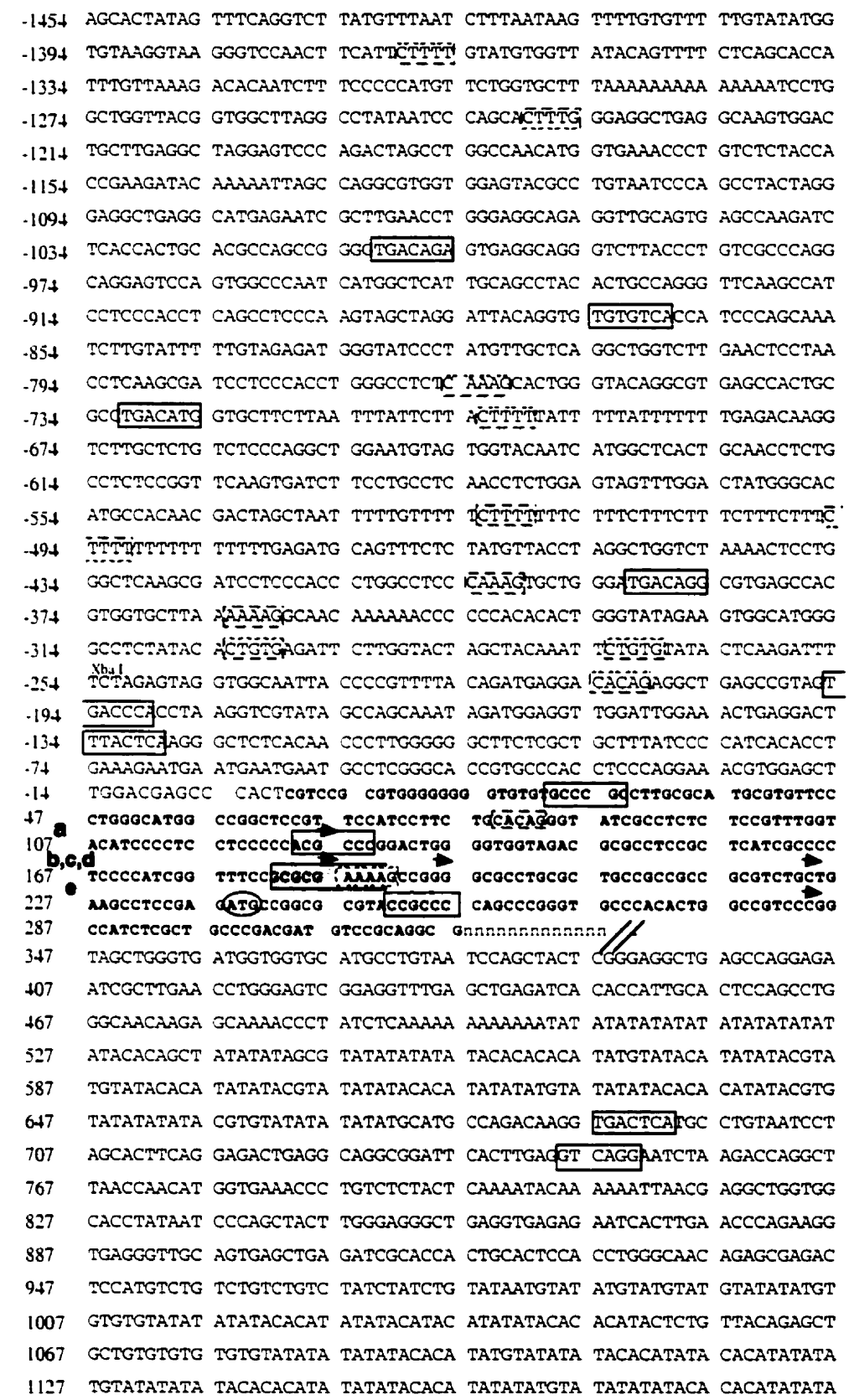

A
ATG
ATG
161
145
B
a
b,c
d, e
f
g
h
1
2a
10 kb
0.5 kb

Figure 2: Nucleotide sequence of 5'-region of human *dnmt1* and the position of the 5' boundaries of the sequenced RACE-PCR products. Bases -1454 to 317 includes exon 1 and 1.5 kb 5' of exon 1. Bases 347 to 3122 represents a 2775 nucleotide sequence containing exons 2, 3 and 4. Restriction enzymes utilized in the construction of different CAT constructs are indicated : Xba I and Nco I. Transcription initiation sites determined by RACE are indicated by arrowheads and indicated by letters in the shaded boxes on the left side of the sequence. Putative AP-1 binding sites are boxed with solid lines, putative TCF-1 sites are boxed with dashed lines, putative E2F site is boxed in grey, putative SP1 sites are shaded. Exons are represented in bold letters.

Figure 2


-1454 AGCACTATAG TTTCAGGTCT TATGTTTAAT CTTTAATAAG TTTTGTGTTT TTGTATATGG
-1394 TGTAAGGTAA GGGTCCAACT TCATTCTTTT GTATGTGGTT ATACAGTTTT CTCAGCACCA
-1334 TTTGTTAAAG ACACAATCTT TCCCCCATGT TCTGGTGCTT TAAAAAAAAA AAAAATCCTG
-1274 GCTGGTTACG GTGGCTTAGG CCTATAATCC CAGCACTTTG GGAGGCTGAG GCAAGTGGAC
-1214 TGCTTGAGGC TAGGAGTCCC AGACTAGCCT GGCCAACATG GTGAAACCCT GTCTCTACCA
-1154 CCGAAGATAC AAAAATTAGC CAGGCGTGGT GGAGTACGCC TGTAATCCCA GCCTACTAGG
-1094 GAGGCTGAGG CATGAGAATC GCTTGAACCT GGGAGGCAGA GGTTGCAGTG AGCCAAGATC
-1034 TCACCACTGC ACGCCAGCCG GGCTGACAGA GTGAGGCAGG GTCTTACCCT GTCGCCCAGG
-974 CAGGAGTCCA GTGGCCCAAT CATGGCTCAT TGCAGCCTAC ACTGCCAGGG TTCAAGCCAT
-914 CCTCCCACCT CAGCCTCCCA AGTAGCTAGG ATTACAGGTG TGTGTCACCA TCCCAGCAAA
-854 TCTTGTATTT TTGTAGAGAT GGGTATCCCT ATGTTGCTCA GGCTGGTCTT GAACTCCTAA
-794 CCTCAAGCGA TCCTCCCACCT GGGCCTCTCC AAAGCACTGG GTACAGGCGT GAGCCACTGC
-734 GCCTGACATG GTGCTTCTTAA TTTATTCTT ACTTTTTTATT TTTATTTTTT TGAGACAAGG
-674 TCTTGCTCTG TCTCCCAGGCT GGAATGTAG TGGTACAATC ATGGCTCACT GCAACCTCTG
-614 CCTCTCCGGT TCAAGTGATCT TCCTGCCTC AACCTCTGGA GTAGTTTGGA CTATGGGCAC
-554 ATGCCACAAC GACTAGCTAAT TTTTGTTTT TCTTTTTTTC TTTCTTTCTT TCTTTCTTTC
-494 TTTTTTTTTTT TTTTTGAGATG CAGTTTCTC TATGTTACCT AGGCTGGTCT AAAACTCCTG
-434 GGCTCAAGCG ATCCTCCCACC CTGGCCTCC CAAAGTGCTG GGATGACAGG CGTGAGCCAC
-374 GTGGTGCTTA AAAAAGGCAAC AAAAAAACCC CCCACACACT GGGTATAGAA GTGGCATGGG
-314 GCCTCTATAC ACTGTGAGATT CTTGGTACT AGCTACAAAT TCTGTGTATA CTCAAGATTT
-254 Xba I TCTAGAGTAG GTGGCAATTA CCCCGTTTTA CAGATGAGGA CACACAGAGGCT GAGCCGTAGT
-194 GACCCACCTA AGGTCGTATA GCCAGCAAAT AGATGGAGGT TGGATTGGAA ACTGAGGACT
-134 TTACTCAAGG GCTCTCACAA CCCTTGGGGG GCTTCTCGCT GCTTTATCCC CATCACACCT
-74 GAAAGAATGA ATGAATGAAT GCCTCGGGCA CCGTGCCCAC CTCCCAGGAA ACGTGGAGCT
-14 TGGACGAGCC CACTCGTCCG CGTGGGGGGG GTGTGTGCCC GCCTTGCGCA TGCGTGTTCC
47 CTGGGCATGG CCGGCTCCGT TCCATCCTTC TGCACAGGGT ATCGCCTCTC TCCGTTTGGT
107 ACATCCCCTC CTCCCCCACG CCCGGACTGG GGTGGTAGAC GCGCCTCCGC TCATCGCCCC
167 TCCCCATCGG TTTCCCCGCG AAAAGCCGGG GCGCCTGCGC TGCCGCCGCC GCGTCTGCTG
227 AAGCCTCCGA GATGCCGGCG CGTACCGCCC CAGCCCGGGT GCCCACACTG GCCGTCCCGG
287 CCATCTCGCT GCCCGACGAT GTCCGCAGGC Gnnnnnnnnnnnnn
347 TAGCTGGGTG ATGGTGGTGC ATGCCTGTAA TCCAGCTACT CGGGAGGCTG AGCCAGGAGA
407 ATCGCTTGAA CCTGGGAGTC GGAGGTTTGA GCTGAGATCA CACCATTGCA CTCCAGCCTG
467 GGCAACAAGA GCAAAACCCT ATCTCAAAAA AAAAAAATAT ATATATATAT ATATATATAT
527 ATACACAGCT ATATATAGCG TATATATATA TACACACACA TATGTATACA TATATACGTA
587 TGTATACACA TATATACGTA TATATACACA TATATATGTA TATATACACA CATATACGTG
647 TATATATATA CGTGTATATA TATATGCATG CCAGACAAGG TGACTCATGC CTGTAATCCT
707 AGCACTTCAG GAGACTGAGG CAGGCGGATT CACTTGAGGT CAGGAATCTA AGACCAGGCT
767 TAACCAACAT GGTGAAACCC TGTCTCTACT CAAAATACAA AAAATTAACG AGGCTGGTGG
827 CACCTATAAT CCCAGCTACT TGGGAGGGCT GAGGTGAGAG AATCACTTGA ACCCAGAAGG
887 TGAGGGTTGC AGTGAGCTGA GATCGCACCA CTGCACTCCA CCTGGGCAAC AGAGCGAGAC
947 TCCATGTCTG TCTGTCTGTC TATCTATCTG TATAATGTAT ATGTATGTAT GTATATATGT
1007 GTGTGTATAT ATATACACAT ATATACATAC ATATATACAC ACATACTCTG TTACAGAGCT
1067 GCTGTGTGTG TGTGTATATA TATATACACA TATGTATATA TACACATATA CACATATATA
1127 TGTATATATA TACACACATA TATATACACA TATATATGTA TATATATACA CACATATATA
a
b,c,d

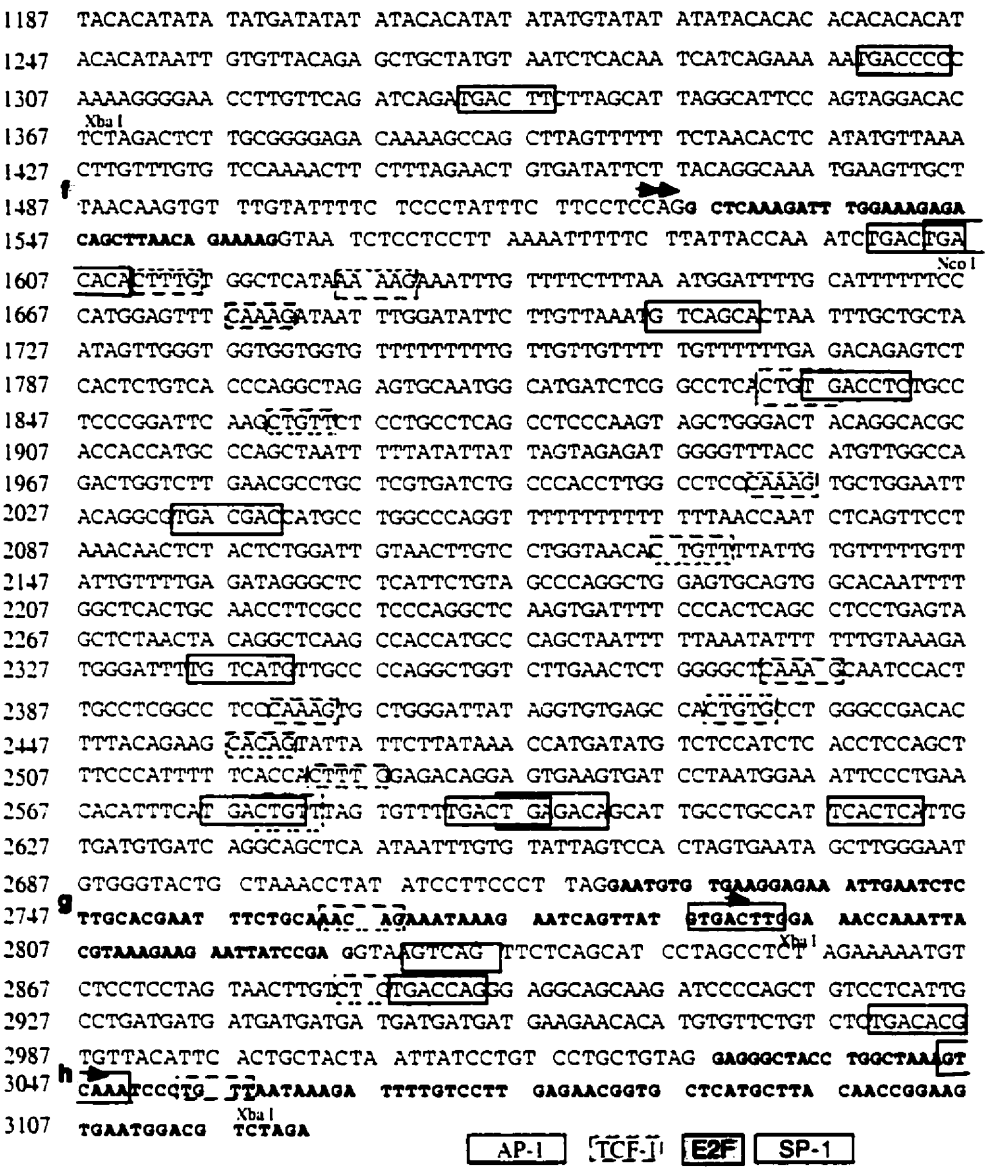
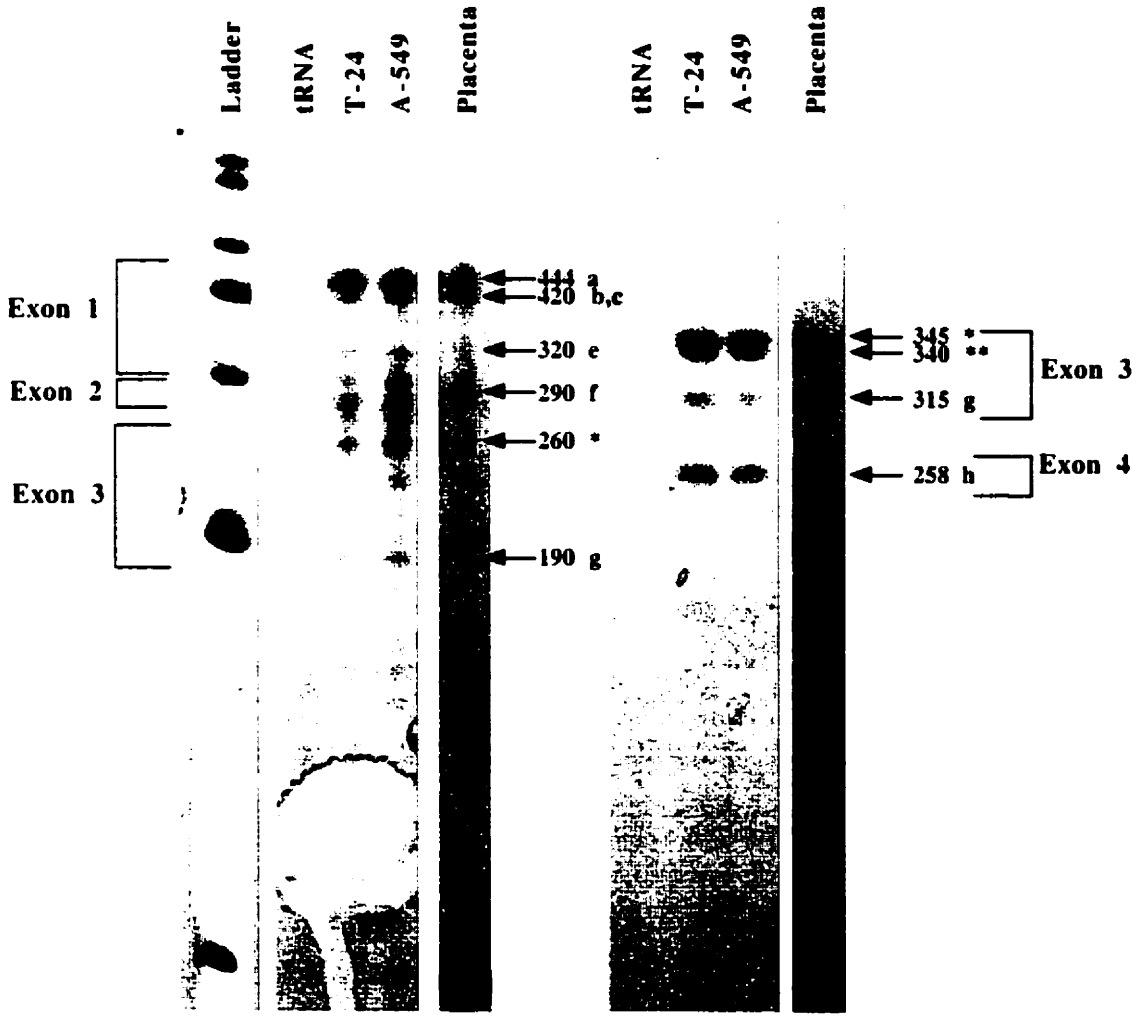

1187 TACACATATA TATGATATAT ATACACATAT ATATGTATAT ATATACACAC ACACACACAT
1247 ACACATAATT GTGTTACAGA GCTGCTATGT AATCTCACAA TCATCAGAAA AATGACCCCC
1307 AAAAGGGGAA CCTTGTTCAG ATCAGATGAC TTCTTAGCAT TAGGCATTCC AGTAGGACAC
Xba I
1367 TCTAGACTCT TGCGGGGAGA CAAAAGCCAG CTTAGTTTTT TCTAACACTC ATATGTTAAA
1427 CTTGTTTGTG TCCAAAACTT CTTTAGAACT GTGATATTCT TACAGGCAAA TGAAGTTGCT
f
1487 TAACAAGTGT TTGTATTTTC TCCCTATTTC TTCCTCCAGG CTCAAAGATT TGGAAAGAGA
1547 CAGCTTAACA GAAAAGGTAA TCTCCTCCTT AAAATTTTTC TTATTACCAA ATCTGACTGA
Nco I
1607 CACACTTTGT GGCTCATAAA AAGAAATTTG TTTTCTTTAA ATGGATTTTG CATTTTTTCC
1667 CATGGAGTTT CAAAGATAAT TTGGATATTC TTGTTAAATG TCAGCACTAA TTTGCTGCTA
1727 ATAGTTGGGT GGTGGTGGTG TTTTTTTTTG TTGTTGTTTT TGTTTTTTGA GACAGAGTCT
1787 CACTCTGTCA CCCAGGCTAG AGTGCAATGG CATGATCTCG GCCTCACTGT GACCTCTGCC
1847 TCCCGGATTC AAGCTGTTCT CCTGCCTCAG CCTCCCAAGT AGCTGGGACT ACAGGCACGC
1907 ACCACCATGC CCAGCTAATT TTTATATTAT TAGTAGAGAT GGGGTTTACC ATGTTGGCCA
1967 GACTGGTCTT GAACGCCTGC TCGTGATCTG CCCACCTTGG CCTCCCAAAG TGCTGGAATT
2027 ACAGGCGTGA CGACCATGCC TGGCCCAGGT TTTTTTTTTT TTTAACCAAT CTCAGTTCCT
2087 AAACAACTCT ACTCTGGATT GTAACTTGTC CTGGTAACAC TGTTTTATTG TGTTTTTGTT
2147 ATTGTTTTGA GATAGGGCTC TCATTCTGTA GCCCAGGCTG GAGTGCAGTG GCACAATTTT
2207 GGCTCACTGC AACCTTCGCC TCCCAGGCTC AAGTGATTTT CCCACTCAGC CTCCTGAGTA
2267 GCTCTAACTA CAGGCTCAAG CCACCATGCC CAGCTAATTT TTAAATATTT TTTGTAAAGA
2327 TGGGATTTTG TCATGTTGCC CCAGGCTGGT CTTGAACTCT GGGGCTCAAA GCAATCCACT
2387 TGCCTCGGCC TCCCAAAGTG CTGGGATTAT AGGTGTGAGC CACTGTGCCT GGGCCGACAC
2447 TTTACAGAAG CACAGTATTA TTCTTATAAA CCATGATATG TCTCCATCTC ACCTCCAGCT
2507 TTCCCATTTT TCACCACTTT CGAGACAGGA GTGAAGTGAT CCTAATGGAA ATTCCCTGAA
2567 CACATTTCAT GACTGTTTAG TGTTTTGACT GAGACAGCAT TGCCTGCCAT TCACTCATTG
2627 TGATGTGATC AGGCAGCTCA ATAATTTGTG TATTAGTCCA CTAGTGAATA GCTTGGGAAT
2687 GTGGGTACTG CTAAACCTAT ATCCTTCCCT TAGGAATGTG TGAAGGAGAA ATTGAATCTC
g
2747 TTGCACGAAT TTCTGCAAAC AGAAATAAAG AATCAGTTAT GTGACTTGGA AACCAAATTA
Xba I
2807 CGTAAAGAAG AATTATCCGA GGTAAGTCAG TTCTCAGCAT CCTAGCCTCT AGAAAAATGT
2867 CTCCTCCTAG TAACTTGTCT GTGACCAGGG AGGCAGCAAG ATCCCCAGCT GTCCTCATTG
2927 CCTGATGATG ATGATGATGA TGATGATGAT GAAGAACACA TGTGTTCTGT CTCTGACACG
2987 TGTTACATTC ACTGCTACTA ATTATCCTGT CCTGCTGTAG GAGGGCTACC TGGCTAAAGT
h
3047 CAAATCCGTG TTAATAAAGA TTTTGTCCTT GAGAACGGTG CTCATGCTTA CAACCGGAAG
Xba I
3107 TGAATGGACG TCTAGA
AP-1
TCF-1
E2F
SP-1

Figure 3. Multiple 5' transcripts of *dnmt1* are detected by an RNase protection assay. RNA prepared from tumor cell lines and human placenta indicated in the figure was subjected to an RNase protection assay using a riboprobe complementary to a cDNA sequence spanning either exons 1 to 4 of the human *dnmt1* (A) or a riboprobe complementary to exons 3 to 5 as described in materials and methods and separated on a sequencing gel next to an RNA molecular weight marker. Arrows indicate the observed fragment and their predicted size. The letters next to the arrows indicate the corresponding RACE-PCR product. whereas a * or ** next to the arrow indicate a start site not identified by RACE-PCR. The indicated fragment can not be a product of the 3 alternatively spliced mRNAs in this region since protection of these splice products would have resulted in fragments of 150 nt or 346 nt. The physical structure of each probe is indicated under the respective autoradiogram. The striped box at the 5' of each probe represents leader sequence originating from the plasmid template. The size in nucleotides of each exon that is included in the probes is indicated on top of the lines. The first line indicates the expected fragment size if the mRNA is initiated at the previously reported site (Tucker *et al.*, 1996; Yoder *et al.*, 1996). Initiation downstream to the previously described initiation site will result in shorter protected fragments as indicated below the top line.



Probe: 168-611 (exon 1-4) 396-740 (exon 3-5)

full probe= 444bases full probe = 345 bases

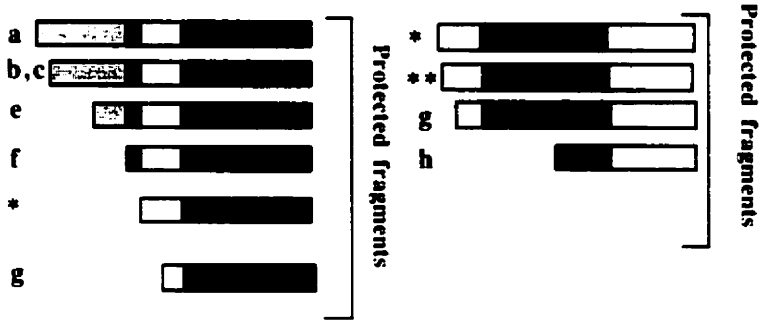
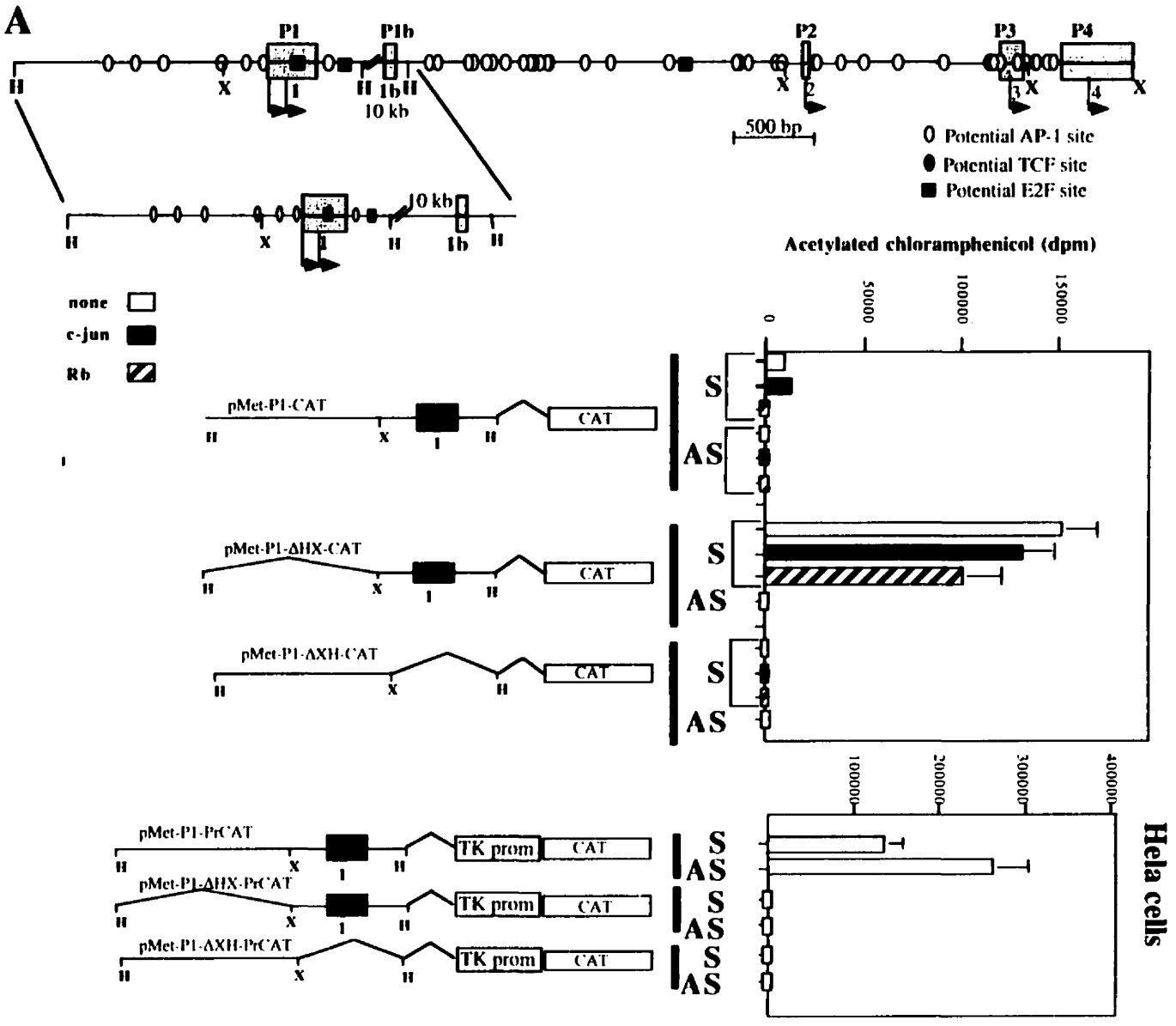
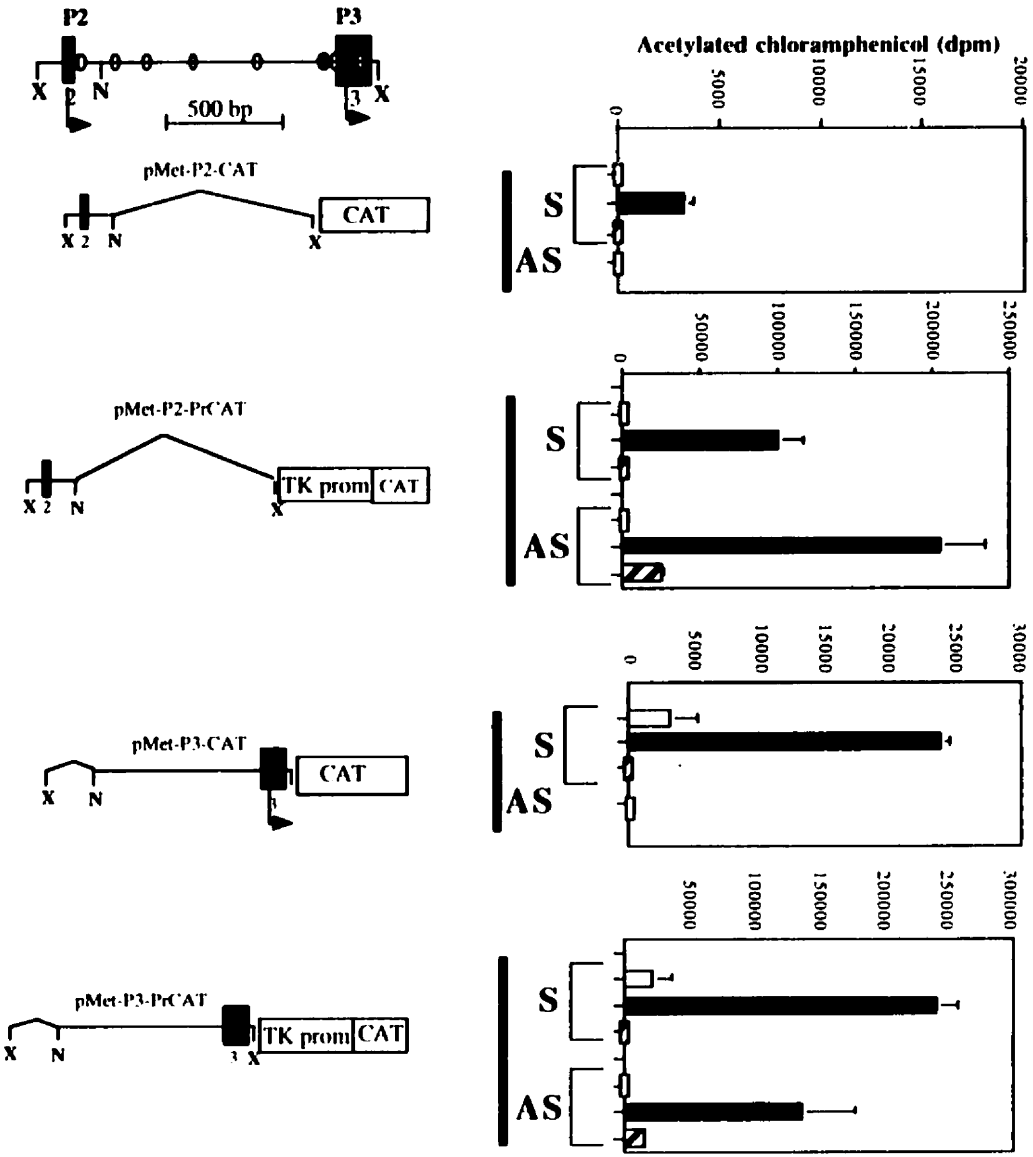


Figure 4 : Analysis of *dnmt1* promoter and enhancer functions. The physical map of *dnmt1* 5'-CAT constructs are shown relative to the *dnmt1* 5' region. Exons and transcription initiation sites are indicated. The predicted transcription initiation clusters are indicated as P1 to P4. Fragments deleted in each construct are indicated by a broken line. Sense (S) and antisense (AS) indicate the orientation of the genomic *dnmt1* insert relative to the CAT gene. Either HeLa or P19 cells were transfected with the indicated plasmids and CAT activity was measured as described in materials and methods. To determine whether transcription activity is controlled by either c-Jun or Rb, the cells were cotransfected as indicated with either control SK plasmid (indicated as an empty box), RSV-c-Jun (Binetruy *et al.*, 1991) indicated as a dark box, or pLRbRNL (ATCC RB1 65003) (indicated by a hatched box). Each value is presented as means $\pm$ S.D. of triplicate independent transfections. Similar results were observed using two different plasmid preparations and cell line cultures. For each transfection, the values obtained for the empty vectors pOCAT or pPr-CAT were subtracted as background. **A** Promoter and enhancer functions of the P1 region. **B.** Promoter and enhancer function of the P2 region. **C.** Promoter and enhancer function of the P3 region. **D.** Promoter and enhancer function of the P4 region.:


A
P1
P1b
P2
P3
P4
10 kb
500 bp
Potential AP-1 site
Potential TCF site
Potential E2F site
none
c-jun
Rb
Acetylated chloramphenicol (dpm)
pMet-P1-CAT
pMet-P1-ΔHX-CAT
pMet-P1-ΔXH-CAT
pMet-P1-PrCAT
pMet-P1-ΔHX-PrCAT
pMet-P1-ΔXH-PrCAT
CAT
TK prom
S
AS
Hela cells

B


P2
P3
X
2
N
500 bp
3
X
pMet-P2-CAT
CAT
pMet-P2-PrCAT
TK prom
CAT
Acetylated chloramphenicol (dpm)
0
5000
10000
15000
20000
50000
100000
150000
200000
250000
S
AS

C

pMet-P3-CAT
CAT
pMet-P3-PrCAT
TK prom
CAT
0
5000
10000
15000
20000
25000
30000
50000
100000
150000
200000
250000
300000
S
AS

D

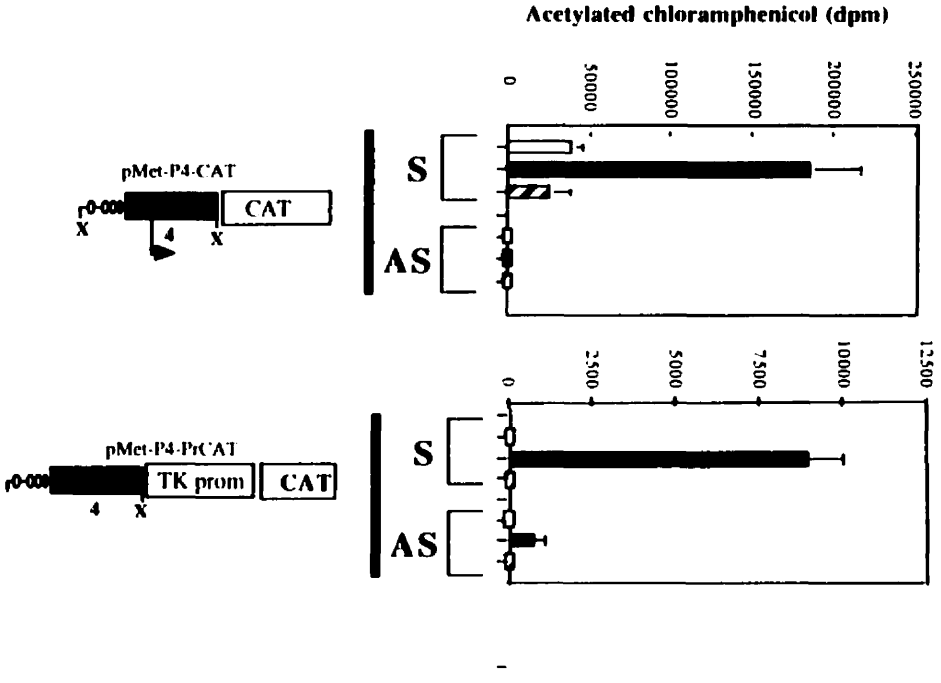
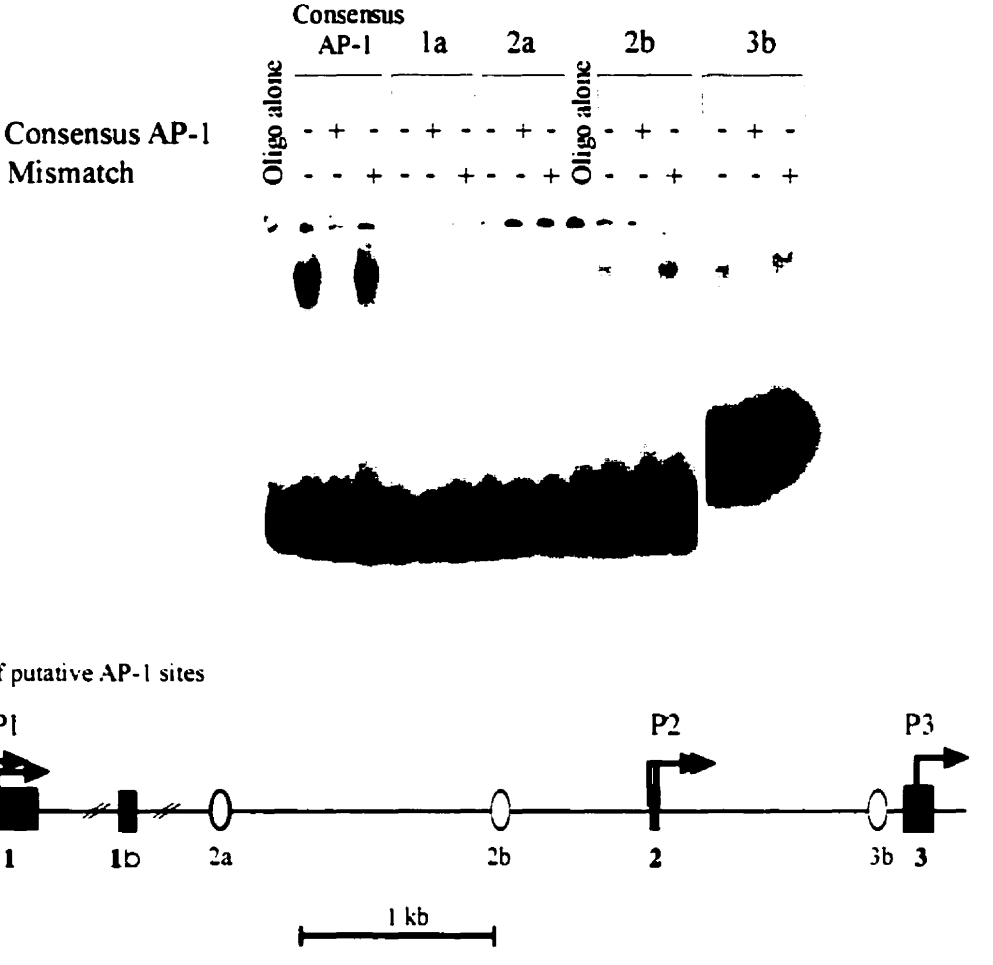

Acetylated chloramphenicol (dpm)
0
50000
100000
150000
200000
250000
pMet-P4-CAT
CAT
X
4
X
S
AS
0
2500
5000
7500
10000
12500
pMet-P4-PrCAT
TK prom
CAT
4
X
S
AS

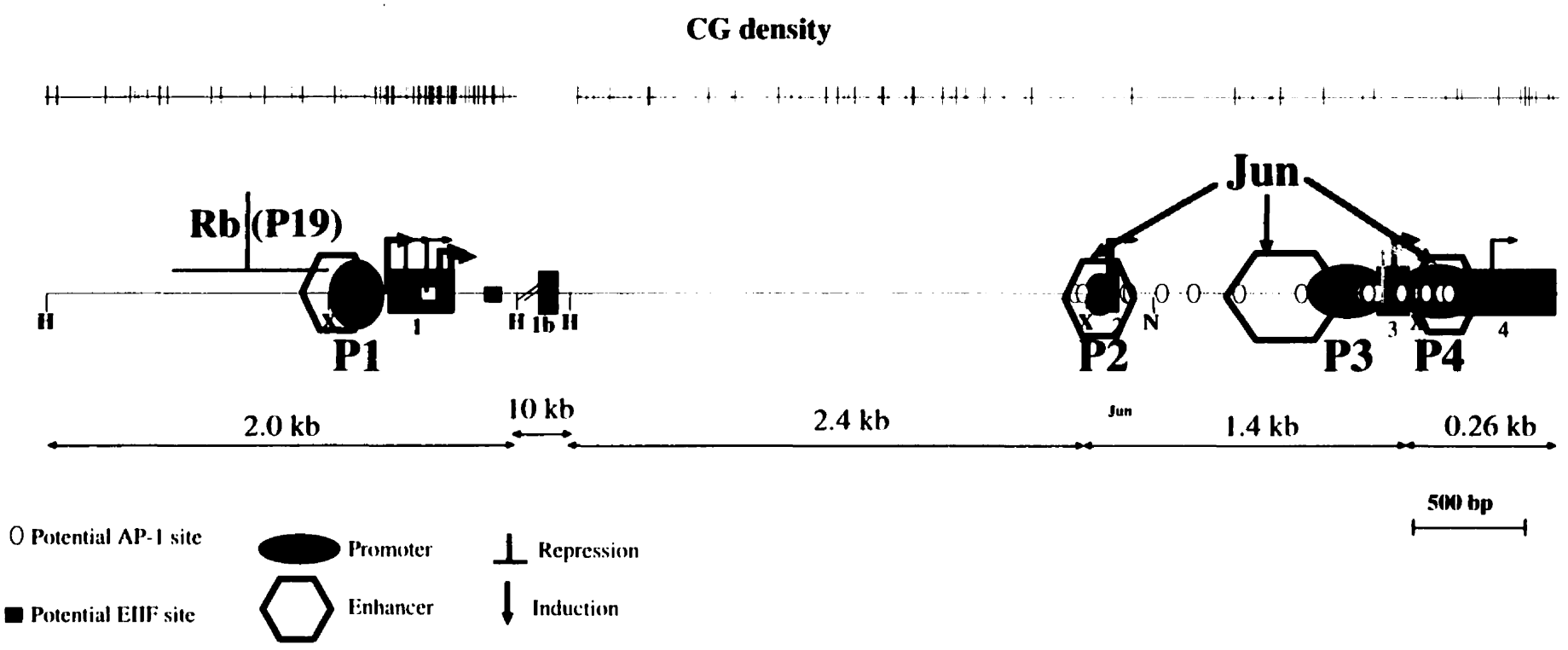
Figure 5: Binding of the AP-1 transcription complex to sequences in the *dnmt1* 5' upstream region. The first line indicates the position of the sequences tested for ability to form an AP-1 specific complex with nuclear extracts prepared from human lung carcinoma H446 cells. ^{32}P-labelled double stranded oligomers bearing either the consensus AP-1 recognition sequence, a putative AP-1 in P1 (1a) : a putative AP-1 site in P2 (2b) and a putative AP-1 site in P3 (3b) were incubated for 20 minutes at room temperature, following which a 300-fold excess of either a consensus AP-1 or a mismatch AP-1 double stranded oligomer competitor was added (as indicated) and the reaction was incubated for 20 additional minutes. The mixtures were then analyzed by electrophoresis on a 4% non-denaturing polyacrylamide gel.


Consensus
AP-1
1a
2a
2b
3b
Oligo alone
Oligo alone
Consensus AP-1
Mismatch
- + - - + - - + -
- - + - - + - - +
- + - - + - - + -
- - + - - + - - +
- + -
- - +
- + -
- - +

Location of putative AP-1 sites
P1
P2
P3
1a
1
1b
2a
2b
2
3b
3
1 kb

Fig. 6. Transcriptional regulation of *dnmt1*: a summary.

The first line shows the CG density of the 5' of the *dnmt1* gene relative to the physical map shown in the second line. Boxes indicate exons and horizontal arrows indicate transcription initiation sites. The position of putative binding sites for AP-1 (open circle) and E2F (shaded box) are indicated. Positions of promoters are indicated by an oval and enhancers by trapezoids. Enhancers that are dependent on c-Jun for activity are indicated. c-Jun activation is indicated by an arrow and Rb repression is indicated by a blunted line. The region upstream to exon 1 bears an enhancer which is active in HeLa cells and a repressor which is active in P19 cells.


CG density
Rb (P19)
Jun
H
X
1
H
1b
H
P1
X
N
P2
3
4
P3
P4
2.0 kb
10 kb
2.4 kb
Jun
1.4 kb
0.26 kb
500 bp
O Potential AP-1 site
Potential EIIF site
Promoter
Enhancer
Repression
Induction

The observations in chapter 2 indicate that both oncogenic and tumor suppressor signalling pathways can control transcriptional expression of *dnmt1.* There is a vast body of evidence connecting the deregulation of both oncogenic and tumor suppressor pathways in cancer, so it stands to reason that deregulation of these pathways is the mechanism responsible for upregulation of *dnmt1* expression in cancer tissue. Chapter 2 describes transcriptional elements that can respond to both classes of pathways, but the question of whether *dnmt1* expression can be unregulated by mechanisms other than transcription still remains. Evidence from non transformed cells indicates that *dnmt1* expression is controlled with the growth state of the cell and that this control is achieved by a posttranscriptional mechanism (Szyf *et al.*, 1991). Recently, it has been demonstrated that cellular transformation mediated by SV 40 large T antigen increases relative *dnmt1* mRNA abundance by a transcription independent mechanism, possibly through a direct interaction with the tumor suppressor protein Rb (Slack *et al.*, 1999). If misregulation of *dnmt1* expression is critical to the cellular transformation process then there is certainly a potential that the mechanism of post-transcriptional control of *dnmt1* can be disrupted in certain pathological states. Chapter 3 is an initial study which elucidates the mechanism of posttranscriptional control of *dnmt1* expression.

Chapter 3

A novel RNA element mediates growth dependent posttranscriptional regulation of DNA methyltrasferase expression

Shyam Ramchandani and Moshe Szyf

(Submitted)

DNA MeTase mRNA is regulated with the state of growth of cells at the posttranscriptional level. To understand the mechanisms responsible for posttranscriptional regulation of DNA MeTase, we determined whether sequences located at the 3' UTR of the DNA MeTase could destabilize a reporter globin mRNA. First, we show that the 3' UTR of DNA MeTase mRNA confers growth regulation on an heterologous stable mRNA b-globin. Second, our results suggest that the 3'UTR is composed of at least two basic components, a destabilizing 54 base highly conserved element that is not growth dependent and additional elements that are responsible for conferring cell-growth dependency. Third, we show using RNA gel shift assays and deletion analysis the presence of a specific RNA-protein complex formed with the 3'UTR sequence in growth-arrested cells and a different specific complex formed in cycling cells. Fourth, we show that treatment of growth arrested cells with cyclohexamide results in induction of DNA MeTase mRNA and disappearance of the growth-arrested specific RNA-protein complex. This observation is consistent with the hypothesis that a labile protein(s) mediates the growth dependent destabilizing of DNA MeTase mRNA. The element described in this study and the factors interacting with it are a new example of elements that confer growth dependent expression upon an mRNA.

DNA methylation is now recognized as an important mechanism of regulation of genome functions (Szyf, 1996). The hallmark of DNA methylation in vertebrates is the fact that not all methylatable CpG dinucleotide sequences are methylated, many sites are differentially methylated in different tissues or in the same cell (Yisraeli and Szyf, 1985). Methylation is believed to mark inactive genes (Nan *et al.*, 1997) and might be involved in other functions of the genome such as replication (Szyf, 1996; Rein, 1997). The pattern of methylation is maintained during cell division by the enzyme DNA methyltransferase (DNA MeTase) which

catalyzes the transfer of a methyl group from S-adenosyl-methionine to 5th position on C residing in the dinucleotide sequence CpG(Wu and Santi, 1985; Razin and Szyf, 1984). It has been proposed that regulation of the level of DNA MeTase activity in a cell is an important control over the pattern of DNA methylation and genome function (Szyf *et al.*, 1984; Szyf, 1991). Therefore the coordination of the rate of DNA synthesis and the level of DNA MeTase activity is critical for maintaining the pattern of methylation (Szyf *et al.*, 1991). If the pattern of methylation is important for genome functions then aberrant expression of DNA MeTase will result in a change in cellular identity. In accordance with this hypothesis it has been shown that elevated levels of DNA MeTase and hypermethylation of tumor suppressor genes are observed in tumors (el-Deiry *et al.*, 1991; Makos *et al.*, 1992) and cancer cell lines (Kautiainen and Jones, 1986), that ectopic elevated expression of DNA MeTase results in alterations in DNA methylation and cellular transformation (Wu *et al.*, 1993), partial inhibition of DNA MeTase by expression of an antisense or applying phosphorothioate modified oligonucleotides to DNA MeTase results in differentiation (Szyf *et al.*, 1992) and inhibition of tumorigenesis (MacLeod and Szyf, 1995; Ramchandani *et al.*, 1997) and that Min - mice which bear only one functional allele of DNA MeTase form polyps at a reduced frequency (Laird *et al.*, 1995).

Based on this data it has been proposed that regulation of DNA MeTase expression must be linked to the main cellular pathways regulating cell growth (Szyf, 1994). In accordance with this hypothesis we have shown that DNA MeTase is transcriptionally regulated by the Ras-Jun signaling pathway linking regulation of DNA MeTase expression to a nodal oncogenic pathway (MacLeod *et al.*, 1995).

It stands to reason that an enzyme that plays a critical role in regulating genomic functions is regulated at multiple levels. Transcriptional regulation alone is insufficient to achieve full control over the level of an enzyme, the cell must possess

mechanisms to remove the existing mRNA or proteins. A long line of evidence has established that regulation of mRNA stability is a very important mechanism involved in orchestrating the expression of a select group of critical genes in development (Surdej *et al.*, 1994), early response genes involved in cellular growth such as c-myc (Jones and Cole, 1987) c-fos(Treisman, 1985) and c-jun (23) as well as cytokines such as GM-CSF (Shaw and Kamen, 1986). Whereas the mechanisms responsible for regulating mRNA stability with developmental and other cellular control points are unclear, it is apparent that both cis-acting signals in the RNA and trans-acting factors interacting with them are responsible for altering mRNA stability (Malter, 1989). Three classes of RNA destabilizing elements have been identified in early response genes which differ by the sequence of the destabilizing motifs and the rate and kinetics of mRNA decay (Chen and Shyu, 1995). All these elements reside in the 3' UTR of the mRNAs and are located in a poly AU rich region (Chen and Shyu, 1995). A number of proteins that can interact with AU rich elements have been identified and some of them were cloned such as AUF1 (Zhang *et al.*, 1993), however their role in mRNA decay is unknown (Malter, 1989).

We have previously shown that the regulation of DNA MeTase expression with the state of cellular growth occurs at the posttranscriptional level (Szyf *et al.*, 1991). Whereas both growth arrested and cycling cells transcribe DNA MeTase at a similar rate, DNA MeTase mRNA is detected only in cycling cells (Szyf *et al.*, 1991). Recently it has been shown that posttranscriptional regulation plays a role in downregulation of DNA MeTase during differentiation of myoblasts in vitro (Liu *et al.*, 1996). The mechanisms responsible for posttranscriptional regulation of DNA MeTase are unknown. It stands to reason that the mechanisms involved in destabilizing DNA MeTase mRNA in arrested cells are linked to basic control points of the cell cycle and are potentially linked to cellular transformation. Similar to transcriptional control (MacLeod *et al.*,1995; Rouleau *et al.*, 1995), deregulated posttranscriptional control of DNA MeTase mRNA might be involved in

hyperactivation of DNA MeTase in cancer cells. We have recently shown that ectopic expression of SV40 T antigen increases the expression of DNA MeTase by increasing the stability of the mRNA[1] . To be able to dissect the mechanisms responsible for growth dependent regulation of DNA MeTase mRNA stability, the cis and trans acting factors responsible should be identified. We report here the identification of a novel RNA element that confers growth dependent regulation on a heterologous stable rabbit β-globin mRNA as well as RNA-protein complexes that specifically interact with these sequences in a growth phase dependent manner. No destabilizing mRNA element that differentiates cycling from growth arrested cells has been identified to date. The element described in this study and the factors interacting with it are a first example of elements that confer growth dependent expression upon an mRNA and are proposed to be first representatives of this class of posttranscriptional signals.

MATERIALS AND METHODS

Cell culture and stable transfection and analysis of mRNA- BALB/c 3T3 cells (ATCC) were maintained as a monolayer in Dulbeccos Modified Medium (GIBCO-BRL) containing 10% fetal calf serum (FCS) (Medicorp, Montreal). Cycling cells were harvested at 50% confluency. To arrest cells at Go, confluent cultures of BALB/c 3T3 were transferred to a low serum medium (1% FCS) for 72 hours. To measure the effects of inhibition of protein synthesis on DNA MeTase mRNA, the protein synthesis inhibitor cyclohexamide (Boehringer Mannheim) was added to Go arrested cells at a final concentration of 10μg/ml for periods of 1 to 8 hours. RNA was prepared by the guanidinium isothiocyanate method (Rouleau *et al.*, 1992). The level of DNA MeTase mRNA was determined by an RNAse protection assay using a genomic DNA MeTase probe encoding the proximal transcription initiation sites of DNA MeTase and exons 3 and 4 as previously described (Rouleau *et al.*, 1992). The levels of b-globin and neomycin resistance mRNA were determined by a Northern blot analysis (using 10 μg of total RNA)

[1] Pinard and Mszyf, unpublished

and hybridization with a ^{32}-P labeled rabbit b- globin probe (a 500 bp XhoI-BamHI fragment from pCG-RBG or a neomycin probe (encoding the neomycin gene). The level of expression of the different mRNAs was quantified by densitometric scanning of the relevant autoradiogram and normalizing each lane for the amount of total RNA transferred in this lane as determined by hybridization with a ^{32}P labeled 18s ribosomal RNA oligonucleotide probe (Szyf *et al.*,1990). Five µg of either, pGC-RBG (a control plasmid bearing the b-rabbit globin gene and a neomycin resistance gene as a selectable marker), pAT-RBG (bearing the 3' UTR of human granulocyte-macrophage-colony-stimulating factor GM-CSF) (both plasmids were a kind gift of Dr. G Shaw) (Shaw and Kamen, 1986) or chimeric constructs bearing different deletions of the human DNA MeTase inserted upstream of the b-rabbit globin 3' UTR, were introduced into BALB/c 3T3 cells by DNA mediated gene transfer using the calcium phosphate protocol (Ausubel *et al.*, 1988). G418-resistant colonies were cloned and propagated in selective medium containing 0.25 mg/ml Geneticin (GIBCO-BRL) and analyzed for expression of the transfected DNA by Northern blot analysis (Ausubel *et al.*, 1988).

Plasmid construct- pCRII-3'UTR- Human DNA MeTase 3'UTR (5090-5408) was amplified by RT-PCR from 1 µg of total RNA prepared from human lung cancer H446 cell line (ATCC). Reverse transcription was performed using random primers (Boehringer Mannheim) and Superscript reverse transcriptase as recommended by the manufacturer (GIBCO-BRL). For PCR amplification with Taq polymerase (Clontech,) 50 ng of the sense primer; 5' TCTGCCCTCCCGTCACCC3' and antisense primer; 5'GGTTTATAGGAGAGATTT3' were used to amplify the 3'UTR from a 5µl of reverse transcribed cDNA in the presence of 1mM dNTPs and the manufacturers amplification buffer supplemented to 2mM $MgCl_2$. Cycling conditions were: 95^{o}C 1min.: 50^{o}C 1 min.: 72^{o}C 1.5 min. for 30 cycles. The amplified fragment was subcloned into pCR II using TA Cloning Kit (Invitrogen) as recommended by manufacturer . To generate pRBG-MET 3'UTR, the human DNA MeTase 3'UTR

was excised from PCRII-3UTR with BamH1 and Xba1 and ligated into a XbaI-SalI digested pRBG -GC the incompatible ends were filled using Klenow DNA polymerase and the blunt ends were ligated.

pRBG-Consensus: sense and antisense oligonucleotides coding for bases 5349 to 5405 bearing 5' Xba1 and 3' Sal1 overhangs were annealed and ligated either into pRBG-GC cut with Xba1 and Sal1 to generate p MET (3'UTR) or ligated into pBluescript SK- which was cut with Xba1 and Sal1 (to generate the gel shift probe template).

3'UTR deletion 1 was generated by PCR amplification using 10 ng of pCR II-MET 3'UTR as a template, the 3'UTR sense primer as above and an antisense primer; 5'GTCGACTTAATTTCCACTCATACAGTGGTAG3'. The amplified sequence was cloned in PCRII, removed from vector by Sal1 digestion and ligated into pBluescript cut with Sal 1 and Sma1 (gel shift probe template).

3' UTR deletion 2 was generated by PCR amplification using PCRII-MET3'UTR as a template and the antisense primer

5'GTCGACTTAGTTGATAAGCGAACCTCACACA3'.

RNA binding gel shift assays- The relevant RNA probes were transcribed using the appropriate plasmid templates, either T7, T3 or Sp6 RNA polymerases and either [a-^{32}P]UTP(3000Ci/mmol) (for gel shift probes) or nonradioactive UTP (for competitor) using the Ambion in vitro transcription kit as recommended by the manufacturer. The labeled probe was diluted to 100,000 cp/µl.

Preparation of whole cell extracts - Ten million BALB/c 3T3 cells were pelleted and resuspended in 100 µl of extraction buffer (20 mM Tris-HCl pH 7.5, 0.4 M KCl, 20% (v/v) glycerol, 2mM DTT, 100 µg PMSF, 10µg aprotinin and 1mM Na_3VO_4), frozen immediately at -80^{o}C for 1 hour and the whole cell extract was isolated by centrifugation at 10000g for 15 min. The supernatant was recovered and used for RNA binding assays. The ^{32}P labeled RNA probe used for binding assays was denatured at 95^{o}C for 5 min. followed by slow cooling to

37°C. RNA gel shift binding assays were incubated on ice for 30 min. in a 15µl reaction mixture using 40µg of whole cell extract, 100000 cpm of probe (4ng RNA), 10µg tRNA (as a non specific RNA competitor), 5X cold competitor probe 20 ng in the following buffer: 10 mM Hepes pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1mM DTT. Following incubation, free unbound ^{32}P -labeled RNA was removed by treating the reaction mixture with RNAse T1 (1 unit/ml) and RNAse A (10mg/ml) for 10 min at room temperature. Heparin 5 µg/ml was added and the reaction was incubated for additional 10 min. at room temperature. The resulting complexes were resolved on a 4% non-denaturing polyacrylamide gel.

RESULTS

Expression of DNA MeTase mRNA is induced by cyclohexamide in growth arrested cells- We have previously shown that even though DNA MeTase is transcribed in growth arrested cells the mRNA is not detectable (and fig. 1A). This data is consistent with the hypothesis that DNA MeTase is destabilized in growth arrested cells. Unstable early response gene mRNAs such as c- myc could be induced by the protein synthesis inhibitor cyclohexamide (Dony *et al.*, 1985). Whereas it is yet unclear whether ongoing translation of an mRNA influences its rate of decay, cyclohexamide is believed to increase the stability of RNA by inhibiting the synthesis of a labile protein which destabilizes the mRNA (Dony *et al.*, 1985). To test whether DNA MeTase mRNA is destabilized in growth arrested cells by a labile protein, we determined using an RNAse protection assay the level of DNA MeTase mRNA in growth arrested BALB/c 3T3 cells treated with cyclohexamide. As observed in Fig. 1B cyclohexamide treatment results in the appearance of detectable levels of DNA MeTase mRNA in growth arrested cells 1 hour after initiation of treatment. DNA MeTase mRNA remains high up to 8 hours post treatment. Whereas this data does not prove that a labile protein destabilizes DNA MeTase mRNA in growth arrested cells, it is consistent with the hypothesis. In summary, similar to other early response genes, DNA MeTase mRNA is stabilized in growth arrested cells by a protein synthesis inhibitor suggesting that

the mechanism of posttranscriptional regulation of DNA MeTase mRNA might be similar to that of other early response genes such as c-myc and GM-CSF.

The 3'UTR region of DNA MeTase mRNA determines its cell growth dependent stability

If a protein is involved in growth regulation of DNA MeTase it must interact with sequences in the mRNA. As most of the sequences controlling RNA decay are located in the 3' UTR, we focussed on this region. The destabilization of early response genes is mediated by an AU rich element (ARE) located in the 3' UTR. Three classes of destabilizing elements have been identified which differ based on the presence of either AUUUA motifs (class I), UUAUUUA(U/A)(U/A) such as c-fos, nonamer motifs (class II) such as GM-CSF or c-fos (Chen and Shyu, 1995) or U rich but no AUUUA motifs such as c-jun (Chen and Shyu, 1995). None of the first two elements are found in DNA MeTase 3' UTR and there is no identifiable homology between DNA MeTase 3' UTR and other UTRs except the fact that it is U rich. Other properties of DNA MeTase posttranscriptional regulation such as the growth dependent stability of the mRNA which is not observed in other early response genes discussed above suggest that its regulation is different from the regulation of early response genes (Chen and Shyu, 1995). To test the hypothesis that growth regulation by DNA MeTase involves cis-acting sequences in the 3'UTR of the DNA MeTase mRNA we tested whether this region could direct growth dependent regulation of the heterologous very stable rabbit b-globin mRNA . The GM-CSF ARE has been shown to destabilize b-globin mRNA when inserted into its 3' UTR (23). We inserted the 3'UTR of DNA MeTase into the rabbit b-globin gene 3'UTR as indicated in Fig. 2B (pMET-RBG) and introduced it into BALB/c 3T3 cells. As a control we used the stable pGC-RBG construct and the pAT-RBG construct bearing the destabilization element from GM-CSF as a positive control. RNA was prepared from growth arrested (A) and cycling (C) transfectants and the relative level of globin mRNA under each condition was quantified relative to 18s rRNA (Fig. 2C and D). The results presented in Fig.

2C and quantified in Fig. 2D demonstrate that the 3' UTR of DNA MeTase can confer growth regulation on a heterologous mRNA whereas the stable pGC-RBG (GC) does not show significant variation with the growth phase (similar results have been observed with other pGC-RBG transfectants). As observed in Fig. 2C, the GM-CSF (AT) destabilizing element destabilize mRNA under both cycling and arrested conditions. The DNA MeTase element described above is a novel element regulating the stability of DNA MeTase mRNA with the growth phase of the cell.

The consensus sequence in the 3'UTR of DNA MeTase is a destabilizing element

A homology comparison of the human, mouse, Xenopus and chicken DNA MeTase 3'UTR reveals a 54 bp stretch showing striking homology of 100% between the human and chicken DNA MeTase (Fig. 2A). Such an homology is unusual in a noncoding region and strongly suggest an important regulatory role for this RNA sequence. The results presented in Fig. 2C, D show that the DNA MeTase 3'UTR consensus sequence acts as a destabilizer when inserted into RBG (CON). The level of expression of globin in a vector bearing the DNA MeTase 3'UTR consensus sequence is dramatically reduced in both cycling an arrested cells as observed with the GM-CSF ARE (pAT-RBG). To account for differences in transfection efficiency between the different clones, the level of expression of globin was normalized to the level of expression of the neomycin resistant gene mRNA which is expressed from the same vector (Fig. 2E). The results shown in Fig. 2E demonstrate a very significant reduction in the level of globin mRNAs bearing either the GM-CSF ARE or the DNA MeTase consensus (CON) when compared to the control rabbit b-globin mRNA. In summary the 3'UTR of DNA MeTase bears a core destabilizer sequence that, similar to other early response genes AREs (Chen and Shyu, 1995) does not show growth dependent regulation. Other sequences in the 3'UTR must mediate growth regulation, stabilizing the message in cycling cells (Fig. 2E MET1, 6) and destabilizing it in arresting cells (Fig. 2D hatched bars MET 1 and 6). The growth

regulation of DNA MeTase mediated by the 3'UTR must be a result of an interaction between the core destabilization element identified here and other sequence that determine cell cycle modulation of the core destabilizing element.

Identification of DNA MeTase 3'UTR RNA-binding activities -Does growth regulation of DNA MeTase mRNA stability involve the formation of growth phase specific RNA-protein complexes? To address this question we performed an RNA gel mobility shift assay using in vitro synthesized ^{32}P labeled DNA MeTase 3'UTR RNA and whole cell extracts prepared from cycling and growth arrested BALB/c 3T3 cells as described in Materials and Methods. The RNA-protein complexes were subjected to RNase digestion to remove free unbound RNA prior to resolving of the complex on a nondenaturing polyacrylamide gel. As observed in Fig. 3 B (U lanes) RNA protein complex A (composed of three closely migrating complexes) is highly abundant in growth arrested cells (A) and present at low levels in cycling cells (C). The fact that a putative growth-arrested-cells specific complex is present at all in whole cells extract prepared from cycling cells is not surprizing since a nonsynchronized population of cycling cells contains a fraction of cells that are not at the synthetic phase. The enrichment of this complex in growth arrested cells is consistent with the hypothesis that the 3' UTR is recognized by specific proteins in growth arrested cells. Complex C is specific for cycling cells and absent in growth arrested cells. The absence of this complex in whole cells extracts prepared from arrested cells is consistent with the fact that a serum deprived cell population does not contain a significant sub-population of dividing cells. These complexes are formed in the presence of excess of nonspecific tRNA suggesting that they mediate specific interactions. In addition, pre incubation of the extracts with a 5 fold excess of cold 3'UTR RNA results in inhibition of complex formation with both complexes A and C (Fig. 3B).

To further test the correlation between growth-dependent destabilization and RNA-protein complexes A and C, we tested whether the 3' consensus sequence (5349-5405) forms these growth dependent complexes. As described above,

while this element is destabilizing (Fig. 1C and E) mRNA in cis it does not do so in a growth-state dependent manner. As observed in Fig. 3B, the 3' UTR bearing the consensus destabilizing region does not form either complexes A or C but does form complex B which is independent of state of growth. This seems to be a low specificity complex since it is not competed out by excess cold full 3'UTR or the consensus sequence. The conclusion that the consensus region does not form complexes A or C is further supported by the fact that whereas the full 3'UTR sequence can efficiently compete the formation of complexes A and C the 3' consensus sequence is incapable of doing so. This data is consistent with either the possibility that the consensus region is not involved in forming complexes A or C or with the prospect that efficient binding requires interaction with a number of additional UTR elements in addition to the consensus element. Thus, the binding data is consistent with the hypothesis that sequences located upstream of the 3' destabilizing sequence are required to mediate growth state specific regulation of RNA-protein interactions and MeTase mRNA stability.

Does the consensus 3' sequence participate in forming the growth-arrest specific complexes A or can one dissociate the destabilizing element from the elements forming complexes A and C? To address this possibility we tested whether a 3'UTR deletion that does not bear the 3' consensus region (D1) can form complex A. As shown in Fig. 3D, deletion of the consensus 3' sequence inhibits the formation of complex A suggesting that the 3' consensus sequence participates in the formation of complex A and it does so in combination with additional elements located upstream in the 3'UTR. Similarly, deletion of the sequences at 5090-5350 (D2) does not precipitate the formation of complex A. Each of the deletion constructs forms lower size RNA-protein complexes which is consistent with the model that complex A is a multi-peptide RNA-protein complex requiring the participation of a number of RNA-protein recognition elements in the 3'UTR. Different complexes are formed in cycling versus arrested cells which might be a consequence of different combinations of growth-state specific and nonspecific

proteins. For example D1 forms a strong lower molecular complex in growth arrested cells (Fig. 3D D1) but a higher molecular weight and weak complex in cycling cells.

Cyclohexamide inhibits the formation of complex A- One of the distinct features of many destabilized early response mRNAs such as c-myc is induction by the protein synthesis inhibitor cyclohexamide (Dony *et al.*, 1985) Different reasons might explain this induction, inhibition of synthesis of a labile transcriptional repressor, dependence of mRNA destabilization on translation of the mRNA, as well as removal of a labile protein involved in destabilization which is the acceptable model for the induction of c-myc by cyclohexamide. DNA MeTase mRNA is also induced following cyclohexamide treatment (Fig. 1). This observation enables us to determine whether complex A is involved in destabilization of DNA MeTase mRNA. If a component of complex A is a labile protein involved in destabilization of DNA MeTase mRNA at G0 as predicted by the mRNA data, then cyclohexamide treatment should result in removal of complex A. Complex B which is present in both cycling and arrested cells should not be affected and could serve as an internal control. As observed in Fig. 3C, a one hour treatment of growth-arrested BALB/c 3T3 cells with cyclohexamide results in disappearance of complex A but complex B remains intact. Thus, complex A formation is correlated with regulation of growth arrested cells and DNA MeTase mRNA instability. The complex is formed in growth arrested cells, it requires the presence of sequences known to be required for growth regulated destabilization and is removed by cyclohexamide which induces DNA MeTase mRNA stability.

DISCUSSION

This report addresses the question of what is the mechanism responsible for regulation of DNA MeTase with the growth status of a cell? Since hyperactivation of DNA MeTase has been suggested to play a causal role in cellular

transformation, understanding how DNA MeTase is regulated with cell growth is of obvious interest. Previous data has suggested that this regulation occurs at the posttranscriptional level (Szyf *et al.*, 1991). A long list of data has shown that control of mRNA stability is an important mechanism of gene regulation during development (MacLeod *et al.*, 1995) and is an important control over expression of proto oncogenes (Dony *et al.*, 1985) and cytokines (Shaw and Kamen, 1986). Elements that can target mRNA for rapid degradation (AREs) were previously identified in AU rich region at the 3' ends of proto oncogenes such as c-fos (Treisman, 1985) c-myc (Jones and Cole, 1987) and c-jun (33). A number of proteins have been described that can bind the AUUUA motifs (Chen and Shyu 1995). An element that is responsible for differential regulation of mRNA stability with cell growth has been not been identified to date. DNA MeTase mRNA is an excellent model for dissecting the mechanisms linking regulation of mRNA stability and the control of cell growth.

In this paper we demonstrate that first, similar to some early response genes that are actively destabilized (Chen and Shyu, 1995), DNA MeTase mRNA levels are induced in growth-arrested cells by the protein synthesis inhibitor cyclohexamide (Fig. 1). It has been previously proposed that cyclohexamide induces unstable mRNAs by inhibiting the synthesis of a putative labile protein involved in destabilization of mRNA (Dony *et al.*, 1985). Cyclohexamide treatment can be therefore utilized as an experimental paradigm to identify proteins responsible for DNA MeTase mRNA destabilization. Second, we show that the 3' UTR of DNA MeTase mRNA confers growth regulation on an heterologous stable mRNA b-globin suggesting that cis-acting sequences in the 3' UTR of DNA MeTase mRNA can direct destabilization of juxtaposed mRNA in growth arrested cells. Third, we identify a 54 base pair highly conserved element in the most 3' region of the DNA MeTase 3'UTR that destabilizes heterologous globin mRNA in both growth arrested and cycling cells. This implies that the 3'UTR is composed of two basic components, a destabilizing element that *per se* is insensitive to the growth state

of the cell and additional elements that are responsible for conferring cell-growth dependency. One possible mechanism is that the additional elements direct the formation of RNA-protein complexes that either promote or inhibit the destabilizing activity. Fourth, in accordance with this postulated mechanism, we show using RNA gel shift assays the presence of a specific RNA-protein complex formed with the 3'UTR sequence in growth-arrested cells and a different specific complex formed in cycling cells. As predicted from the lack of growth regulation by the destabilizing sequence *per se*, the 54 bp destabilizing sequence does not form a growth state specific RNA-protein specific complex. This suggests that the formation of the growth-arrest specific complex requires additional elements to the consensus destabilizing sequence. The destabilizing sequence is required however for forming of both the growth-arrest and cycling specific complexes since deletion of the destabilizing sequence results in the absence of these complexes. The deletion analysis suggests that a combination of a number of elements in the 3'UTR is required to build these RNA-protein complexes. Since the different deletions can form smaller RNA-protein complexes when assayed by themselves, the most plausible explanation is that the growth state specific RNA-protein complexes formed with the 3'UTR are combinations of multiple RNA-binding proteins interacting with the different elements in the 3'UTR. Fifth, we show that treatment of growth arrested cells with cyclohexamide results in disappearance of the growth-arrested specific RNA-protein complex which is consistent with the hypothesis that a labile protein(s) mediates the destabilizing of DNA MeTase mRNA and that the complex observed in the growth arrested cells plays a role in destabilizing DNA MeTase mRNA.

The data presented here identify a novel element regulating DNA MeTase mRNA stability in a growth dependent manner. This element is composed of a core destabilizing sequence which is 100% conserved between human and chicken that is independent of the growth state of the cell and additional sequences that interact with RNA-binding proteins to form growth-state specific complexes. We

hypothesize that these complexes can either promote or inactivate the destabilizing activity. Our data does not demonstrate a causal link between the presence of RNA-protein complexes and mRNA stability. However, our results do show good correlation between DNA MeTase mRNA stability and the appearance of specific RNA-protein complexes. Additional experiments will be required to demonstrate whether and how these complexes play a role in determining mRNA stability. This bi modal mechanism of action of the DNA MeTase 3'UTR can serve as a general model for other growth-state-specific regulations of mRNA stability. The fact that the destabilizing core sequence *per se* does not differentiate between arrested and cycling cells is consistent with what is observed with other AREs such as c-fos (Chen and Shyu, 1994) and c-myc (Jones and Cole, 1987). It is possible that similar to DNA MeTase additional elements are present in the 3'UTR of other proto oncogenes that differentially regulate the activity of the core destabilizing sequence. The conserved destabilizing sequence identified in our study does not bear the AUUUA motif which is found in class I AREs or the nonamer UUAUUUA(U/A)(U/A) found in class II AREs. It is however AU rich and might be a member of the non-AUUUA family of AREs which includes the proto oncogene c-jun (34).

Regulation of mRNA stability of key mRNAs is most probably a critical point in the control of cell growth. Deregulation of this mechanism could possibly lead to cellular transformation. Our non published results suggest that ectopic expression of the viral oncogene SV40 T antigen alters the stability of DNA MeTase mRNA and that this plays a causal role in cellular transformation[2]. Identification of the RNA elements that determine the stability of DNA MeTase mRNA with the growth state of the cells and the trans acting factors that interact with them is critical for understanding the molecular links between the nodal points of control of cell growth and the control of mRNA stability. Uncovering these links may provide us with a better understanding of the processes of cellular growth and cellular transformation.

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[2] Pinard and Szyf, non published

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Fig. 1. Induction of DNA MeTase mRNA by cyclohexamide in growth arrested BALB/c 3T3 cells.

A. RNA was extracted from serum depleted and cycling BALB/c 3T3 cells and subjected (5µg) to an RNAse protection assay using a 700-bp riboprobe (probe A) (30) encoding the DNA MeTase genomic sequences containing exons 3 and 4 and the proximal initiation sites (30). The major protected bands (92-99 bp) are indicated by arrows. To evaluate the amount of total RNA in each sample a riboprobe bearing the coding sequence of 18s ribosomal RNA was included in the RNase protection assay.

B. BALB/c 3T3 cells were arrested by serum depletion as described in materials and methods and treated with 10µg/ml of cyclohexamide for the indicated time points. RNA was prepared at each of the time points and subjected to an RNase protection assay using a DNA MeTase and 18s riboprobes.

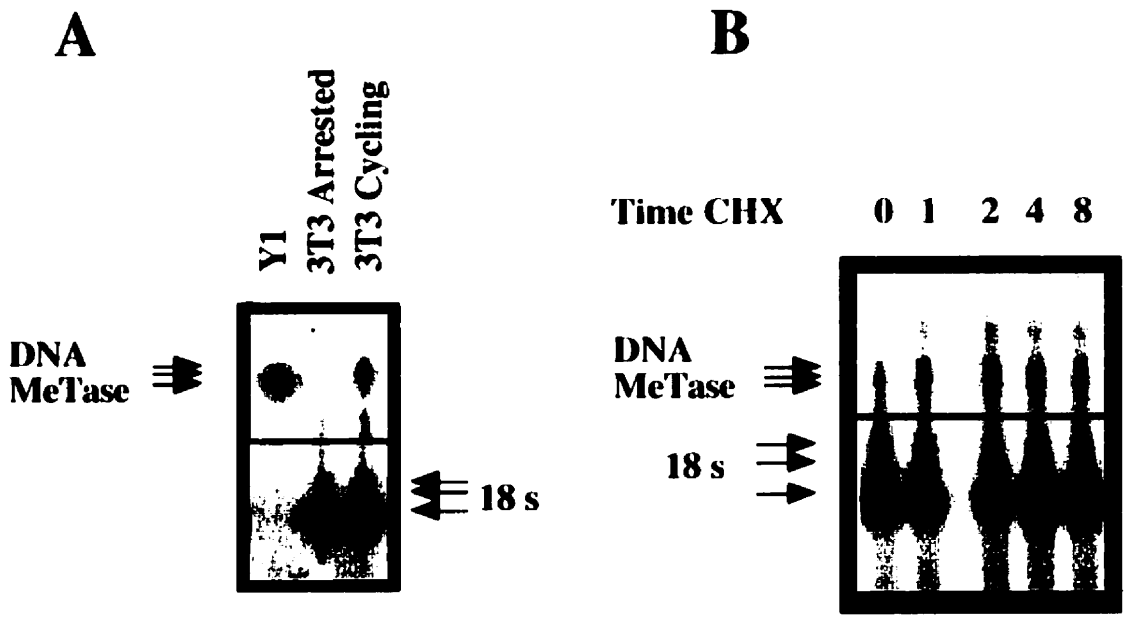

A
Y1
3T3 Arrested
3T3 Cycling
DNA
MeTase
18 s
B
Time CHX
0 1 2 4 8
DNA
MeTase
18 s

Figure 2. The 3' UTR of DNA MeTase mRNA destabilizes β-globin mRNA in a growth dependent manner.

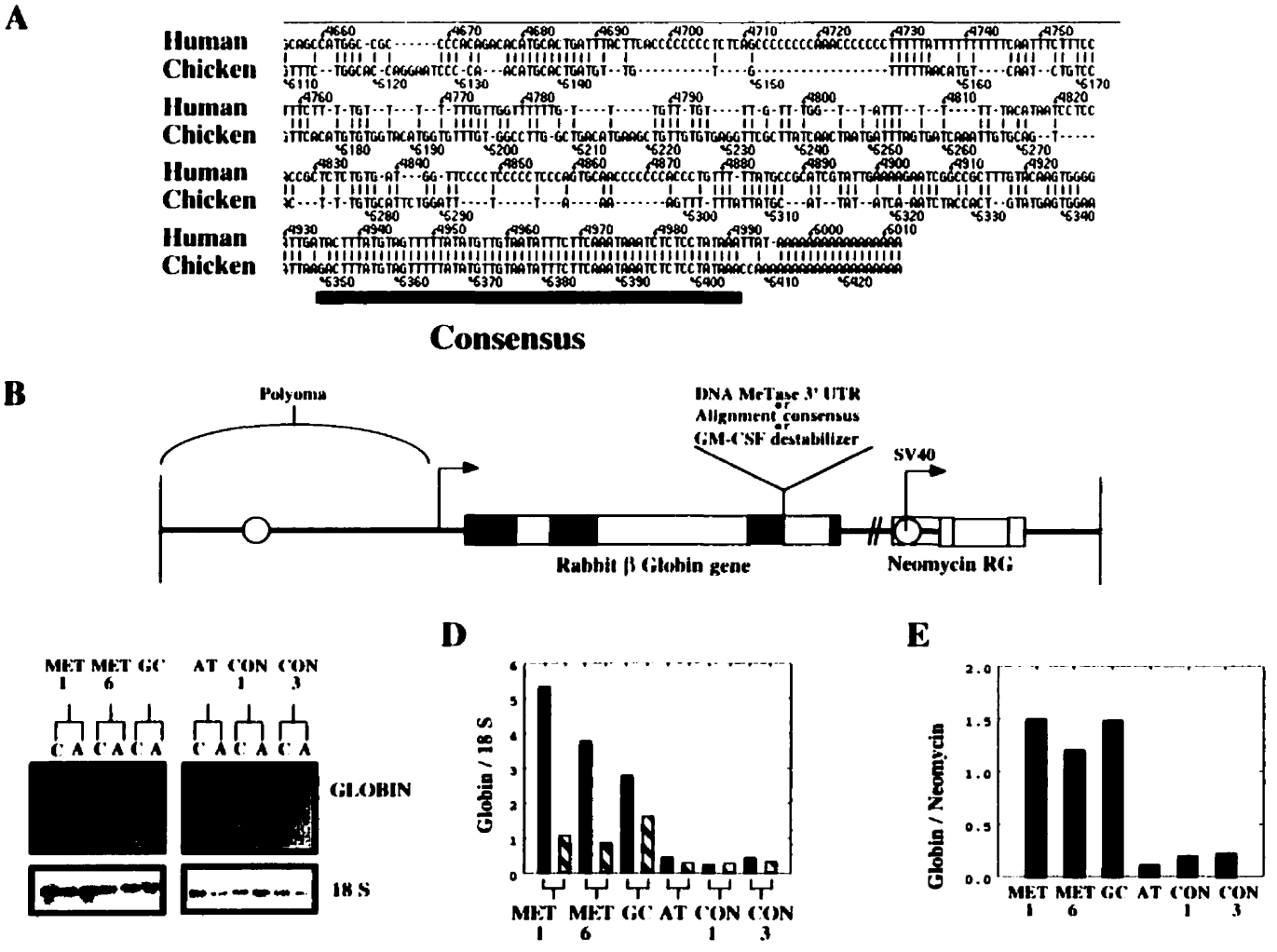
A. Homology between the 3'UTR of the human and chicken DNA MeTase. The 3' sequences that are 100% homologous are indicated by a line and are defined as consensus.

B. Map of the RBG construct. The site of insertion of the 3' UTR is indicated.

C. BALB/c 3T3 cells were transfected with either one of the following plasmids: pMET-RBG (MET), pGC-RBG (GC), pAT-RBG (AT) and pCON-RBG (CON) and stable G418 resistant transfectants were isolated as described in materials and methods. RNA was extracted from the indicated transfectants and subjected to a Northern blot (Hybond+ (Amersham) analysis using standard protocols and sequential hybridization to a labeled globin probe, stripping and hybridization with a 18 s probe followed by neomycin resistance probe.

D. The amount of globin mRNA as well as neomycin resistance expressed in each of the transfectants was quantified by scanning densitometry . The amount of globin expressed was normalized to the signal obtained following hybridization with an 18 s probe as an internal control, solid bars for cycling cells, hatched bars for arrested cells.

E. To compare the expression of the globin in the different transfectants we normalized the obtained signal for globin to the signal obtained with a neomycin resistance probe.

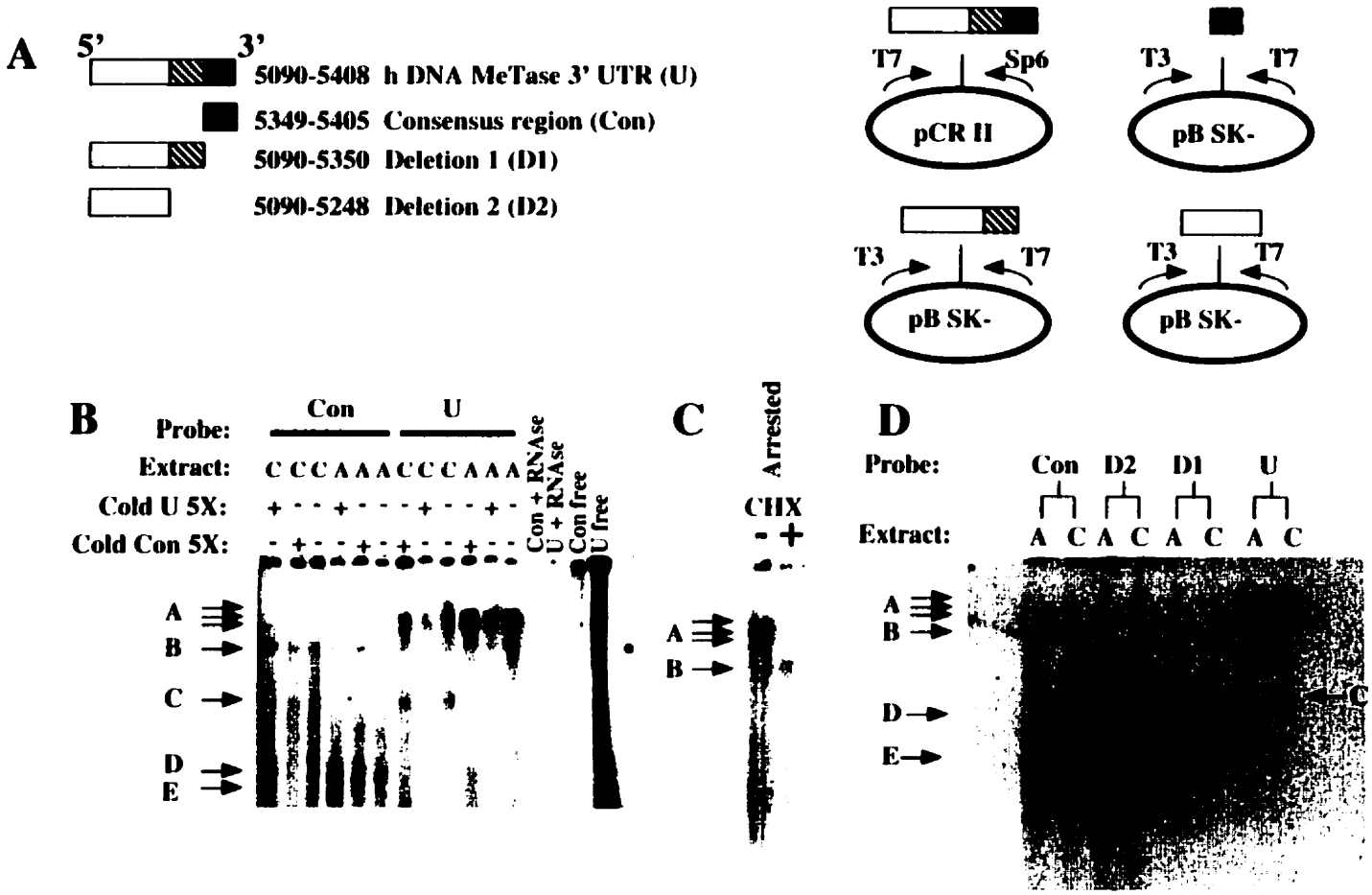

A
Human
Chicken
Consensus
B
Polyoma
DNA MeTase 3' UTR
or
Alignment consensus
or
GM-CSF destabilizer
SV40
Rabbit β Globin gene
Neomycin RG
C
MET MET GC AT CON CON
1 6 1 3
C A C A C A C A C A C A
GLOBIN
18 S
D
Globin / 18 S
MET MET GC AT CON CON
1 6 1 3
E
Globin / Neomycin
MET MET GC AT CON CON
1 6 1 3

3. Extracts prepared from growth arrested and cycling cells form different complexes with the 3' UTR of DNA MeTase as determined by RNA gel shift assay. A. The different deletion fragments used to construct the plasmids are indicated. The orientation of the different promoters is indicated.

B. An RNA gel shift assays were performed using ^{32}P labeled in vitro transcribed DNA MeTase 3' UTR (U) or the 3' consensus sequence (Con) and extracts prepared from either arrested (A) or cycling (C) BALB/c 3T3 cells. As a control for specificity, the reaction mixture included non labeled (cold) mRNA in the indicated samples (UTR or Con). The complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. The different complexes formed are indicated by arrows. The first two lanes in the right contain the nonreacted labeled probes. The next two lanes are the non reacted RNase digested probes.

C. Growth arrested BALB/c 3T3 control cells and cycling cells were treated with cyclohexamide (+) (10µg/ml) or without (-) for 1 hour. Whole cell extracts were prepared from the control and cyclohexamide treatment, reacted with the labeled 3'UTR and resolved on a nondenaturing polyacrylamide gel. The position of the complexes are indicated.

D. RNA gel shift assay with labeled RNA bearing different deletions of the 3'UTR as indicated in A. RNA gel shift assay was performed as above and subjected to nondenaturing polyacrylamide gel electrophoresis. The position of the different complexes are indicated.


A
5'
3'
5090-5408 h DNA MeTase 3' UTR (U)
5349-5405 Consensus region (Con)
5090-5350 Deletion 1 (D1)
5090-5248 Deletion 2 (D2)
T7
Sp6
pCR II
T3
T7
pB SK-
T3
T7
pB SK-
T3
T7
pB SK-
B
Probe:
Con
U
Extract:
C C C A A A C C C A A A
Cold U 5X:
Cold Con 5X:
Con + RNAse
U + RNAse
Con free
U free
A
B
C
D
E
C
Arrested
CHX
A
B
D
Probe:
Con
D2
D1
U
Extract:
A C A C A C A C
A
B
D
E
C

The observations from chapters 2 and 3 describe mechanisms of control of the expression of *dnmt1*. These are, potentially, also the mechanisms that the cell takes advantage of in the process of cellular transformation, which will lead to cancerous cell growth. Is the upregulation of *dnmt1* required for cellular transformation or is it just a consequence of the deregulation of oncogenic and tumor suppressor pathways? Inhibition of cellular transformation of tumorigenic cells can be accomplished by inhibiting *dnmt1* expression (MacLeod and Szyf, 1995) suggesting that one of the key goals of deregulation of both oncogenic and tumor suppressor pathways in the process of cellular transformation is to up regulate *dnmt1* expression. Can inhibition of *dnmt1* expression *in vivo* inhibit cellular transformation? Mice which bear the homologous mutation found in human familial colon cancer of the *apc* gene develop spontaneous colorectal polyps which lead to cancerous lesions. A genetic cross of mice bearing this mutation with mice that are heterozygous null for *dnmt1* (*dnmt* $^{+/-}$) produces mice that develop far fewer polyps and cancerous lesions in the intestine (Laird *et al.*, 1995). Analysis of *dnmt1* $^{+/-}$ mice demonstrates that they express approximately half of the DNA MeTase activity that wildtype mice do (Li *et al.*, 1992). These data suggest that inhibition of *dnmt1* expression by pharmacological means will have positive therapeutic implications in the treatment of cancer. The following manuscript (Ramchandani *et al.*, 1997) tests this hypothesis by using a phosphorothioate modified antisense oligonucleotide directed against the *dnmt1* mRNA sequence to treat mice which were implanted with tumorigenic cells. This manuscript provides the initial preclinical basis for the development of antisense oligonucleotides directed against *dnmt1* as a viable therapeutic strategy against human cancer.

Chapter 4

Inhibition of tumorigenesis by a cytosine-DNA methyltransferase antisense oligodeoxynucleotide.

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This paper tests the hypothesis that cytosine DNA methyltransferase (DNA MeTase) is a candidate target for anticancer therapy. Several observations have recently suggested that hyperactivation of DNA MeTase plays a critical role in initiation and progression of cancer and that its upregulation is a component of the Ras oncogenic signaling pathway. We show that a phosphorothioate modified antisense oligodeoxynucleotide directed against the translation initiation domain of the DNA MeTase mRNA reduces the level of DNA MeTase mRNA, inhibits DNA MeTase activity and inhibits anchorage independent growth of Y1 adrenocortical carcinoma cells *ex vivo* in a dose dependent manner. Injection of DNA MeTase antisense oligodeoxynucleotides intraperitoneally inhibits the growth of Y1 tumors in syngeneic LAF1 mice, reduces the level of DNA MeTase, induces demethylation of the adrenocortical specific gene C21 and its expression in tumors *in vivo*. These results support the hypothesis that an increase in DNA MeTase activity is critical for tumorigenesis and is reversible by pharmacologic inhibition of DNA MeTase.

Modification of DNA by methylation is now recognized as an important mechanism of epigenetic regulation of genomic functions (Razin and Riggs, 1980; Razin and Cedar, 1991; Tate and Bird, 1993). Methylation of DNA is a post replication event catalyzed by the DNA methyltransferase (MeTase) enzyme using S-adenosyl methionine as a methyl donor (Adams *et al*, 1979). About eighty per cent of cytosines located in the CpG dinucleotide sequence are methylated in the genome of most vertebrate cells but the distribution of methylated sites is cell- and tissue-specific (Yisraeli and Szyf, 1984). Patterns of methylation are generated during development by enzymatic *de novo* methylation and demethylation processes (Razin and Riggs, 1980; Razin and Cedar, 1991; Tate and Bird, 1993; Adams *et al*, 1979; Yisraeli and Szyf, 1984; Razin and Kafri, 1994; Szyf, 1991) and maintained in somatic cells.

A number of observations have suggested that the pattern of DNA methylation is disrupted in cancer cells (Gama-Sosa *et al.*, 1983a; Feinberg and Vogelstein, 1983). Both hypomethylation (Feinberg and Vogelstein, 1983) and hypermethylation (de Bustros*et al.*, 1988; Nelkin *et al.*, 1991; Baylin *et al.*, 1991) of different CpG sites in cancer cells and tissues relative to the cognate normal tissue have been documented. Some of the sites which are hypermethylated in tumors are located in tumor-suppressor loci such as p16 (Merlo*et al.*, 1995) Rb (Ohtani-Fujita *et al.*, 1993) VHL (Herman *et al.*, 1994) and WT (Royer-Pokora and Schneider, 1992) and recently, a new candidate tumor-suppressor gene was cloned by molecular analysis of the hypermethylated region in chromosome 17p13.3 (Makos-Wales *et al.*, 1995). One possible mechanism that has been proposed to explain the changes in DNA methylation observed in cancer cells is that they are the end result of a change in the enzymatic machinery controlling DNA methylation in the cell (Szyf, 1991; Baylin *et al.*, 1991; Szyf, 1994; Szyf, 1996, Szyf *et al.*, 1984). In accordance with this hypothesis, cancer cell lines (Kautiainen and Jones, 1986) and human tumors (el-Deiry *et al.*, 1991) have been shown to express elevated levels of DNA MeTase. Recently Belinsky *et al.*, have shown that increased DNA MeTase activity is an early event in carcinogen initiated lung

cancer in the mouse (Belinsky *et al.*, 1996). Forced expression of DNA MeTase cDNA in murine NIH 3T3 cells leads to genomic hypermethylation and neoplastic transformation (Wu *et al.*, 1993) and expression of an antisense mRNA to the DNA MeTase leads to loss of tumorigenicity of adrenocortical carcinoma cell line Y1 (MacLeod and Szyf, 1995).

Many stimuli may account for increased DNA MeTase activity in tumors. One possible molecular mechanism explaining this elevation of DNA MeTase in cancer cells is that the expression of the DNA MeTase gene is regulated by oncogenic signaling pathways such as the Ras-Jun signaling pathway (Szyf, 1994; Szyf, 1996). The 5' region of the murine DNA MeTase gene bears AP-1 sites, these sites can mediate induction of DNA MeTase expression by the Ras-Jun signaling pathway and modulation of this pathway can alter DNA MeTase expression and DNA methylation (Rouleau *et al.*, 1992; Rouleau *et al.*, 1995; MacLeod *et al.*, 1995). Similarly ectopic expression of *Ha-ras* leads to induction of demethylation activity in P19 cells (Szyf *et al.*, 1995) which can explain (Szyf, 1994) the observed hypomethylation of some CpG sites in cancer cells (Gama-Sosa, 1983a; Feinberg and Vogelstein, 1983).

If hyperactivity of DNA MeTase is a critical downstream component of oncogenic programs (Rouleau *et al.*, 1992; Rouleau *et al.*, 1995; MacLeod *et al.*, 1995; MacLeod and Szyf, 1995), it should be an excellent target for anticancer therapy (Szyf, 1996). One critical question is whether it is possible to differentially inhibit the oncogenic effects of hyperactivity of DNA MeTase without compromizing the regular function of DNA MeTase in normal tissues. To test this hypothesis in an animal model, specific inhibitors of DNA MeTase are required. The only DNA MeTase inhibitor that has been available to date is the nucleoside analog 5-azadeoxycytidine (5-azadC) (Jones, 1985). Although 5-azadC is an effective inhibitor of DNA methylation (Jones, 1985), it has many side effects that might compromise the interpretation of the experimental data and limit its clinical utility (Szyf, 1996; Tamame *et al.*, 1983; Juttermann *et al.*, 1994). The advent of antisense oligodeoxynucleotides as specific inhibitors of protein expression in

whole animal systems offers new opportunities in approaching this hypothesis (Dean and McKay, 1994).

Y1 cells offer a model to test our hypothesis. First, this line which was isolated from a naturally occurring adrenocortical tumor in an LAF1 mouse (Yasumura *et al.*, 1966) bears a 30- to 60- fold amplification of the cellular proto oncogene c-Ki-ras (Schwab *et al.*, 1983). Second, the molecular link between hyperactivation of Ras, DNA MeTase hyperactivity and DNA methylation and the state of cellular transformation has been recently demonstrated (MacLeod and Szyf, 1995; MacLeod *et al.*, 1995). Third, identification of effective antisense oligodeoxynucleotide inhibitors requires screening of a number of potential candidates. This could only be done effectively *ex vivo*. Y1 cells could be grown and tested for tumorigenic characteristics *ex vivo* as well as implanted in syngeneic LAF1 mice (MacLeod and Szyf, 1995) *in vivo* thus enabling the study of the effects of inhibition of DNA methylation in a whole animal system.

In this paper we demonstrate that treatment with antisense phosphorothioate modified oligodeoxynucleotides directed against the translation initiation region of the murine DNA MeTase leads to a reduction in DNA MeTase mRNA, DNA methylation activity and tumorigenicity in Y1 cells *ex vivo* and in Y1 cells implanted in syngeneic LAF1 mice *in vivo*. These data are consistent with the hypothesis that DNA MeTase is an anticancer drug target.

MATERIALS AND METHODS

Cell Culture, *ex vivo* oligodeoxynucleotide treatment and tumorigenicity assays. Y1 cells were maintained as monolayers in F-10 medium which was supplemented with 7.25% heat inactivated horse serum and 2.5% heat inactivated fetal calf serum (Immunocorp, Montreal). All other media and reagents for cell culture were obtained from GIBCO-BRL. Y1 cells ($1X10^6$) were plated on a 150 mm dish (Nunc) 15 hours before oligo treatment. The sequences of the oligodeoxynucleotides used in this paper are: antisense

(HYB101584): 5'-TCT ATT TGA GTC TGC CAT TT-3' corresponding to bases -2 to +18 in the murine DNA MeTase mRNA (relative to the putative translation initiation site (9), the scrambled sequence corresponding to the antisense sequence (HYB102277): 5'-TGT GAT TCT CCT TAT TCG AT-3' and the reverse sequence (HYB101585): 5'-TTT ACC GTC TGA GTT TAT CT-3' . Phosphorothioate oligodeoxynucleotides were synthesized using phosphoramadite chemistry on a Biosearch 8700 automated synthesizer and purified by high pressure liquid chromatography using a phenyl sepharose column followed by DEAE 5PW anion exchange chromatography. The purity of all oligonucleotides was greater than 98% as determined by ion exchange chromatography. The oligodeoxynucleotides dissolved in phosphate buffered saline at a concentration of 1mM were added to the medium and were replenished daily for a period of thirteen days at which time the cells were harvested and RNA and nuclear proteins were extracted as previously described (Szyf *et al.*, 1985; Szyf *et al.*, 1991). These experiments were performed in the absence of any lipid carrier to avoid nonspecific effects of the carrier in long term treatments and to recapitulate the situation *in vivo,* where no carrier was used. This experimental paradigm required using oligodeoxynucleotides at the micromolar concentration range which is higher than the concentrations required when lipid carriers are used. For analysis of growth in soft-agar, $1X10^3$ cells were seeded in triplicate onto a six well dish (Falcon) with 4 ml of F-10 medium containing 7.5% horse serum, 2.5% FCS, 0.25 mg/ml G418 (for transfectants) and 0.33% agar solution at 37°C (Freedman and Shin, 1974). Cells were fed with 2 ml of medium every two days. Growth was scored as colonies containing >10 cells, 21 days after plating.

DNA and RNA analyses. Genomic DNA was prepared from pelleted nuclei and total cellular RNA was prepared from cytosolic fractions according to standard protocols (Ausubel *et al.*, 1988). To purify nuclei from tumors, the tumors were first homogenized in a glass homogenizer in buffer A (10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 5mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.5% Nonident P40) and then processed similar to extracts prepared from cell lines as we

previously described (Szyf *et al.*, 1985; Szyf *et al.*, 1991). Genomic DNA was first digested with HindIII followed by ethanol precipitation and was then subjected to MspI or HpaII restriction enzyme digestion (Boehringer Mannheim) (2.5 units/μg for 8 h at 37°C). Radionucleotides (3000 mCi/mmol) were purchased from Amersham and the probes were labeled by the random prime method (Boehringer Mannheim). To determine the relative abundance of the different HpaII fragments, the autoradiograms were scanned with a Scanalytics scanner (one D analysis) and the intensity of each fragment relative to the total signal in all the scanned fragments per lane was determined. To quantify the relative abundance of DNA MeTase mRNA, total RNA (3μg) was subjected to an RNAase protection assay as described in reference (Rouleau *et al.*, 1992) using a 0.7-kb HindIII-BamHI fragment (-0.39- to +0.318 kb) as a riboprobe [probe A in Rouleau *et al.*, 1992] and an 18s RNAase protection probe for 18s ribosomal RNA (provided by Ambion Inc. Austin TX) to normalize for total RNA loading. The autoradiograms were scanned and the signal at each data point was determined and normalized to the amount of total RNA at the same point.

Western Blot Analysis of DNA MeTase. Rabbit polyclonal antibodies were raised (by Pocono Rabbit Farm Laboratory, Canadensis Pennsylvania) against a peptide sequence consisting of amino acids 1107-1125 of the mouse DNA MeTase (1101-1119 of the human DNA MeTase). The specificity of the polyclonal serum was tested by competition with the antigen peptide. Fifty μg of nuclear extracts were resolved on a 5% S.D.S P.A.G.E., transferred onto PVDF membrane (Amersham) and subjected to immunodetection for the DNA MeTase according to standard protocols using 1:2000 dilution of primary antibody and ECL detection kit (Amersham) (Mayer and Walker, 1987). The membrane was then subjected to staining for total protein with Amido black dye according to standard protocols (Mayer and Walker, 1987). The intensity of the DNA MeTase signal and total protein was determined by densitometry (Scanalytics) and the level of DNA MeTase was normalized to the amount of total nuclear protein.

Assay of DNA MeTase activity. To determine nuclear DNA MeTase

levels, cells were maintained at a nonconfluent state and fed with fresh medium every 24 hours. DNA MeTase activity (3µg) was assayed by incubating 3µg of nuclear extract with a synthetic hemimethylated double-stranded oligodeoxynucleotide (Szyf *et al.*, 1991) substrate and S-[*methyl* -^{3}H]-S-adenosyl -L- methionine (78.9 Ci/mmol Amersham) as a methyl donor for 3 hours at 37^{o}C as previously described (Szyf *et al.*, 1985).

Assay of C21 mRNA by RT PCR. The expression of the C21 gene was determined using our previously described primers and amplification conditions (Szyf *et al.*, 1990) with the following modifications: RNA (1µg) was treated with DNAase I 10U (Boehringer-Mannheim) for 30 minutes at 37^{o}C along with 1U RNAase Inhibitor (Boehringer-Mannheim). The RNA was then reverse transcribed with murine leukemia virus reverse transcriptase (200U) (Superscript, GIBCO-BRL) using random hexamer primers (1:40 dilution) (Boehringer-Mannheim) as recommended by the manufacturer. Two µl of the reverse transcribed cDNA was amplified with the primers described in reference (Szyf *et al.*, 1990a) using Taq polymerase (Promega) in the presence of 10^{-18} to 10^{-22} µg of plasmid DNA bearing C21 genomic sequences pC21(Szyf *et al.*, 1990a) (to control for amplification by the primers, the amplification primers flank two exons and an intron so that the control genomic sequence and cDNA could easily be distinguished) for 30 cycles of: 94 C 30 seconds, 55 C 30 seconds, 72 C 2 minutes. The reaction products were separated on an agarose gel, Southern blotted onto Hybond N+ filter and hybridized with a ^{32}P labeled internal oligodeoxynucleotide as in reference (Szyf *et al.*, 1990a).

Tumorigenicity assays. LAF-1 mice (Bar-Harbor) (16-20 week old males) were injected subcutaneously (in the flank area) with 2x10^{6} Y1 cells randomized and divided into 4 color coded treatment groups (antisense=blue, reverse sense=purple, scrambled=brown, and PBS=yellow) of 10. Blind, intraperitoneal injections of the different color coded solutions (5mg/kg in 100µl

PBS solution) per group were initiated 3 days post implantation and were repeated every 48 hours for 29 days. On each injection day mice were palpated for tumors and were measured for average diameter. Tumor volume was calculated as previously described (Plumb *et al.*, 1994). Mice were sacrificed on day 30, tumors were dissected and weighed.

Ten tumor bearing mice treated with PBS over the course of 30 days as above were treated with 5mg/kg anti sense, or scrambled oligodeoxynucleotides for 3 consecutive days (sub cutaneous injections, 1 cm proximal to the tumor). Tumors were dissected, weighed and DNA, RNA, and nuclear extracts were prepared using standard protocols (Ausubel*et al.*, 1988).

RESULTS

Antisense oligodeoxynucleotides to the translation initiation region of the murine DNA MeTase inhibits DNA MeTase mRNA, DNA MeTase activity and tumorigenesis *ex vivo*.

We have previously shown that expression of a 600 bp fragment bearing sequences encoding the translation initiation domain of the DNA MeTase mRNA in the antisense orientation can inhibit DNA methylation and induce both cellular differentiation of 10T 1/2 cells (Szyf *et al.*, 1992) as well as reversal of transformation of Y1 cells (MacLeod and Szyf, 1995). Antisense expression vectors could not be easily utilized to study the function and therapeutic potential of inhibiting DNA MeTase *in vivo*. We therefore tested the possibility that shorter antisense oligodeoxynucleotides directed against the same region of the mRNA could recapitulate these effects. An antisense oligodeoxynucleotide [+18 to -2 (sequence as in Materials and Methods), when the translation initiation site is indicated as 1(Bestor *et al.*, 1988)] was found to be active in a preliminary screen and we further determined its mechanism of action.

One of the possible mechanisms of action of antisense oligodeoxynucleotides is targeting RNAase H activity to the RNA-DNA duplex

resulting in degradation of the mRNA (Walder and Walder, 1988). We first determined the dose response relationship of DNA MeTase mRNA abundance and DNA MeTase antisense oligodeoxynucleotide concentration at one time point. Y1 cells (10^6 cells) were treated with different concentrations (0, 10 and 20μM) of antisense oligodeoxynucleotides and scrambled controls for 48h. Cellular RNA was subjected to an RNAase protection assay as described in the methods. The results presented in Figure 1A demonstrate a sharp decrease in abundance of DNA MeTase mRNA following incubation of the cells with 20μM of the DNA MeTase antisense oligodeoxynucleotides which is not observed following treatment with scrambled oligodeoxynucleotides. We then defined the time dependence of reduction in DNA MeTase activity at the inhibitory concentration of the antisense oligodeoxynucleotide, 20 μM. The results presented in Figure 1B (RNA) and C (MeTase activity) show that both DNA MeTase activity and mRNA are reduced by ten to hundred fold after 6 days of treatment. Some fluctuations are observed in the levels of DNA MeTase in Y1 cells treated with control oligodeoxynucleotides (two fold) as well as antisense oligodeoxynucleotides (such as the relatively high levels of DNA MeTase at 4 days). These oscillations in DNA MeTase mRNA expression might reflect changes in the cell-cycle kinetics of the cells at different time points as DNA MeTase levels are regulated with the cell cycle (Szyf *et al.*, 1985; Szyf *et al.*, 1991). Alternatively, they might result from nonspecific effects of oligodeoxynucleotides on different cellular parameters or reflect some inaccuracies in our measurements. However, an overall reduction in DNA MeTase activity is established after six to nine days of treatment with the antisense oligodeoxynucleotides.

Can DNA MeTase antisense oligodeoxynucleotides induce a dose-dependent inhibition of tumorigenicity *ex vivo* as measured by anchorage-independent growth on soft agar? Y1 cells were treated with a range of concentrations of antisense and scrambled oligodeoxynucleotides (0-20μM) for 13 days. The cells were harvested and plated onto soft-agar as described in the methods. The results presented in Fig. 1D demonstrate a dose dependent

inhibition of colony formation on soft agar in antisense treated cells versus the scrambled control. The drop in the number of colonies formed on soft agar between 10-20μM corresponds to the precipitous drop in DNA MeTase mRNA at this concentration of antisense oligodeoxynucleotide (Fig. 1A).

Inhibition of anchorage-independent growth of antisense treated cells was observed even though the soft-agar medium was not supplemented with antisense oligodeoxynucleotides suggesting that the changes in the level of tumorigenicity of antisense treated cells were irreversible. This is consistent with the hypothesis that once DNA MeTase is inhibited, the cells are reprogrammed to a less transformed state (Szyf, 1994; MacLeod and Szyf, 1995).

The experiments described above demonstrate that antisense oligodeoxynucleotides can inhibit DNA MeTase activity *ex vivo* and that this inhibition corresponds to a dose dependent inhibition of tumorigenicity.

Inhibition of tumor growth and DNA MeTase *in vivo* by a DNA MeTase antisense oligodeoxynucleotide.

To test the hypothesis that inhibition of DNA MeTase *in vivo* can result in inhibition of tumor growth and to determine the general toxic effects of DNA MeTase antisense treatment, Y1 cells ($1x10^6$) were implanted in the flank of the syngeneic mouse strain LAF1 and were treated by intrapertinoneal injections every 48 hours with PBS, antisense oligodeoxynucleotide or two control oligodeoxynucleotides: a scrambled version of the antisense oligodeoxynucleotide and a reverse sequence (see Materials and Methods for sequence). Preliminary experiments with a small number of animals per group (n=3) established a dose dependent relationship between oligodeoxynucleotide concentrations and tumor growth. No effects were observed at 0.5 mg/kg whereas inhibition of tumor appearance and growth was observed at 1-5mg/kg range. At 20 mg/kg nonspecific effects were observed with the scrambled oligodeoxynucleotides in two out of three experiments whereas a statistically significant reduction in tumor growth with antisense oligodeoxynucleotides versus controls was observed in one experiment (data not shown). Forty LAF1 mice

were implanted with Y1 cells, randomized and divided into color coded groups of 10 mice each and treated and evaluated as follows in a double-blinded fashion . Three days post implantation the mice were injected intraperitoneally (i.p.)with 100 µl of PBS, or PBS containing 5 mg/kg of either antisense, scrambled or reverse oligodeoxynucleotides. Injections were repeated every 48 hours and tumor diameter measurements were taken at each time point. Thirty days post injection the animals were sacrificed, tumors were excised and weighed. The results described in Figure 2 show that tumor growth was inhibited by injection of DNA MeTase antisense oligodeoxynucleotides relative to control oligodeoxynucleotides as determined by the rate of increase in the average tumor volume (Fig. 2B) as well as by the final weight and size of the tumors (Fig. 2A,C). The difference in the average tumor volume between the antisense treated group and either of the different control groups (PBS, scrambled and reverse) at 29 days is highly statistically significant as determined by a student *t* test ($p<0.005$) whereas the difference between the different control oligodeoxynucleotide treated groups and PBS is not statistically significant. Similarly, the difference in average final tumor weight at thirty days between the antisense and control oligo treated group(s) is highly statistically significant ($p<0.001$). One of the antisense treated animals did not develop tumors whereas all the control groups developed tumors (one mouse of the reverse group died with a heavy tumor load before termination of the experiment).

We determined the general toxic effects of *in vivo* DNA MeTase antisense oligodeoxynucleotide treatment versus the control oligodeoxynucleotides. Blood parameters and weight loss of antisense, reverse and scrambled injected (20mg/kg) tumor bearing LAF1 mice (n=5) were assayed. As observed in Table 1 there were no significant reductions in red blood cell count, hematocrit or % hemoglobin in DNA MeTase antisense treated animals versus controls. Similarly, platelets and white blood cell count was not increased but rather slightly decreased in antisense treated animals (Table 1). There was no significant weight loss even though tumor load was decreased significantly in this experiment by DNA MeTase

antisense oligodeoxynucleotides.

These experiments demonstrate that *in vivo* treatment of tumor bearing LAF1 mice with DNA MeTase antisense oligodeoxynucleotides can inhibit tumor growth supporting the hypothesis that DNA MeTase is a critical component in maintaining the transformed state and that *in vivo* treatment with an antisense based inhibitor of DNA MeTase can inhibit tumor growth.

DNA MeTase antisense oligodeoxynucleotide inhibits DNA MeTase levels, induces limited demethylation of the adrenocortical specific C21 gene and reactivates it.

To determine whether injection of DNA MeTase antisense oligodeoxynucleotide can inhibit DNA MeTase activity, we treated tumor bearing LAF1 mice for three days with either DNA MeTase antisense oligodeoxynucleotide (n=5, 5 mg/kg) or the scrambled oligodeoxynucleotide (n=4, 5 mg/kg) by subcutaneous injection near the tumor (1 cm) for three days. To limit as much as possible complicating indirect factors that might have clouded the interpretation of data, we did not look at DNA methylation in tumors that were chronically treated. Tumors were harvested, nuclear extracts were prepared and DNA MeTase levels in the nuclear extracts were determined by a Western blot analysis as described in the methods. The results of such an analysis are demonstrated in Fig. 2D and the normalized average levels of DNA MeTase in each of the treatment groups plotted in Fig. 2E demonstrate a statistically significant reduction in DNA MeTase levels in antisense treated animals ($p<0.05$). The level of inhibition varies, however, from 90% inhibition in mouse number 3 in the group treated with antisense (3 in Figure 2D) to no detectable inhibition in mouse number 5 (5 in figure 2D).

C21 is specifically expressed in the adrenal cortex, the enzyme encoded by this gene, steroid 21 hydroxylase, is required for the synthesis of glucocorticoids which is the main normal function of this tissue. The gene is expressed at very high levels in the adrenal cortex but is totally repressed and heavily methylated in Y1 tumor cells (Szyf *et al.*, 1990a). No C21 mRNA is detected in Y1 cells even when the most sensitive assays such as RT-PCR are

used (Szyf *et al.*, 1990a). We have not observed any expression of C21 in Y1 cells in multiple Y1 cultures in the last decade under any conditions. We have previously suggested that this is a consequence of the increase in *de novo* DNA methylation activity in these cancer cells (Szyf *et al.*, 1990a). Reexpression of C21 could serve as a good marker of demethylation and the reprogramming of Y1 cells to a nontransformed state.

To address this question, we performed an RT-PCR analysis of C21 expression on RNA prepared from the following samples: Y1 cells treated with either antisense DNA MeTase or scrambled oligodeoxynucleotides (20μM) *ex vivo*, a tumor isolated from a mouse treated with antisense oligodeoxynucleotides *in vivo* for three days (antisense 3 exhibited the highest reduction in DNA MeTase activity, 90%) and Y1 cells transfected with hGAP [which attenuates Ras signaling pathway resulting in inhibition of DNA MeTase activity and partial demethylation of the C21 gene (MacLeod *et al.*, 1995)]. C21 expression was induced under all these conditions (Figure 3A). This is the first induction of C21 reexpression in Y1 cells under any conditions observed in our laboratory. These results strongly support the hypothesis that DNA MeTase antisense oligodeoxynucleotides induce a partial demethylation and reprogramming of gene expression in Y1 cells which is similar to that observed following attenuation of the Ras signaling pathway.

To determine whether the 5' promoter region of the C21 gene was demethylated in tumor DNA following antisense treatment, tumor DNA was subjected to MspI/HpaII restriction enzyme analysis, Southern blotting and hybridization with a 5' C21 probe [0.36 Kb XbaI-BamHI fragment encoding the promoter region of the C21 gene(Szyf *et al.*, 1990a)]. Hypomethylation of the two HpaII sites in the promoter region will result in a 0.36 kb fragment. As observed in Fig. 3B, the Y1 tumor that was extracted from a mouse (antisense 3) that was injected with antisense oligodeoxynucleotides *in vivo* exhibits an increase in the abundance (as determined by densitometric analysis, Fig. 3C) of the 0.36 kb HpaII fragment relative to the partially methylated fragments at 1.9, 2.5 and 4 kb as compared with the control tumor. Demethylation of C21 is observed in other

tumors injected with antisense (data not shown).

CpG island containing genes are *de novo* methylated in tumor cells (Merlo *et al.*, 1995; Jones *et al.*, 1990; Issa *et al.*, 1996; Herman *et al.*, 1996). We therefore determined the state of methylation of a generally expressed CpG island containing gene, *thy-1* in mice treated with either antisense or control oligodeoxynucleotides. There is an increase in the relative abundance of the 600 bp HpaII fragment contained in the 5' *thy*-1 CpG island (Szyf *et al.*, 1990b) (Fig. 3B) and a decrease in the relative abundance of the partial HpaII fragments (~3 kb to ~5.5 kb) in tumor extracted from antisense treated mice (a) relative to the pattern observed in the control tumor (s), (see Figure 3C for quantification). These experiments demonstrate limited hypomethylation in tumor DNA in response to DNA MeTase antisense treatment *in vivo.*

DISCUSSION

The goal of this paper is to test the hypothesis that tumorigenesis could be reversed by pharmacological inhibition of DNA MeTase activity and to suggest that DNA MeTase inhibitors could serve as potential anticancer agents. This paper demonstrates that an antisense oligodeoxynucleotide directed against a region in DNA MeTase mRNA encoding the translation initiation codon can inhibit, in a dose dependent manner, DNA MeTase mRNA expression, DNA MeTase activity, and tumorigenesis *ex vivo.* Similar effects are not observed when a scrambled sequence is used. This is consistent with the hypothesis that the observed effects are a result of reduction in the level of DNA MeTase. The sequences used in our experiments do not bear CG sites or G quartets that have been previously shown to bear nonantisense related immunogenic and antitelomerase effects (Zahler *et al.*, 1991; Krieg *et al.*, 1995). Whereas it is clear that phosphorothioate oligodeoxynucleotides might exhibit nonspecific antitumorigenic effects, our experiments reveal that the nonspecific and sequence specific effects could be differentiated. Utilizing the fact that Y1 cells could be implanted *in vivo* in a syngeneic mouse, we demonstrate that a DNA MeTase antisense

oligodeoxynucleotide could reduce DNA MeTase levels in the tumor, induce demethylation of certain sites, induce demethylation and reexpression of an adrenal specific gene that is heavily methylated and repressed in Y1 cells and inhibit the growth of Y1 tumors *in vivo*. Taken together these results are consistent with the hypothesis that induction of DNA MeTase is critical for carcinogenesis and that inhibition of DNA MeTase by pharmacological agents such as DNA MeTase antisense inhibits tumor growth. One important question is whether inhibition of DNA MeTase could reverse the growth of established tumors or whether it will just prevent the establishment of tumors. The experimental paradigm used in this paper utilizes a long established tumor cell line and treatment is intiated only three days after implantation. The slower growth of the tumors when treated with DNA MeTase inhibitors (Fig. 2B) is consistent with the model that inhibition of methylation can inhibit the growth of established tumor cells *in vivo*. One interesting question that has not been addressed by this experiment is whether there is a critical size or level of tumor organization that is not treatable by DNA MeTase antisense inhibitors. Future studies will directly address this question.

Why are elevated levels of DNA MeTase critical for maintaining the cancer state? Three models have been previously suggested: a. Elevated levels of DNA MeTase might result in disruption of the appropriate gene expression profile of a cell leading to inactivation of tumor suppressor genes (Makos-Wales *et al.*, 1995) and other genes that are characteristic of the differentiated state of the cell such as C21 in Y1 cells (Szyf *et al.*, 1990a). Inhibition of DNA MeTase might result in activation of tumor suppressor genes and other differentiated functions. The induction of C21 (Fig. 3A) observed in our antisense treated cells and tumors is consistent with this hypothesis. b. High levels of DNA MeTase might have a direct effect on origins of replication (Szyf, 1994; Szyf, 1996). Some origins of replication were shown to be densely methylated in both CG and other dinucleotide sequences (Tasheva and Roufa, 1994). It is possible that putative cancer-specific origins are methylated only when high levels of DNA MeTase are present in the cell (Szyf, 1996). c. Methylated cytosines are hot-spots for

mutation, deamination of methylated cytosines will result in a C-T transition mutation which becomes permanent if not fixed by the GT repair system before a second round of replication (Rideout *et al.*, 1990).

Whereas more data is required to determine which of these mechanisms is involved in the genesis and maintenance of cancer, two issues are critical for the pharmacological and therapeutic application of DNA MeTase inhibitors. First, are the changes caused by aberrant methylation in carcinogenesis irreversible as has been previously suggested (Laird *et al.*,1995) or are they reversible by pharmacological intervention. *Min* mice bearing a mutation in the homolog of the human repair associated tumor suppressor gene *APC* are protected from formation of adenopolyps in the intestines when treated prophylactically with 5-azadC early after birth (Laird *et al.*,1995). The development of polyps could not be reversed when 5-azadC is applied later suggesting an irreversible mechanism.

Second, is the aberrant methylation observed in cancer a consequence of the enhanced levels of DNA MeTase and therefore reversible by reducing the level of DNA MeTase (Szyf, 1994; Szyf, 1996). Although additional experiments will be required to demonstrate that similar results to those reported here will be obtained with cancers formed in the animal rather than implanted tumors, our results lend support to the hypothesis that the effects of DNA MeTase induction are reversible and therefore suggest that the DNA MeTase is a target for anticancer intervention.

Acknowledgements: We thank Dr. Kirk Field for critical reading of the manuscript and his thoughtful suggestions. We also thank Deanna Collier, Vera Bozovic and Johanne Theberge for excellent technical assistance. This research was supported by the National Cancer Institute Canada, a contract with Hybridon Inc. Worcester MA. and MethylGene Inc. Montreal.

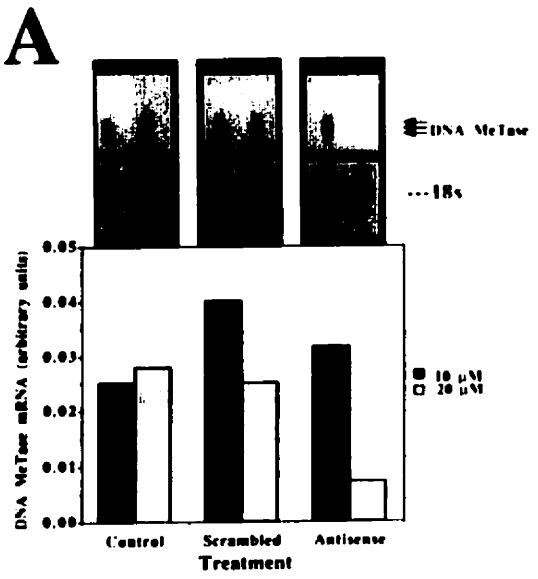
Figure 1. DNA MeTase antisense oligodeoxynucleotides inhibit DNA MeTase mRNA, DNA MeTase activity and anchorage independent growth ex vivo.

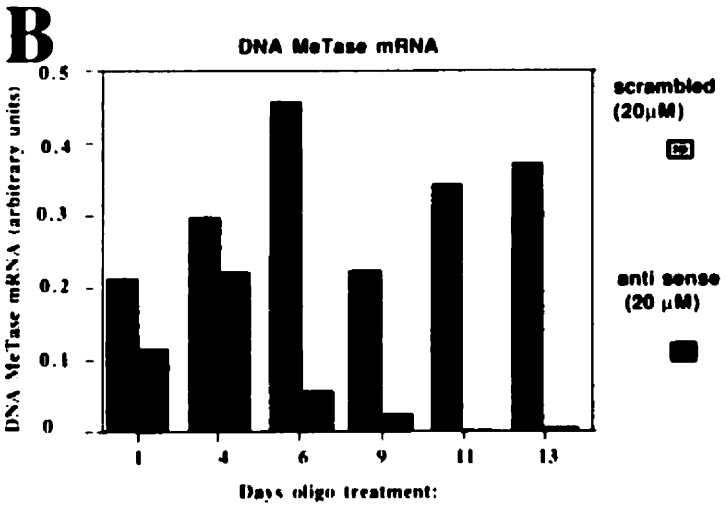
A. RNAase protection analysis of DNA MeTase mRNA in Y1 cells treated with control scrambled and antisense oligodeoxynucleotides. Y1 cells were cultured in the presence of different concentrations of scrambled and antisense oligodeoxynucleotides (sequence shown in Materials and Methods) as indicated in the figure for 48 hours. RNA (3µg) extracted from the cells was subjected to an RNAase protection assay as previously described (Rouleau *et al.*, 1992) using a 700 bp riboprobe [probe A in reference (Rouleau *et al.*, 1992)] encoding the DNA MeTase genomic sequence from -0.39 to +318. The major bands representing the two major initiation sites are indicated (92,90) as well as the the first exon which gives a 99 bp protected fragment.

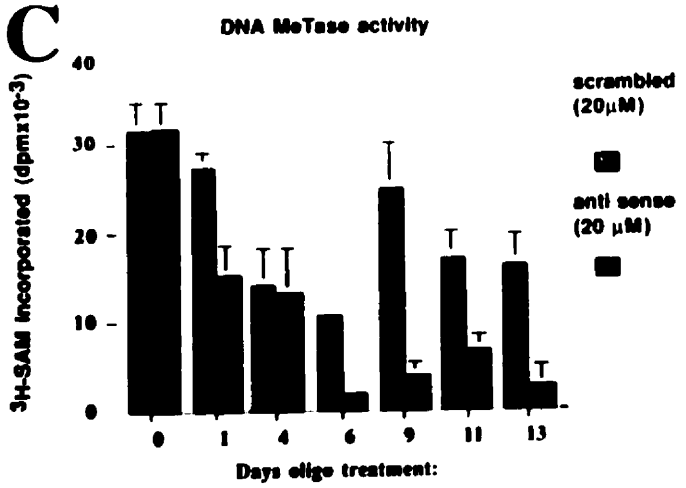
B. Time course of inhibition of DNA MeTase mRNA by antisense oligodeoxynucleotides. Y1 cells were incubated in the presence of 20 µM of either antisense or scrambled oligodeoxynucleotides and the medium was replaced with oligodeoxynucleotide containing medium every 24 hours. Cells were harvested at the indicated time points, RNA and nuclear extracts were prepared as previously described. RNA was subjected to RNAase protection assay as described in A. An autoradiogram similar to the one presented in A was scanned and the amount of DNA MeTase mRNA at each point was normalized to the signal obtained for 18s ribosomal RNA.

C. Nuclear extracts prepared from oligo treated Y1 cells described in B were assayed for DNA MeTase activity as described in the methods. The results represent an average of triplicate determination +/- S.D.

D. Y1 cells were treated with scrambled and antisense oligodeoxynucleotides as described in B and seeded onto soft-agar for determination of anchorage independent growth as described in the methods. The results represent an average of triplicate determinations +/- S.D.


A
DNA MeTase
18s
DNA MeTase mRNA (arbitrary units)
0.05
0.04
0.03
0.02
0.01
0.00
10 μM
20 μM
Control
Scrambled
Antisense
Treatment


B
DNA MeTase mRNA
DNA MeTase mRNA (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0
scrambled
(20μM)
anti sense
(20 μM)
1
4
6
9
11
13
Days oligo treatment:


C
DNA MeTase activity
3H-SAM incorporated (dpmx10-3)
40
30
20
10
0
scrambled
(20μM)
anti sense
(20 μM)
0
1
4
6
9
11
13
Days oligo treatment:

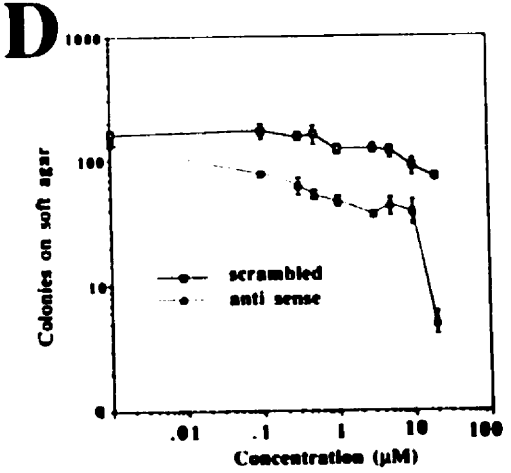

D
Colonies on soft agar
1000
100
10
0
scrambled
anti sense
.01
.1
1
10
100
Concentration (μM)

Fig. 2 DNA MeTase antisense oligodeoxynucleotide inhibits tumor growth in vivo

A. The average weight of tumors isolated from LAF1 mice bearing Y1 tumors which were injected with antisense, scrambled or reverse oligodeoxynucleotide (5mg/kg) every 48 hours for 29 days. The results are presented as an average +/- SEM. The statistical significance of the difference between the scrambled or reverse groups and the antisense group was determined by a student *t* test to be $p<0.001$. There was no statistically significant difference between the two control groups $p>0.5$

B. Average volume of tumors determined as described at the indicated time points post implantation (determined as described in Plumb *et al.*, 1994) .

C. A photograph of the tumors removed from the antisense, reverse and scrambled oligodeoxynucleotides treated mice described above.

D. LAF1 mice bearing Y1 tumors were injected with 5mg/kg scrambled (n=4) or antisense (n=5) oligodeoxynucleotides three times every 24 hours subcutaneously. Tumors were removed from each mouse (indicated in the figure by serial numbers from 1 to 4 for the scrambled group and 1-5 for the antisense group) and nuclear extracts prepared from the tumors were subjected to a Western blot analysis as described in the methods. The band corresponding to the DNA MeTase is indicated by an arrow. The amount of signal corresponding to the DNA MeTase (O.D arbitrary units) was normalized to the level of total protein transferred onto the membrane as determined by Amido black staining and quantified by scanning (O.D. arbitrary units). The values obtained (O.D. of DNA MeTase signal divided by O.D. of the total protein staining) for the tumors extracted from each of the treated mice (serial number of mice in bold) were as follows: scrambled: **1**. 2.2; **2**. 3.1; **3.** 2.7; **4:** 2.5: antisense: **1.** 0.6; **2.** 0.5; **3.** 0.16; **4**. 1.0; **5**. 2.9.

E. The average DNA MeTase level per group is plotted with the SEM. The difference between the scrambled and antisense groups was determined by a student *t* test to be statistically significant, $p<0.05$.

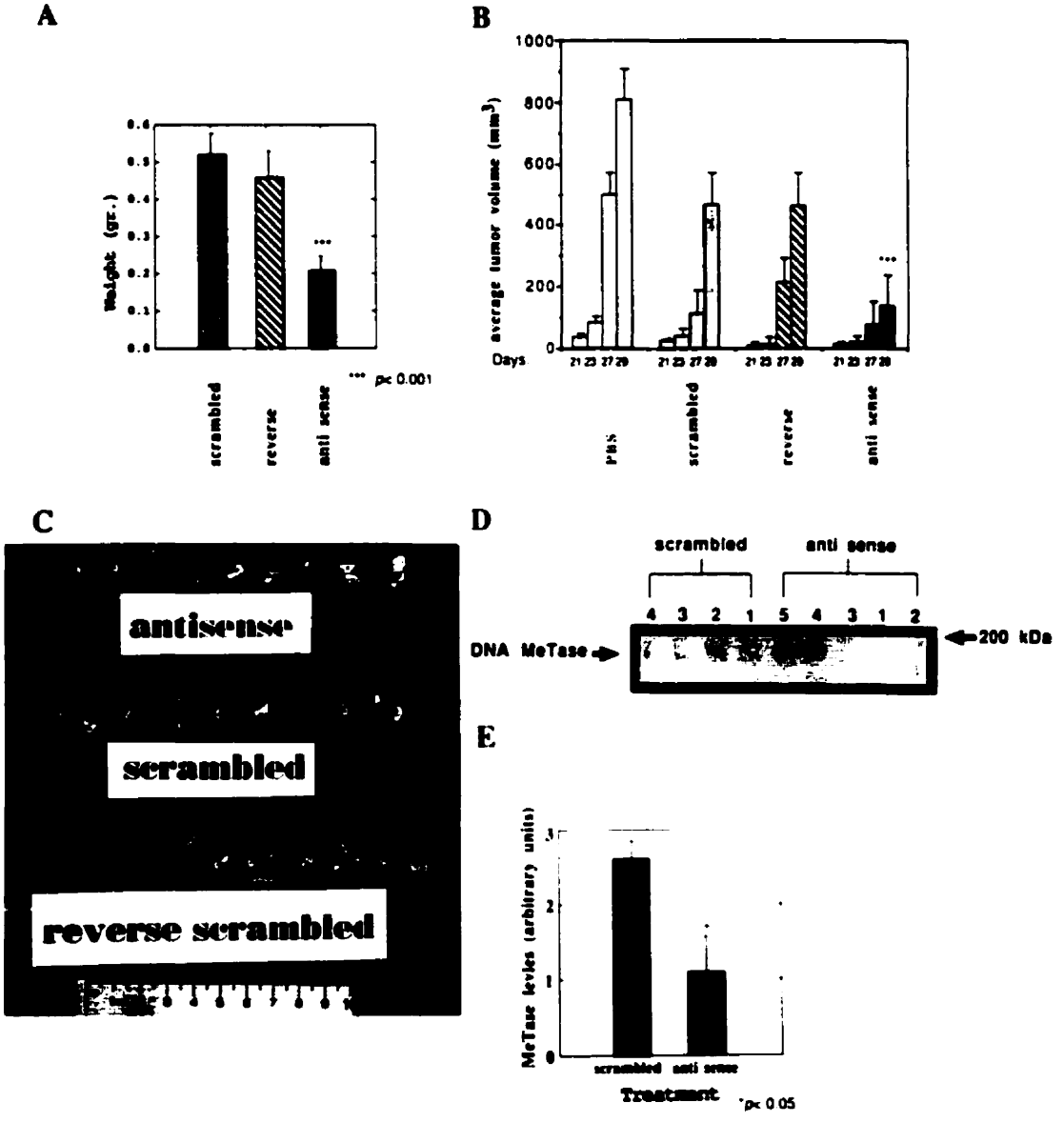

A
B
C
D
E
scrambled
reverse
anti sense
*** pc 0.001
average tumor volume (mm3)
Days
PBS
antisense
scrambled
reverse scrambled
anti sense
DNA MeTase
200 kDa
MeTase levels (arbitrary units)
Treatment
*pc 0.05

Figure 3. Expression and demethylation of the C21 gene in Y1 tumors isolated from LAF1 mice treated with antisense oligodeoxynucleotides. **A.** C21 expression was determined by RT-PCR amplification with C21 specific primers of total RNA isolated from Y1 cells, Y1GAP transfectants expressing hGAP (GTPase activator protein), an attenuator of Ras activity (MacLeod *et al.*, 1995), Y1 cells treated with 20 μM of either scrambled oligodeoxynucleotide or DNA MeTase antisense oligodeoxynucleotides *(ex vivo* as indicated in the figure), Y1 tumors from LAF1 mice injected with either 5mg/kg of scrambled (s) or DNA MeTase antisense (a) oligodeoxynucleotides (*in vivo*) as well as adrenal RNA. C21 plasmid DNA encoding the C21 gene (Szyf *et al.*, 1989) was included in the amplification reaction to control for nonspecific inhibition of amplification. The expected genomic and C21 mRNA amplification products are indicated by arrows. **B**. DNA was extracted from Y1 tumors isolated from LAF1 mice injected with either scrambled oligodeoxynucleotides (scrambled 4, indicated as **s**) or antisense oligodeoxynucleotides (antisense 3, indicated as **a**) for three days as described in the methods were subjected to HindIII digestion followed by either HpaII (H) (which cleaves the sequence CCGG when the internal C is not methylated) or MspI (M) (which cleaves the sequence CCGG even when the internal C is methylated), agarose gel fractionation (2.5%), Southern blotting and hybridization with the indicated probes.

The promoter region of the C21 gene. Complete digestion of the gene should result in a 0.36 kb fragment (Szyf *et al.*, 1989) as indicated by the arrow (dark). The partially methylated fragments are indicated by shaded arrows. The partial cleavage with MspI is a consequence of the fact that the MspI sites are nested within a HaeIII site. These sites are highly resistant to cleavage by MspI when fully or partially methylated as previously described (Keshet and Cedar, 1983).

Thy-1. DNA prepared from the tumors indicated in the figure were subjected to a similar HpaII-MspI restriction enzyme analysis and hybridization with a 0.36 probe from the 5' region of the *thy-1* gene (Szyf *et al.*, 1990b). The expected HpaII fragment is indicated by an arrow (dark). Partially methylated fragments are

indicated by shaded arrows.

Physical maps of the sequences analyzed for their methylation state are presented in the bottom panel. The first exons of the three genes are shown and are indicated as filled boxes, the probes used are indicated as thick lines and thin line indicates the expected nonmethylated and partially methylated HpaII fragments. (XbaI- X, BamHI-B).

C. The relative abundance of the HpaII fragments was determined by densitometry as described in Materials and Methods. The size in kb of the scanned fragments is indicated. The results are presented as intensity of a specific fragment as a percentage of the total intensity in all scanned fragments per lane.

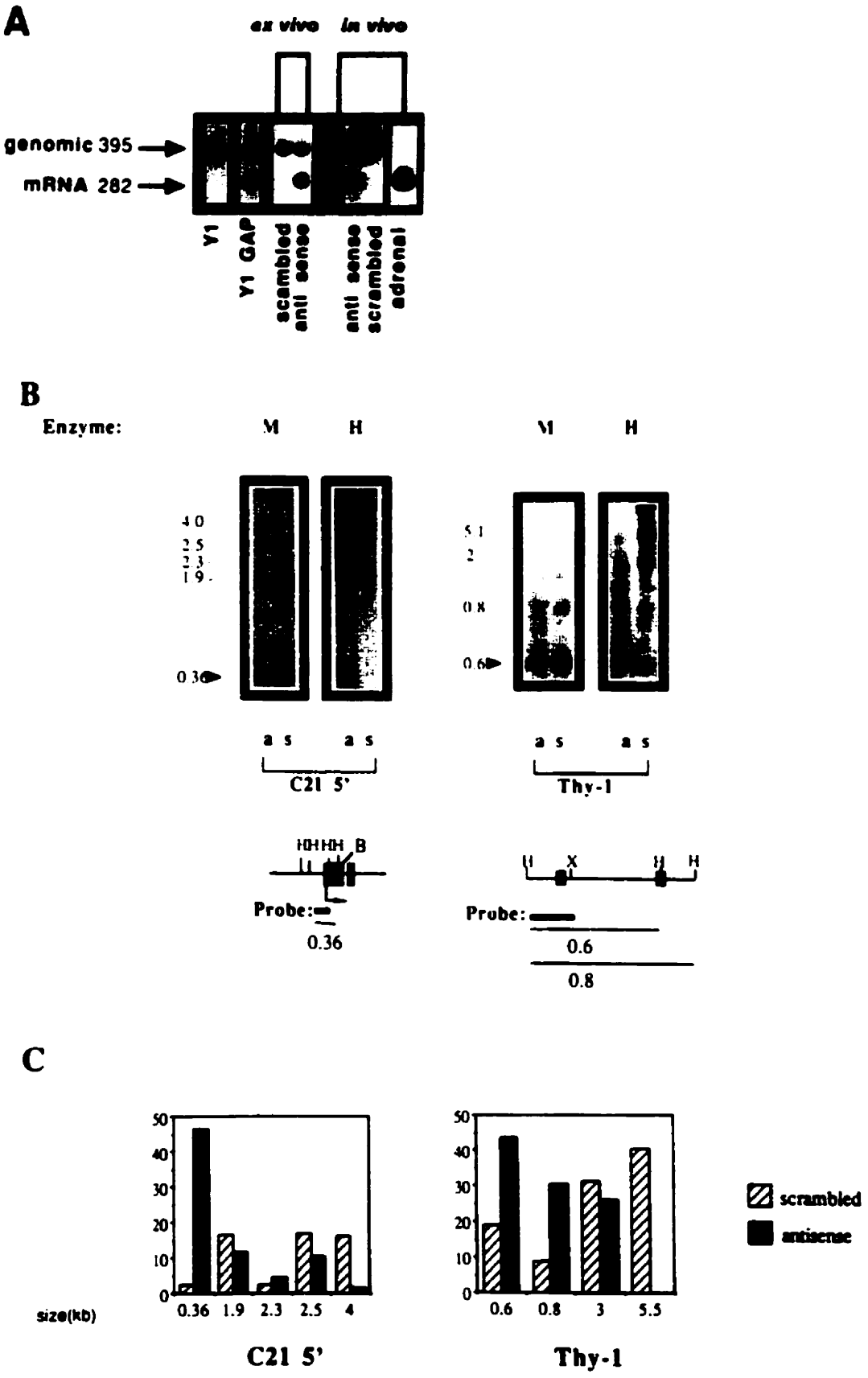

A
ex vivo
in vivo
genomic 395
mRNA 282
Y1
Y1 GAP
scambled anti sense
anti sense scrambled adrenal
B
Enzyme:
M
H
M
H
40
25
23
19
0.36
5.1
2
0.8
0.6
a s
a s
a s
a s
C21 5'
Thy-1
Probe:
0.36
Probe:
0.6
0.8
C
50
40
30
20
10
0
0.36 1.9 2.3 2.5 4
size(kb)
C21 5'
0.6 0.8 3 5.5
Thy-1
scrambled
antisense

Table 1: Hematological analysis of LAF1 mice treated with antisense or control oligodeoxynucleotides (20 mg/kg) for 30 days (n=5).

Treatment	Hematocrit	%Hemoglobin	WBC	RBC	Platelets
REVERSE	17.2 (9.2)	6.4 (3.3)	59.6 (2.19)	3.4 (2.19)	514 (291)
SCRAMBLED	16.1 (2.5)	6.16 (0.9)	71.8 (21.9)	2.99 (.45)	503.2 (104)
ANTISENSE	21.9 (9.5)	7.44 (3.8)	50.7 (33)	4.4(1.8)	302 (95)

White blood cell count (WBC), red blood cell count (RBC), hematocrit in g/dcl. The numbers represent the mean and standard deviation in brackets.

The first four chapters of this thesis have focused on the role of the enzyme that adds methyl moieties to DNA, DNA MeTase. If regulation of DNA methylation is important for proper cellular function then it stands to reason that the regulation of the process of DNA demethylation would be just as important. However, for the last 40-50 years it has been believed that the modification of DNA by methylation is not directly reversible. This notion has prevailed because it has always been thought that the energy required to directly break the carbon-carbon bond between the methyl group and the cytosine ring was too high to achieve in a biological setting (I apologise, but there appears to be no documentation of this hypothesis). Therefore the mechanisms of observed DNA demethylation were explained by: a) replication of DNA in the absence of DNA MeTase enzymatic activity, where demethylation is achieved in a passive manner (Razin and Riggs, 1980) or b) methyl-cytosine excision and replacement with with a non methylated cytosine (Razin *et al.*, 1986; Vairapandi and Duker, 1993; Jost *et al.*, 1995; Weiss *et al.*, 1996; Fremont *et al.*, 1997).

The basic building blocks of any cell consist of fats, proteins, and carbohydrates. These moieties are used in the production of fundamental cellular structures; membranes, receptors, enzymes, transcription factors, histones, ribosomes, DNA and RNA. However, these fundamental structures do not remain static, as pillars supporting a platform, but are in a dynamic equilibrium and poised to respond to incoming stimuli. Each of these structures can exist in alternative forms, marked by covalent modifications. These modifications impart modulation of each particular structure's function and allow each structure to communicate with the other structures in order to mount a coordinated, whole cell, response to any stimuli. In most all of these cases the modifications are easily reversible by separate enzymatic machinery. Kinases phosphorylate, while phosphatases dephosphorylate. Proteins mediate the exchange of GDP for GTP and separate proteins catalyse the hydrolysis of GTP to GDP. If DNA can be methylated in response to biological stimuli (Rouleau, *et al.*, 1995; MacLeod *et al.*, 1995; Deng and Szyf, 1999a,b; Chapter 2) then logic would predict that it can also be

demethylated in response to biological signals. It has been demonstrated that over expression of the *ras* oncogene can induce a specific DNA demethylating activity in embryonal cells (Szyf *et al.*, 1995). A genome wide demethylation event occurs in preimplantation embryos (Kafri, *et al.*, 1993). The rapid rate of the demethylation rules out passive demethylation, and the potential mutagenic danger that the extreme DNA damaging nature of an excision replacement activity strongly suggests that this mechanism of demethylation is not active at this stage. There must be another DNA demethylation activity that can directly remove methyl groups from DNA. The manuscripts that make up chapters 5 and 6 test this hypothesis and provide a foundation for re-examination of all previous observations regarding DNA demethylation.

Chapter 5

A mammalian protein with specific mCpG DNA demethylase activity

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*These authors contributed equally to the preparation of the manuscript.

DNA methylation patterns play an important role in regulating genome functions. The nature of the enzyme responsible for demethylation has been a mystery. A mammalian cDNA encoding a methyl-CpG binding domain (MBD) bears demethylase activity, releases the methyl groups as methanol, transforms methylated cytosines to cytosines and demethylates a methylated plasmid when it is *in vitro* translated or transiently transfected into human embryonal kidney cells, demonstrating that it encodes a *bona fide* DNA demethylase (dMTase). The identification of demethylase cDNA provides a conclusive molecular proof for a *bona fide* demethylase activity and forms a template for a molecular and developmental analysis of the biological role of DNA methylation and demethylation.

Many lines of evidence suggest that DNA methylation plays a critical role in differential control of gene expression (Siegfried and Cedar, 1997). The molecular mechanisms through which methylation of cytosines residing in the dinucleotide CpG repress transcription are now being elucidated (Nan *et al.*, 1997; Nan *et al.*, 1998; Jones *et al.*, 1998). The process that determines which CpGs are methylated is obviously critical for proper development (Li *et al.*, 1992) and possibly maintenance of somatic biological functions. It is clear that two enzymatic processes are involved in laying down the pattern of methylation during development, methylation and demethylation (Razin and Riggs, 1980; Brandeis *et al.*, 1993; Kafri *et al.*, 1993; Monk *et al.*, 1987). Whereas the enzyme responsible for methylation, DNA Methyltransferase (DNA MeTase) has been purified and cloned from a number of organisms (Bestor *et al.*, 1988; Bestor and Verdine, 1994), the enzymatic process responsible for demethylation has remained controversial. Since removal of methyl groups from cytosines has been presumed to be energetically unlikely, different alternative biochemical pathways have been proposed to explain active demethylation such as glycosylase (Razin *et al.*, 1986; Vairapandi and Duker, 1993; Jost *et al.*, 1995) and an RNA catalysed replacement of methyl cytosine with cytosine (Wiess *et al.*, 1996). It is clear that

this issue will not be resolved until the molecular identity of the enzyme responsible for demethylation is unravelled. We have now been able to prove that mammalian cells bear an enzyme that truly demethylates DNA by catalyzing the replacement of the methyl group on the 5 position of C, residing in the dinucleotide sequence mdCpdG, releasing the methyl group in the form of methanol (Ramchandani et al., 1999 Chapter 6) . However, the identification of the gene encoding demethylase and characterization of its molecular properties is essential for establishing its presence in vertebrate biology and for further understanding of its role in development and formation of the DNA methylation pattern as well as its possible role in pathological states.

Identification of a lead DNA demethylase candidate by homology search of dbEST

As the purification of demethylase suggests that the demethylase is of very low abundance (Ramchandani et al., 1999 Chapter 6), we opted for cloning the demethylase based on the fact that it specifically demethylates methylated CG dinucleotides (Ramchandani et al., 1999 Chapter 6). We therefore assumed that it should bear the ability to recognise methylated CG dinucleotides. Previous reports have shown that proteins interacting with methylated DNA share a common domain (MBD) (Cross *et al.*, 1997). A TBLASTN (Boguski *et al.*, 1995) search of the dbEST database using the MBD of MeCP2 identified a novel expression tag cDNA (from a T-cell lymphoma Homo sapiens cDNA 5' end) (gb/AA361957/AA361957 EST71295) and the mouse homologue ((gb/W97165/W97165 mf90g05.r1) from Soares mouse embryo NbME13.5) with unknown function that bears homology to the MBD of MeCP2 (Fig.1a) . Following submission of this report the same cDNA was cloned and identified as methylated DNA binding protein MBD2b (Hendrich and Bird, 1998) . Alignment of the novel EST and MeCP2 and MeCP1 associated protein has revealed no homology beyond the previously characterized MBD which is consistent with a different function for this methylated DNA binding protein. A 1.36 kb cDNA was

cloned from Hela cells. A virtual translation of the protein identified an open reading frame (ORF) of 262 amino acids (Fig. 1b). The ORF may extend further 5' as no in frame stop codon was found upstream of this ATG. A longer cDNA encoding MBD2 has been recently cloned from the mouse (Hendrich and Bird, 1998).

A BLAST search (Corpet *et al.*, 1998; Altschul *et al.*, 1990) of the candidate protein using the Predict protein server (Bairoch *et al.*, 1997) against a database of protein domain families has identified only the MBD domain. No other functional motifs were identified by the Prosite analysis (Bairoch *et al.*, 1997; Rost, 1996). This is consistent with a novel biochemical function for this protein. A coiled coil prediction (Lupas *et al.*, 1991) of the sequence identified a coiled coil domain which is known to play a role in protein protein interactions (O'Shea *et al.*, 1989).

The identified cDNA encodes an mRNA that is broadly expressed in human cells as revealed by a Northern blot analysis of human poly A+ mRNA (Fig. 1c) as one major transcript of ~ 1.6 kb which is close to the size of the cloned cDNA, verifying that the cloned cDNA does not represent a highly repetitive RNA but rather a mRNA encoded by a single or low copy number gene.

In vitro translated candidate cDNA bears demethylase activity

A conclusive proof for the existence of a single protein that *bona fide* demethylates DNA is to demonstrate that an *in vitro* translated candidate cDNA can transform a methyl cytosine to cytosine in an isolated system. The candidate demethylase cDNA was subcloned it into a pcDNA3.1/His Xpress (INVITROGEN) expression vector in the putative translation frame (pcDNA3.1His A) and in a single base frame shift (pcDNA3.1His B), and was *in vitro* transcribed and translated in the presence of ^{35}S-methionine and the resulting translation products were resolved by SDS-PAGE. Autoradiography revealed a ~40KDa protein (Fig. 2a).

To establish that the candidate cDNA encodes a *bona fide* demethylase we demonstrate that *in vitro* translated protein (purified on a Ni^{2+} charged agarose

resin) can transform CH_3-cytosine residing in [^{32}P]-a-dGTP labeled plasmid DNA or in [methyl-dC^{32}pdG]$_n$ double stranded oligomer DNA to cytosine, whereas a frame shift *in vitro* translated demethylase does not demethylate DNA (Fig.2b). This demonstrates that the demethylase activity is dependent on the demethylase translation product and not a contaminating activity found in the *in vitro* translation kit that copurifies with the putative demethylase .

Transiently transfected demethylase cDNA demethylates DNA

Demethylase cDNA (dMTase) and the misframe demethylase cDNA were transiently transfected into human embryonal kidney cells to test whether the cDNA can direct expression of demethylase activity in human cells. The His-tagged proteins were bound to Ni^{2+} agarose resin and eluted from the resin with 700-1000mM of imidazole. The expression of the transfected demethylase was verified by a Western blot analysis (Fig. 3a). The transiently expressed demethylase transforms methylated cytosine in DNA to cytosine residing in two different substrates (Fig. 3b & 3c), in a protein dependent manner (fig. 3b & 3d). The transiently transfected demethylase can demethylate a methylated SK plasmid as indicated by its sensitivity to HpaII following incubation with demethylase but not after incubation in the presence of the protease k pretreated demethylase or buffer alone (fig. 3d). The reaction displays substrate dependence and saturability (fig. 3e). Transiently expressed demethylase was loaded on a non denaturing glycerol gradient to determine its native MW. Similar to demethylase purified from human cells (Ramchandani et al., 1999 Chapter 6), cloned and purified demethylase activity fractionated at the 160-190 KDa range (data not shown). This is consistent with self association of cloned demethylase possibly mediated by the coiled-coil domain.

A *bona fide* demethylase activity could be purified from human cancer cells

To verify that an activity like the one shown by the synthetic protein could be isolated from human cells, we have subjected nuclear extracts from non-small cell human lung carcinoma A549 cells to sequential chromatography as described in the materials and methods. As shown in fig. 4a, an active demethylase fraction can transform methyl-cytosine residing in a [methyl-dC^{32}pdG]$_n$ double stranded oligomer DNA to cytosine in DNA. To further substantiate this conclusion, we subjected demethylase treated DNA to remethylation with the CpG MeTase M.*Sss* I which can transfer a methyl group exclusively to deoxy-cytosine (Nur *et al.*, 1985). The results presented in Fig. 4a show that the demethylated product of demethylase is deoxy-cytosine since it is completely remethylated with M.*Sss* I. The identity of the demethylated product as dC was further established by a two-dimension TLC analysis demonstrating that the product of demethylase comigrates with a cold dCMP standard in both dimensions (Fig. 4b).

Demethylase does not release a nucleotide, a phosphorylated base or phosphate from methylated DNA when incubated with a [^{32}pmdCpdG]$_n$ substrate which included a labelled ^{32}P 5' to mdC or our standard methylated substrate where ^{32}P is 3' to the m5dC (Fig.4c). Nuclear extracts which obviously contain a number of glycosylases and nucleases release phosphorylated derivatives in the same assay (Fig. 4c). Demethylase transforms the methyl cytosine in the [^{32}pmdCpdG]$_n$ substrate to cytosine as demonstrated when the reacted DNA is digested to 5' mononucleotides (Fig. 4c +V PDS) and analyzed by TLC. Since this reaction does not involve release of a ^{32}P derivative (Fig. 4c - V PDS), it demonstrates that demethylase transforms methylated cytosines to cytosines on DNA without disrupting the integrity of the DNA substrate by glycosylase or nuclease activity. These results establish that mammalian cells

express a *bona fide* demethylase activity, however it is not yet established whether the cloned cDNA encodes the activity purified from A549 cells.

Discussion

Understanding how methylation patterns are created, maintained and modified and how they play a role in development and pathology has been stifled by the longstanding uncertainty surrounding the identity of the enzymes involved in demethylation. We report here the cloning of a single cDNA that encodes a 40 KDa protein in both *in vitro* translation and transient transfection systems. This single polypeptide demethylates DNA in a defined *in vitro* system, thus providing conclusive proof for the existence of a *bona fide* demethylation machinery in mammals and presents us with the molecular tools to dissect its biological functions. Whereas our data suggests that a single protein can bear demethylation activities, it is possible that this protein acts in a homomeric complex as suggested by the sizing of native transiently transfected cloned cDNA.

In this paper we demonstrate that a general mdCpdG *bona fide* demethylase exists and that this novel enzyme can perform the activities that are necessary for demethylation *in vivo*. Our results identify a novel enzyme and a novel biochemical reaction that has not yet been described in any organism. The proposed reactants of the new reaction are H_2O and mdCpdG bearing DNA, the products of the reaction are dCpdG bearing DNA and methanol (Ramchandani, *et al.*, 1999). We have demonstrated that cytosine is the product of demethylase reaction by direct TLC analysis (Fig.2b, 3b,c and 4) and restriction enzyme analysis (Fig. 3d). The DNA is intact following demethylase reaction as evidenced by the lack of exonuclease activity (Fig. 4c). We propose that the demethylase catalyzes the hydrolysis of the methyl residue from the carbon at the 5 position of cytosine. Hydrolytic enzymes that can catalyze the cleavage of covalent bonds under neutral conditions at 37^{o}C are widely distributed in nature such as peptidases. Whereas enzymes can generally reduce high activation energy

barriers by positioning a more favourable transition state, they can not catalyze a reaction that is not thermodynamically favourable without an exogenous source of energy. Since cleavage of a carbon-carbon bond requires high energy, demethylation of 5-methyl-cytosine *per se* has been believed to be thermodynamically unfavourable. The discovery of the reactants and products of the demethylation reaction (Ramchandani *et al.*, 1999) suggests that the reaction is thermodynamically favorable.

Our data suggests that similar to DNA MeTase, which exhibits a general recognition of CG dinucleotides, demethylase is an enzyme that can generally recognize methylated CGs. The presence of demethylase mRNA in somatic cells (Fig. 1c) may allow for plasticity in the methylation pattern even in fully developed cells. Altering the level of demethylase in response to extracellular signals (Szyf *et al.*, 1995) or modulating its accessibility to specific sites by transacting factors can allow for modulation of the covalent modification pattern of the genome in response to physiological signals and might allow for reversible modulation of gene expression by methylation (Szyf, 1994).

Whereas future experiments will be required to demonstrate the role of demethylase in development and in somatic and cancer tissues, the data presented here provides conclusive evidence that demethylase exists and reveals its primary molecular characteristics. The cloned cDNA will enable the further dissection of the biological roles and involvements of demethylase.

Methods

Purification of demethylase from cells and tissues

Nuclear extracts were prepared from A549 (ATCC: CCL 185) cultures at near confluence as previously described (Szyf *et al*, 1995). A freshly prepared nuclear extract (1 ml, 6 mg) was diluted to a conductivity equivalent to 0.2 M NaCl in buffer L (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) and applied onto a DEAE-

Sephadex A-50 column (Pharmacia) (2.0 x 1 cm) that was preequilibrated with buffer L at a flow rate of 1 ml/min. Following a 15 ml wash with buffer L, proteins were eluted with a 5 ml linear gradient of NaCl (0.2-5.0 M). 0.5 ml fractions were collected and assayed for demethylase activity. Demethylase eluted between 4.9-5.0 M NaCl. Demethylase was purified 8000 fold following the DEAE step. Active DEAE-Sephadex column fractions were pooled, adjusted to 0.2 M NaCl by dilution and loaded onto an SP-Sepharose column (Pharmacia) (2.0 x1 cm). Following washing of the column as described above, the proteins were eluted with 5 ml of a linear NaCl gradient (0.2-5.0M). 0.5 ml fractions were collected and assayed for demethylase activity. Active fractions were pooled, adjusted to 0.2 M NaCl by dilution and applied onto a Q-sepharose (Pharmacia) column (2.0 x1 cm) and proteins were eluted as described above. The demethylase activity eluted around 4.8-5.0 M NaCl. The pooled fractions of Q-Sepharose column were loaded onto a 2.0 x 2.0 cm DEAE-Sephacel column (Pharmacia) and eluted with 10 ml of buffer L. The activity was detected at fraction 4, which is very near the void volume. A batch of 20 purified column fractions were pooled and subjected to cold vacuum evaporation followed by dialysis in one liter buffer L (no salt) at 4^{o}C with three changes at 6 hour intervals. One unit of demethylase activity was defined as the amount of purified enzyme that produced 1 picomole of cytosine using 1 ng of methylated ^{32}pCpG substrate in 1 hour at 37^{o}C.

Assay of demethylase activity

To assay the conversion of methyl-dCMP (mdCMP) to dCMP, we used a previously described method (Szyf *et al.*, 1995). One ng of a^{32}P labelled, fully methylated poly[mdC^{32}PdG]$_n$ or a control non methylated poly[dC^{32}pdG]$_n$ substrate was prepared as previously described (Szyf *et al.*, 1995), reacted in a 50 µl reaction volume in buffer L containing 10µg of RNase (all demethylase reactions were done in the presence of RNase unless indicated otherwise) with the

relevant demethylase fraction for 3 h at 37°C , purified by phenol/chloroform extraction and subjected to micrococcal nuclease (Pharmacia) digestion (100 µg at 10 µl) and calf spleen phosphodiesterase (2µg) (Boehringer) to 3' mononucleotides for 15 hours at 37°C. The digestion products were loaded onto a thin layer chromatography plate (TLC) (Kodak, 13255 Cellulose), developed in medium containing, 132 ml Isobutyric acid:40 ml water: 4 ml ammonia solution, and autoradiographed.

Coupled *in vitro* transcription translation

Demethylase cDNA was cloned from a Hela cell cDNA library in lTriplEx phage (Clontech) according to standard procedures. The positive insert in the pTriplEx plasmid was excised from the phage according to manufacturer's protocols and the identity of the insert was verified by DNA sequencing. The insert was excised by NotI restriction and subcloned into pcDNA3.1/His XpressA vector (INVITROGEN) to generate histidine tagged demethylase (dMTase) and pcDNA3.1/His XpressB to generate a 1 base frame shift (misframe).

The plasmids encoded by the pcDNA 3.1/His Xpress demethylase constructs described above were transcribed and translated by coupled transcription-translation using Promega T7 coupled-TNT rabbit reticulocyte lysate kit (according to manufacturer's protocol). The translation products were bound to a Pro bond nickel column (INVITROGEN) and demethylase was eluted according to the manufacturer's protocol with increasing concentrations of imidazole 50-1000 mM (100µl per step). The imidazole eluted fractions were dialysed against buffer L as previously described. To assay demethylase activity 4 *in vitro* translation reactions were pooled and purified as above.

Transfection and expression of demethylase in vertebrate cells

Ten µg of either pHis-dMTase A (dMTase) or pHis-dMTaseB (misframe) were

introduced into HEK 293 cells (10, 100 mm plates per plasmid at early log phase density) using calcium-phosphate precipitation method. Total cell extracts were prepared 48 h after transfection, bound to a Pro Bond nickel resin (INVITROGEN) and eluted with 500µl of increasing concentration steps of imidazole (50-1000 mM) according to the manufacturer's recommendations. The imidazole eluted fractions were dialysed against buffer L.

Acknowledgements

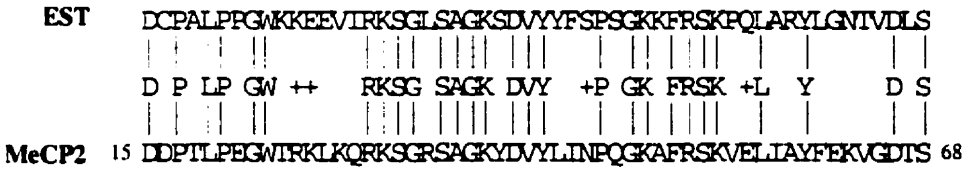
We would like to thank Benjamin ten Oever and Hoi-Ying Shiu for superior technical assistance and Dr. O. A. Mamer from the McGill Biomedical Mass Spectrometry Unit. This research was supported by the National Cancer Institute of Canada.
Correspondence should be addressed to M.S. (e-mail:mszyf@pharma.mcgill.ca).

The accession number of the sequence reported here is: AF 072242.

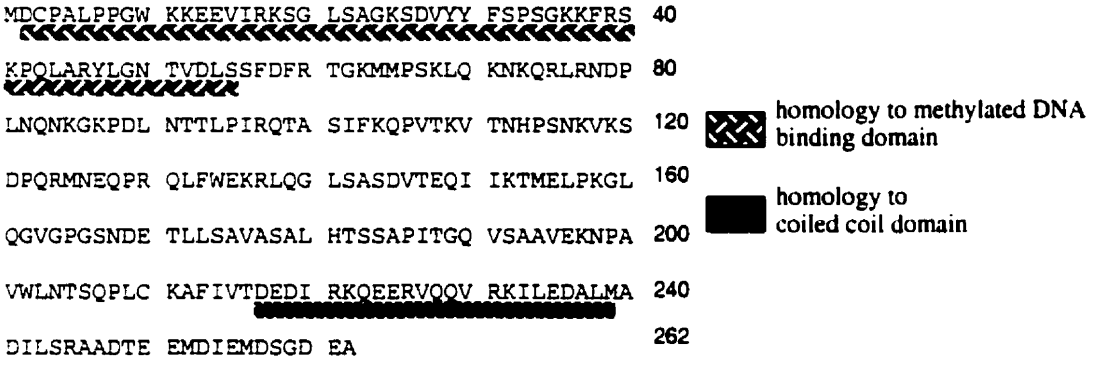
Fig. 1 Human demethylase (dMTase) cDNA.

a, Homology between the methylated DNA binding domain (MBD) of MeCP2 (amino acids 15-68) and demethylase (EST). **b**, The sequence of the putative demethylase cDNA was determined and virtually translated by DNASTAR. The 262 amino acid open reading frame is shown. The region containing homology to the MBD of MeCP-2 is highlighted at the N-termimal of the protein sequence and a predicted coiled-coil domain is highlighted at the C-terminal. **c**. A northern blot analysis of 2µg of polyA mRNA prepared from different human tissues (Origene Technologies Inc.) hybridized with the demethylase cDNA as a probe.

a


EST
DCPALPPGWKKEEVIRKSGLSAGKSDVYYFSPSGKKFRSKPQLARYLGNTVDLS
D P LP GW ++ RKSG SAGK DVY +P GK FRSK +L Y D S
MeCP2 15 DDPTLPEGWIRKLKQRKSGRSAGKYDVYLINPQGKAFRSKVELIAYFEKVGDTS 68

b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KPQLARYLGN TVDLSSFDFR TGKMMPSKLQ KNKQRLRNDP 80
LNQNKGKPDL NTTLPIRQTA SIFKQPVTKV TNHPSNKVKS 120
DPQRMNEQPR QLFWEKRLQG LSASDVTEQI IKTMELPKGL 160
QGVGPGSNDE TLLSAVASAL HTSSAPITGQ VSAAVEKNPA 200
VWLNTSQPLC KAFIVTDEDI RKQEERVQQV RKILEDALMA 240
DILSRAADTE EMDIEMDSGD EA 262
homology to methylated DNA binding domain
homology to coiled coil domain

c

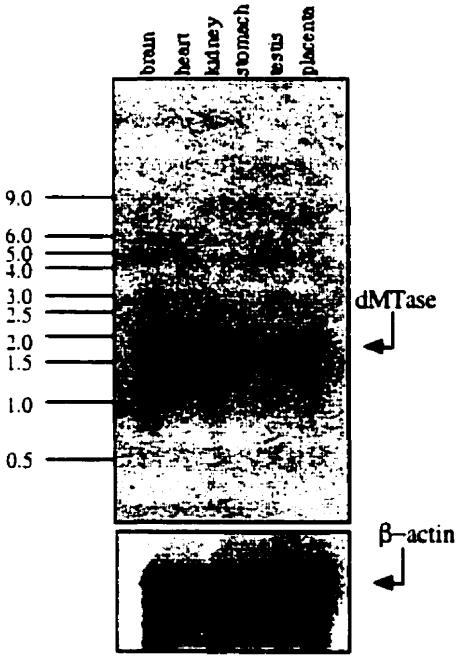
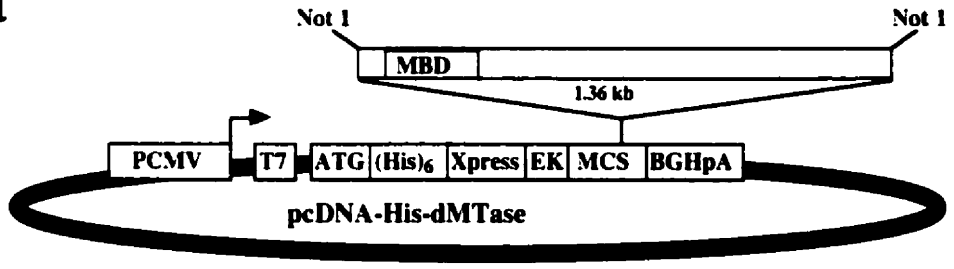

brain
heart
kidney
stomach
testis
placenta
9.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5
dMTase
β–actin

Fig. 2 *in vitro* expression and demethylase activity of cloned human demethylase

a, T7 coupled-histidine$_{(6)}$ tagged mammalian expression constructs containing the entire cloned demethylase cDNA in frame (dMTase) or one base pair frame shift (misframe) were *in vitro* transcribed and translated revealing an in frame polypeptide with an apparent size of ~40 KDa. **b**, Demethylation of 2 ng of a^{32}P-dGTP labelled methylated SK DNA with 10 ml of Probond purified (700mM imidazole fraction) *in vitro* translated demethylase with varying time. Plasmid pBluescript SK (5 ng) was methylated with M.*Sss* I methylase. *In vitro* methylated pBluescript SK was used as template for DNA synthesis using DNA polymerase and a^{32}P-dGTP, mdCTP, dTTP, dATP and hexanucleotide primers. The treated DNA was digested to 3' mononucleotides with micrococcal nuclease and subjected to thin layer chromatography (TLC). The resulting mdCMP (methylated cytosine) and dCMP (nonmethylated cytosine) mononucleotides are shown.

a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Not 1
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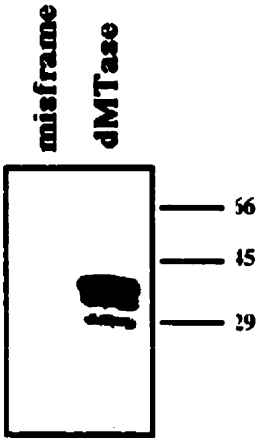
PCMV
T7
ATG
(His)6
Xpress
EK
MCS
BGHpA
pcDNA-His-dMTase


misframe
dMTase
56
45
29

b

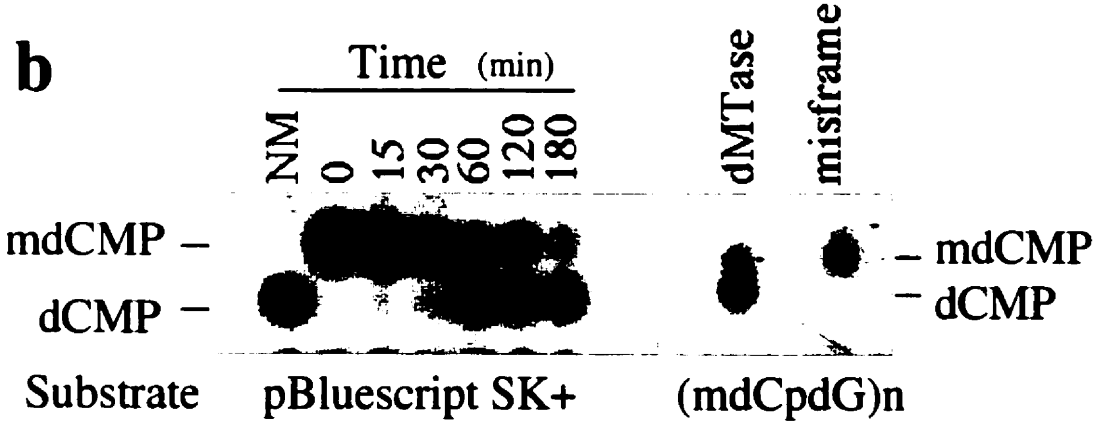

Time (min)
NM
0
15
30
60
120
180
dMTase
misframe
mdCMP
dCMP
mdCMP
dCMP
Substrate
pBluescript SK+
(mdCpdG)n

Fig.3 Expression of cloned demethylase cDNA in HEK cells

a, A Western blot of Probond fraction 700 mM purified DNA demethylase (dMTase) or the vector control pcDN3.1HisC (empty vector). The Western blot was reacted with the anti-Xpress epitope antibody (INVITROGEN). **b**, Demethylation of $[mC^{32}pG]_n$ DNA treated with 10 µl of either Probond purified demethylase (fraction 700 mM) (dMTase), Probond purified misframe demethylase (fraction 700 mM) (misframe), or Probond purified demethylase (fraction 700 mM) pretreated with protease K (20µg at 50°C for 2h) (dMTAse + protease K). NM, non methylated substrate control; ME, untreated methylated DNA control. The positions of mdCMP, methylated cytosine and dCMP non methylated cytosine are indicated. **c**, Demethylation of $a^{32}P$-dGTP labelled SK DNA treated with 10 µl Probond purified demethylase (fraction 700 mM) (dMTase). NM, non methylated substrate control; ME, untreated methylated DNA control. The positions of mdCMP, methylated cytosine and dCMP non methylated cytosine are indicated. **d**, Southern blot analysis of 5 ng of methylated SK DNA treated with either 5 µl Probond purified demethylase (fraction 700 mM) (dMTase), Probond purified demethylase (fraction 700 mM) pretreated with protease K (dMTase+ Protease K), or treated in incubation buffer alone (Buffer L). Demethylation using these concentrations (5 ml of fractionated demethylase) is not complete. We have consistently observed that under partial demethylation conditions some molecules are fully demethylated whereas others remain fully methylated. This is consistent with a processive mechanism for the enzyme (Cervoni et al., unpublished data). **e**, Dependence of demethylation activity on substrate concentration. One ng of methylated $[dC^{32}pdG]$ DNA was supplemented with non radioactive, methylated [dCpdG] DNA to the indicated concentrations, and then incubated with 10 ml of Probond purified demethylase (fraction 700 mM) in a 100 µl reaction volume in buffer L for three hours at 37°C and then analysed by TLC. The rate of transformation of methyl-cytosine to

cytosine was determined following quantification of the corresponding spots on a phosphorimager.

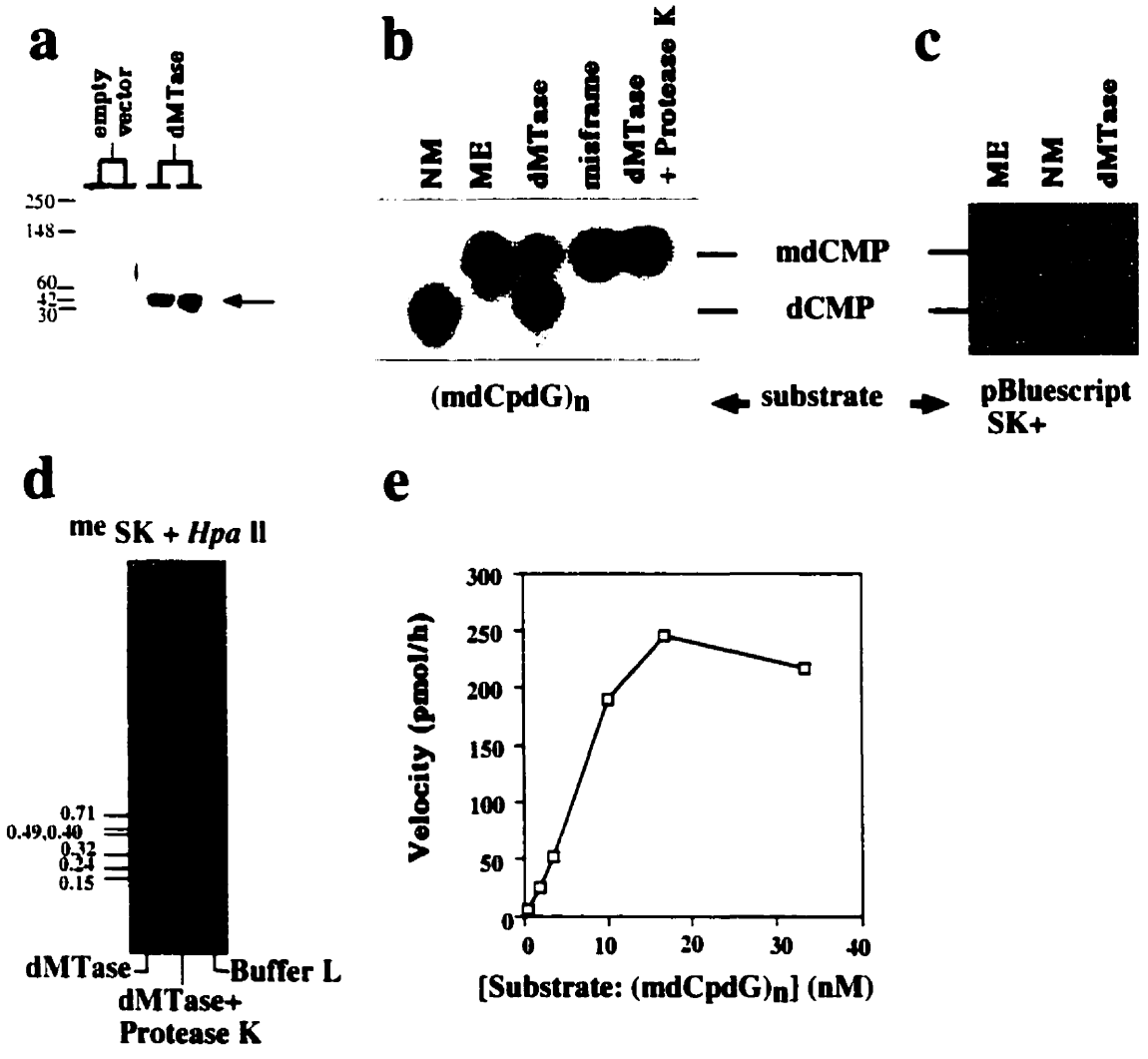
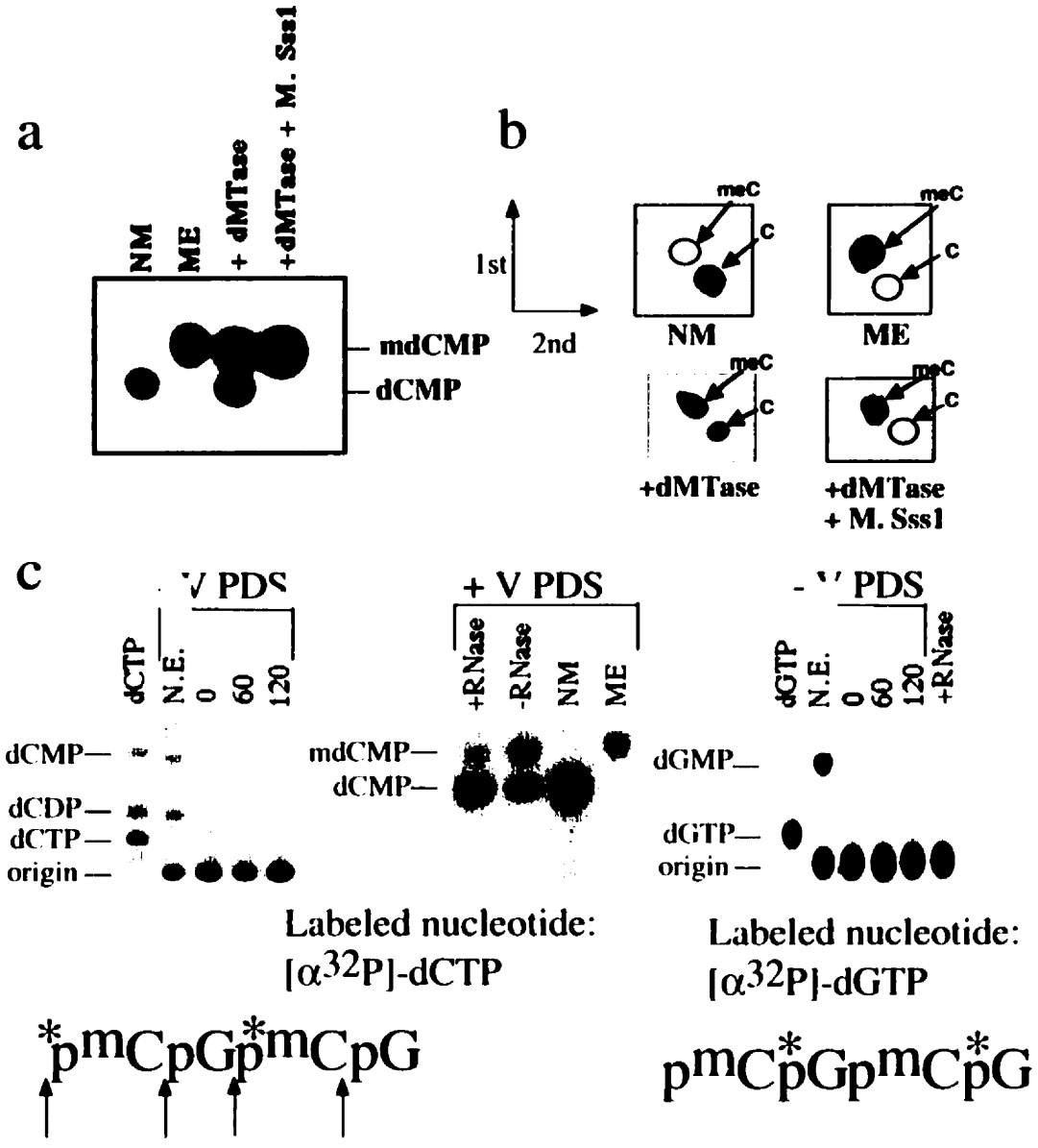

a
empty vector
dMTase
250
148
60
42
30
b
NM
ME
dMTase
misframe
dMTase + Protease K
mdCMP
dCMP
(mdCpdG)n
substrate
c
ME
NM
dMTase
pBluescript SK+
d
me SK + Hpa II
0.71
0.49,0.40
0.32
0.24
0.15
dMTase
Buffer L
dMTase+ Protease K
e
Velocity (pmol/h)
300
250
200
150
100
50
0
0
10
20
30
40
[Substrate: (mdCpdG)n] (nM)

Figure 4 The product of DNA demethylase activity purified from human cancer cells is cytosine and it exhibits no exonuclease or glycosylase activity. a, DEAE Sephacel demethylase fraction (0.5 unit) was incubated with 1 nanogram of $[mC^{32}pG]_n$ substrate in 50 µl total reaction volume as described in the methods and was divided into two 20 µl aliquots. One aliquot was subjected to methylation with 10 units of *Sss*I methylase (+dMTase +M. *Sss*1). The remethylated (+dMTase +M. *Sss*1) aliquot, nonremethylated aliquot (+dMTase), $[mC^{32}pG]_n$ (ME) substrate that was not treated with demethylase and a nonmethylated $[C^{32}pG]_n$ substrate (NM) were digested to 3' mononucleotides and analyzed by TLC. **b**, Two dimensional TLC analysis of the above reaction products in the presence of cold dNMP standards (solvent 1: iso-butyric acid; 66: H_2O; 20: NH_3; 1) (solvent 2: $(NH_4)_2SO_4$; 80: Isopropyl alcohol; 2: NaAcetate; 18:). The position of the cold dCMP or mdCMP spots were identified by shining a UV light on the plate and are indicated in both the one dimensional and two dimensional analyses. **c,** Left Panel: DEAE Sephacel purified demethylase (1unit) was incubated with 1 nanogram of $[^{32}pmdCpdG]_n$ substrate in 50 µl total reaction volume containing buffer L and an aliquot of 3 µl was withdrawn at different time intervals as indicated and analysed for the presence of excised ^{32}P residues by TLC. As a control, the DNA was incubated for 120 minutes with A549 nuclear extract. ^{32}P-a-dCTP was run as a control for the absence of nonincorporated dCTP in the labelled DNA as indicated. Centre Panel: To demonstrate that demethylase demethylated the DNA in this reaction the reaction products were digested with a 5' venom phosphodiesterase (+ V PDS) after 2 hours of incubation in the presence or absence of 100µg/ml RNase as indicated. $[^{32}pmdCpdG]_n$ that was incubated in the absence of demethylase (ME) and nonmethylated $[^{32}pdCpdG]_n$ (NM) were run as controls. Right panel: DNA demethylase (dMTase) was incubated with $[mdC^{32}pdG]_n$ substrate and aliquots

were withdrawn at different time intervals and subjected to TLC analysis and venom phosphodiesterase treatment (data not shown) as described for the middle panel. Bottom of c: pictorial of labelled DNA substrates and and cleavage sites, * delineates the labelled phosphate, arrows delineate cleavage sites of venom phosphodiesterase.


a
NM
ME
+ dMTase
+dMTase + M. Sss1
mdCMP
dCMP
b
1st
2nd
meC
c
NM
ME
+dMTase
+dMTase
+ M. Sss1
c
+ V PDS
dCTP
N.E.
0
60
120
+RNase
-RNase
NM
ME
dCMP—
dCDP—
dCTP—
origin —
mdCMP—
dCMP—
Labeled nucleotide:
[α32P]-dCTP
*pmCpGp*mCpG
- V PDS
dGTP
N.E.
0
60
120
+RNase
dGMP—
dGTP—
origin —
Labeled nucleotide:
[α32P]-dGTP
pmCp*GpmCp*G

Chapter 6

DNA methylation is a reversible biological signal

Shyam Ramchandani*, Sanjoy K. Bhattacharya*, Nadia Cervoni and Moshe Szyf

* Both authors made an equal contribution in the preparation of the manuscript

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The pattern of DNA methylation plays an important role in regulating different genome functions. To test the hypothesis that DNA methylation is a reversible biochemical process, we purified a *bona fide* demethylase from human cells which catalyses the cleavage of a methyl residue from 5-methylcytosine and its release as methanol. We show that similar to DNA methyltransferase, demethylase shows CpG dinucleotide specificity, can demethylate mdCpdG sites in different sequence contexts and demethylates both fully methylated and hemimethylated DNA. Thus contrary to the commonly accepted model, DNA methylation is a reversible signal similar to other physiological biochemical modifications.

Introduction

Many lines of evidence have established that modification of cytosine moieties residing in the dinucleotide sequence CpG in vertebrate genomes plays a role in regulating a number of genome functions such as parental imprinting (Lalande, 1996; Efstratiadis, 1994), X- inactivation (Beard *et al.*, 1995; Mohandas *et al.*, 1981), suppression of expression of ectopic genes (Challita *et al.*, 1995; Shinar *et al.*, 1989) and differential gene expression (Razin and Riggs, 1980; Eden and Cedar, 1994; Nan *et al.*, 1997; Siegfried and Cedar, 1997). DNA methylation can perform its function of differentially marking genes because CpG dinucleotides are differentially methylated, forming a pattern of methylation (Razin and Szyf, 1984). It is clear that the pattern of methylation is fashioned by a sequence of methylation and demethylation events (Brandeis *et al.*, 1993; Kafri *et al.*, 1993; Monk *et al.*, 1987; Shemer *et al.*, 1991) during development and is maintained in the fully differentiated cell (Razin and Riggs, 1980; Eden and Cedar, 1994; Nan *et al.*, 1997; Siegfried and Cedar, 1997; Stein *et al.*, 1982).

Most biological modifications such as protein phosphorylation are reversible, and enzymes that can either catalyse the modification or its removal

exist. This is essential for their functioning as biological signals that can respond to changing physiological cues. DNA methylation has been considered to be an exception since removal of a methyl group from DNA must involve a cleavage of a carbon-carbon bond which has been considered an unlikely reaction. Whereas it has been accepted that demethylation must occur during development, indirect mechanisms involving base excision and repair have been previously proposed to be responsible for removal of methyl groups from DNA at different stages of development (Razin *et al.*, 1986; Vairapandi and Duker, 1993; Jost *et al.*, 1995; Weiss *et al.*, 1996; Fremont *et al.*, 1997). Since it has been believed that removal of methyl groups from DNA is a cumbersome process, the accepted model has been that DNA methylation is a heritable and stable signal (Razin and Riggs, 1980; Eden and Cedar, 1994; Nan *et al.*, 1997; Siegfried and Cedar, 1997; Stein *et al.*, 1982). We have recently cloned a human cDNA that encodes a *bona fide* demethylase activity that can catalyze the cleavage of a methyl residue from 5-methyl cytosine (Chapter 5). This has provided the first molecular proof that true demethylation of DNA is biochemically feasible. If biological DNA methylation is a truly a reversible process in living cells, nuclei of mammalian cells should bear an activity that removes the specific products of the DNA methylation reaction. This manuscript tests this hypothesis by purifying a *bona fide* demethylase from nuclear extracts of human cells and defining its substrate and sequence specificity.

Materials and Methods

Purification of DNA demethylase activity- Nuclear extracts were prepared from A549 (ATCC: CCL 185) cultures at near confluence as previously described (Szyf *et al.* 1995). A freshly prepared nuclear extract (1 ml, 6 mg) was diluted to a conductivity equivalent to 0.2 M NaCl in buffer L (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) and applied onto a DEAE-Sephadex A-50 column (Pharmacia) (2.0 x 1 cm) that was preequilibrated with buffer L at a flow rate of 1 ml/min. Following a 15 ml wash with buffer L, proteins were eluted with a 5 ml linear gradient of NaCl (0.2-

5.0 M). 0.5 ml fractions were collected and assayed for demethylase activity. Demethylase eluted between 4.9-5.0 M NaCl. Active DEAE-Sephadex column fractions were pooled, adjusted to 0.2 M NaCl by dilution and loaded onto an SP-Sepharose column (Pharmacia) (2.0 x1 cm). Following washing of the column as described above, the proteins were eluted with 5 ml of a linear NaCl gradient (0.2-5.0M). 0.5 ml fractions were collected and assayed for demethylase activity. Demethylase activity eluted around 5.0 M NaCl. Active fractions were pooled, adjusted to 0.2 M NaCl by dilution and applied onto a Q-sepharose (Pharmacia) column (2.0 x1 cm) and proteins were eluted as described above. The demethylase activity eluted around 4.8-5.0 M NaCl. The pooled fractions of Q-Sepharose column were loaded onto a 2.0 x 2.0 cm DEAE-Sephacel column (Pharmacia) and eluted with 10 ml of buffer L. The activity was detected at fraction 4, which is very near the void volume. A batch of 40 purified column fractions were pooled and subjected to cold vacuum evaporation followed by dialysis in one liter buffer L (no salt) at 4^{o}C with three changes at every 6 hour interval. The demethylase from 40 preparations were suspended in 0.1 ml buffer L (no NaCl), preparations so obtained were assayed for enzyme activity and were used for subsequent experiments.

Assay of the conversion of methyl-dCMP (mdCMP) to dCMP, we used a previously described method (Szyf *et al.*, 1995). One ng of a^{32}P labelled, fully methylated poly[mdC^{32}pdG]$_n$ or a control nonmethylated poly[dC^{32}pdG]$_n$ substrate was prepared as described (Szyf *et al.*, 1995) , reacted with 50 µl of each columns fraction for 3 h at 37^{o}C , purified by phenol/chloroform extraction and subjected to micrococcal nuclease digestion (100 µg at 10 µl) (Pharmacia) to 3' mononucleotides for 15 hours at 37^{o}C and calf spleen phosphodiesterase (2µg) (Boehringer) for 2 hours at 37^{o}C. The digestion products were loaded onto a thin layer chromatography plate (TLC) (Kodak, 13255 Cellulose), developed in

medium containing, 132 ml Isobutyric acid:40 ml water: 4 ml ammonia solution, autoradiographed, and the intensity of the different spots was determined using a phosphorimager (Fuji, BAS 2000). Poly [mdC^{32}pdA] and [mdC^{32}pdT] substrates were prepared as follows. About 0.5 µg of 20 mer oligonucleotides 5'$(GG)_{10}$3', 5'$(GT)_{10}$3' and 5'$(GA)_{10}$3' were boiled and annealed at room temperature with oligonucleotide 5'CCCCCC3', 5'CACACA3' and 5'CTCTCT3' respectively. The complementary strand was extended with Klenow fragment using m5dCTP (Boehringer Mannheim) and either [a^{32}P] dATP (100µCi, 3000Ci/mmol) or [a^{32}P] dTTP (100 µCi, 3000 Ci/mmol) respectively.

Gas Chromatographic/Mass Spectrometric analysis- The demethylation reactions (volume 50 ml) were run in conical vials having a total internal volume of 350 microlitres. The vials were closed with a teflon-lined screw cap and left at room temperature for 18 h. The vials were cooled in an ice bath, opened and 10 mg of NaCl and 50 microlitres of toluene were added. The vials were frequently shaken over a period of 1 h. The toluene phases were pipetted into clean vials in a manner to rigorously exclude water carry over. Anhydrous sodium sulfate (5 mg) was added to the toluene extracts to remove water, and the toluene phases were pipetted into autoinjector vials for GC/MS analysis. Aliqouts of 3 microlitres were analyzed under the following instrumental conditions: Instrument: Hewlett-Packard 5988A; Column: 30 m x 0.25 mm i.d. fused quartz capillary with 0.25 micron DB-1 liquid phase, programmed after an initial hold for 1 min at 70 deg at 5 deg/min to 80 deg, then ramped ballistically to 280 deg for bake-out for 5 min; Injector and interface temperatures: 250 deg; Helium flow rate 1.5 ml/min; Mass spectrometer: ion source 200 deg, 70 eV electron impact ionization, scanning from m/z 10 to 50 in full scan mode was begun 6 s after injection, and ceased at 1.5 min to avoid acquisition of the intense toluene solvent peak.

Bisulphite treated DNA methylation analysis-The primers used for the DNA

MeTase genomic region (GenBank accession M84387) were: MET5'1, 5'-ggattttggtttatagtattgt-3' MET 5' (nested) 5'-ggaattttaggtttttatatgtt-3' . MET3' 1 5'-ctcttcataaactaaatattataa-3' and MET3' (nested) 5'-tccaaaactcaacataaaaaaat-3'.

Results

Purification of a DNA demethylase activity from mammalian nuclei

Since it has been previously proposed that the extensive hypomethylation observed in cancer cells might be a consequence of activation of demethylase activity by oncogenic pathways (Szyf, 1994; Szyf *et al.*, 1995), we have utilized the human lung carcinoma cell line A549 as a possible source for purification of a *bona fide* DNA demethylation activity. To directly measure the conversion of 5-mdCMP in DNA to dCMP, we have utilized a completely methylated ^{32}P labelled $[mdC^{32}pdG]_n$ double stranded oligomer which we had previously described (Szyf *et al.*, 1995). Following incubation with the different fractions collected from chromatographic separation of nuclear extracts of A549 cells, the DNA is purified and subjected to cleavage with micrococcal nuclease to 3' mononucleotides. The 3' labelled mdCMP and dCMP are separated by thin layer chromatography (TLC) and the conversion of mdCMP to dCMP is directly determined (Szyf *et al.*, 1995). This assay provides a stringent test for *bona fide* demethylation and discriminates it from previously described 5mCpG replacement activities (Razin *et al.*, 1986; Vairapandi and Duker, 1993; Jost *et al.*, 1995; Weiss *et al.*, 1996; Fremont *et al.*, 1997).

Nuclear extracts prepared from A549 cells were subjected to a sequence of protein purification steps using DEAE-Sephadex,Sp-Sepharose, Q-Sepharose and DEAE-Sephacel matrix chromatography and active demethylase fractions were identified (Fig. 1A and Table 1). The purified DNA demethylase activity is a protein since it is abolished after proteinase K treatment and is not inhibited but rather enhanced following RNase treatment. 500 μM of ddCTP which inhibits DNA

polymerase b (Fisher *et al.*, 1979; Copeland and Wang, 1991) does not inhibit demethylation of the $[mdC^{32}pdG]_n$ substrate, nor is it inhibited by high concentrations of methyl-dCTP (500 μM) (Fig. 1A), which is consistent with the hypothesis that demethylation does not involve an excision and replacement mechanism. If a replacement mechanism is involved in demethylation, the presence of mdCTP should result in incorporation of methylated cytosines and essential inhibition of demethylation. Thus, the DNA demethylase identified here is a protein and not an RNA and is unequivocally different from the previously published glycosylase based demethylase activities (Razin *et al.*, 1986; Vairapandi and Duker, 1993; Jost *et al.*, 1995; Weiss *et al.*, 1996; Fremont *et al.*, 1997).

The DNA demethylase reaction proceeds without any requirement for additional substrates such as dCTP, redox factors such as NADH and NADPH or energy sources such as ATP (data not shown). As observed in Fig. 1B, the DNA demethylase reaction maintains its initial velocity up to 90 minutes and continues up to 120 minutes. This time course is inconsistent with dependence on enzyme-bound additional nonreplenishable substrates such as dCTP or ATP or a nonreplenishable redox factor such as NADH or NADPH. Exhausting the nonreplenishable substrate or redox factor would have resulted in rapid deceleration of the initial velocity.

As shown in Fig.1C, a clear peak of DNA demethylase activity is eluted at the high salt fraction 10. The first chromatography step purified the DNA demethylase activity from the bulk of nuclear protein (Fig. 1C) and separated DNA demethylase away from the DNA methyltransferase suggesting that they are independent proteins. DEAE-Sephadex chromatography also purifies dCTP away from the DNA demethylase at least $1x10^6$ fold (Fig. 1C) thus excluding the possibility that the demethylation activity in fraction 10 is a glycosylase-apyrimidine nuclease-repair activity. If any dCTP is present in the nuclear extract, the remaining concentration after fractionation on DEAE is well below the Kms of the known DNA

polymerases (Fisher *et al.*, 1979; Copeland and Wang, 1991).

DNA demethylase reaction produces methanol

To verify that the purified demethylase activity is a true demethylase we identified the leaving group. As illustrated in Fig. 2., incubation of methylated DNA with DNA demethylase (dMTase+ME-DNA) results in release of a peak with the retention time and mass spectrum (peaks are identified at 32 and 29 atomic mass which are the atomic masses of methanol and ionized methanol respectively) which is consistent with its identification as methanol. Incubation of DNA demethylase with nonmethylated DNA does not release methanol indicating that methanol is a product of the demethylation reaction. No methanol is released when the samples are incubated with DNA demethylase treated with protease K indicating that the release of methanol from methylated DNA is catalyzed by an enzymatic activity. These results demonstrate that we have purified a true demethylase from a human cell.

DNA demethylase displays mCpG sequence specificity

If DNA methylation is truly a reversible process the demethylase should be able to remove the specific products of the reaction catalysed by DNA methyltransferase. DNA methyltransferase catalyzes the transfer of methyl groups to CG dinucleotides residing in many possible sequence contexts generating either hemimethylated or fully methylated DNA (Gruenbaum *et al.*, 1982; Bestor *et al.*, 1988). The results presented in Fig.3 demonstrate that DNA demethylase is a general DNA demethylase activity that demethylates fully or hemimethylated dCpdG in DNA flanked by a variety of sequences which are distributed at different frequencies, but does not demethylate methylated adenines or methylated cytosines that do not reside in the dinucleotide CG. First, as shown in Fig. 3A, a plasmid DNA methylated *in vitro* at all dCpdG sites with M.*Sss* I and all d*CpdCpdGpdG sites with M. *Msp* I (which methylates the external C in the

sequence *CCGG, thus enabling the determination of demethylation at the CC dinucleotide) and *in vivo* with the *E. coli* DCM methyltransferase at dCmdCdA/dTdGdG sites and with the DAM methyltransferase at dGmdAdTdC sites (adenine methylated) was treated with DNA demethylase, and the state of methylation of the plasmid was determined using the indicated methylation sensitive restriction enzymes. DNA demethylase demethylates C*G methylated sites as indicated by the sensitivity of the DNA demethylase treated plasmid to *Hpa* II and *Hha* I but does not demethylate C*C,C*A or C*T methylated sites as indicated by the resistance to *Msp* I and *Eco* RII restriction enzymes, or adenine methylation as indicated by its sensitivity to *Dpn* I. Second, bisulfite mapping analysis of methylation of 5 methylated C*G sites residing in a M.*Sss* I *in vitro* methylated pMetCAT plasmid following DNA demethylase treatment shows that all C*G sites are demethylated irrespective of their flanking sequences thus excluding the possibility that demethylation is limited to CCGG or CGCG sequences (Fig. 3B). Third, DNA demethylase does not demethylate two fully methylated cytosine bearing oligomers $[dmC^{32}pdA]_n$,$[mdC^{32}pdT]_n$ demonstrating that mdCpdA and mdCpdT are not demethylated by DNA demethylase (Fig. 3D). Fourth, DNA demethylase demethylates a hemimethylated synthetic substrate $[dCpdG]_n$·$[mdC^{32}pdG]_n$ (Fig. 3D). Demethylation of the plasmid DNA is complete under these conditions (Fig. 3A) whereas demethylation of a methylated $[mdCpdG]_n$ substrate is not complete under the same conditions (Fig. 3D). This can reflect differences in the sequence composition of the substrate and the frequency of methylated cytosines. The $[mdCpdG]_n$ contains on average 16 fold more methylated cytosines per molecule than plasmid DNA. Alternatively, these differences might reflect discrepancies in the assays used; restriction enzyme digestion versus a nearest neighbor analysis. To address this discrepancy we have labelled a fully methylated plasmid DNA with [a^{32}P]dGTP, 5-methyl-dCTP, dATP and dTTP, subjected it to DNA demethylase treatment and digested it to mononucleotides at different time points following the initiation of the reaction and

subjected the samples to a TLC analysis. As shown in Fig. 3c, the plasmid DNA is fully demethylated by 3 hours which is consistent with the results obtained with methylation sensitive restriction enzymes (Fig. 3A).

The K_m of DNA demethylase for hemimethylated and fully methylated DNA was determined by measuring the initial velocity of the reaction at different concentrations of substrate (Table 2). The calculated K_m for hemimethylated DNA is 6 nM which is two fold higher than the K_m for DNA methylated on both strands, 2.5-3 nM (Table 2). It is unclear yet whether this small difference in affinity to the substrate has any significance in a cellular context. Thus, similar to the DNA methyltransferase, DNA demethylase shows dinucleotide sequence selectivity, but differs from DNA methyltransferase (which has preference for hemimethylated substrates), because it prefers fully methylated DNA, which is consistent with a role for DNA demethylase in altering established methylation patterns.

Discussion

This enzyme performs the reverse reaction to DNA methyltransferase and is an excellent candidate to be one of its important partners in shaping the methylation pattern of genomes. It remains to be seen whether lower organisms that bear DNA MeTases also harbour DNA demethylases or whether this activity is unique to organisms where DNA demethylation plays a critical role in development and regulation of genome function.

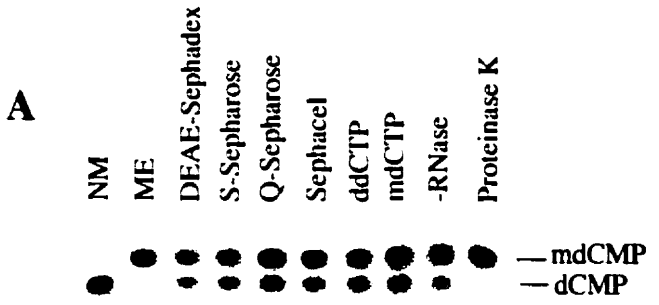
Methylation patterns exhibit site specificity, yet one remaining question is how can a general enzyme demethylate certain sites and not others? One possible hypothesis that has previously been proposed is that an interplay between local factors such as transcription factors and the general availability of DNA demethylase and methyltransferase determines the pattern of methylation (Szyf, 1994; Szyf *et al.*, 1995; Szyf, 1991; Szyf *et al.*, 1990b). The identification of DNA demethylase enables future experiments to test this hypothesis.

The existence of a general DNA demethylase(s) adds unexpected potential plasticity to DNA methylation patterns. The presence of a general enzyme that could remove well established methylation patterns and the potential for modulation of its activity by general signals (Siegfried and Cedar, 1997) points to the possibility that the covalent structure of the genome could be modified postmitotically in response to extracellular signals, pathological as well as physiological.

Fig. 1: Purification of a *bona fide* DNA demethylase from human cells.

A. 1 ng of $[mdC^{32}pdG]_n$ double stranded oligomer was incubated for 1 hour at 37°C with either buffer L (ME), 0.3 mg of active DNA demethylase fractions from the different chromatography steps in the presence of 100µg/ml RNase as indicated, with DEAE-Sephacel DNA demethylase fraction in the presence of RNase and either ddCTP (500 µM) or mdCTP (500µM), with DEAE-Sephacel DNA demethylase fraction in the absence of RNase, or with DEAE-Sephacel DNA demethylase fraction following pre incubation for 30 min. with Proteinase K (200 µg/ml). The reaction products were digested to 3' mononucleotides and analyzed on TLC (24). Methylated (ME) and nonmethylated (NM) $[dC^{32}pdG]_n$ substrates were digested to 3' mononucleotides and loaded on the TLC plate as controls. **B**, Kinetics of DNA demethylase activity. 1 ng of $[mdC^{32}pdG]_n$ double stranded oligomer was incubated with either DEAE-Sephacel DNA demethylase fraction (0.3 mg) for different time points and analyzed for demethylation as above. A representative chromatogram is shown. Chromatograms were quantified by a phosphorimager and the rate of transformation of mdCMP to dCMP was calculated and plotted. The results are a summary of three independent determinations +/- S. D. **C**, Nuclear extracts were fractionated on a DEAE-Sephadex column. Fifty microliter samples from each fraction (total fraction volume was 500 ml) were incubated with 1 ng of [mdC32pdG]n double stranded oligomer, digested to 3' mononucleotides and analyzed on TLC (24). Control methylated (ME) and nonmethylated (NM) $[dC^{32}pdG]_n$ substrates were digested to 3' mononucleotides and loaded on the TLC plate to indicate the expected position of dCMP and mdCMP(methyl-dCMP). The active fraction is indicated by an arrow. DNA methyltransferase activity was determined using a hemimethylated DNA substrate and [^{3}H]-AdoMet[33] (o -o). One unit equals the amount of enzyme that catalyses transfer of one picomole of tritiated methyl group on hemimethylated DNA per minute.

The results are an average of three independent determinations. Protein concentration was determined using the Bio-Rad Bradford kit (□ - □). To test whether dCTP copurifies with DNA demethylase, the elution profile of 20 μM of [^{32}P]-a-dCTP ($1.1x10^6$ dpm) incubated with the protein was determined by scintillation counting of the different DEAE fractions (Δ - Δ) and presented as fraction of dCTP loaded on the column.


A
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ME
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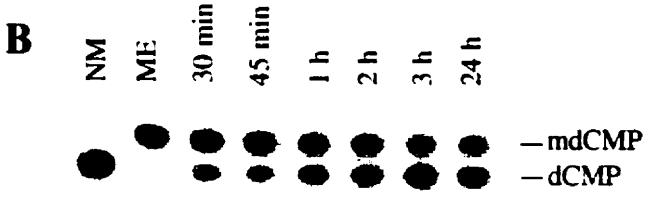
—dCMP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ME
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1 h
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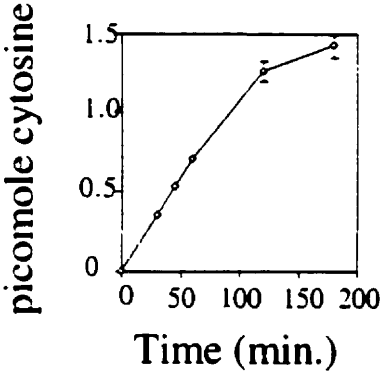
—dCMP


picomole cytosine
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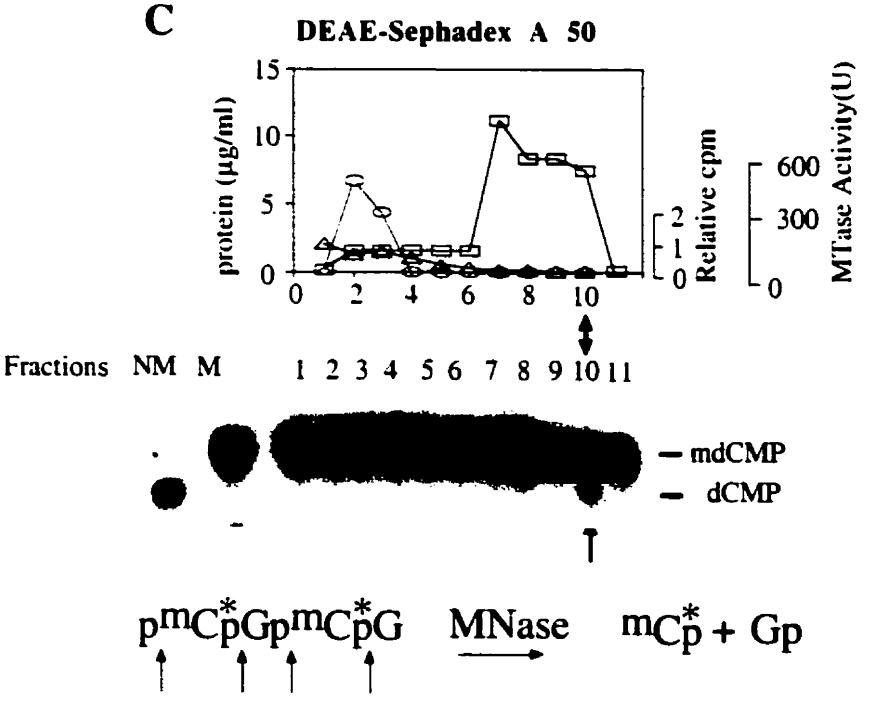
1.0
0.5
0
0 50 100 150 200
Time (min.)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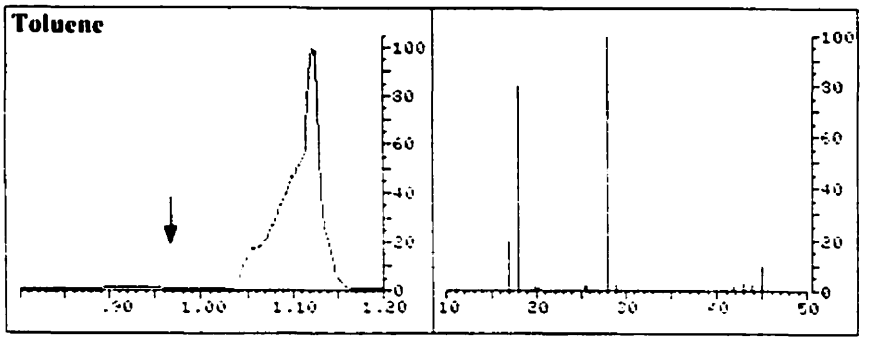
10
5
0
0 2 4 6 8 10
Relative cpm
2
1
0
MTase Activity(U)
600
300
0
Fractions NM M 1 2 3 4 5 6 7 8 9 10 11
—mdCMP
— dCMP
pmC*pGpmC*pG MNase mC*p + Gp

Figure 2: Purified DNA demethylase releases the methyl group in the form of methanol. GC/MS analyses of reference materials and incubated samples for methanol . The left half of each panel represents a portion of the gas chromatogram produced by the injected sample. The abscissa is the retention time in minutes and the ordinate is relative intensity in arbitrary units. The right half of each panel reproduces the mass spectrum measured at the retention time of methanol (marked with an arrow in the left panels). The abscissa is the mass/charge (m/z) ratio for the ions detected, while the ordinate is the relative intensity of the ions detected. The large irregularly-shaped peaks in the gas chromatograms is due to hydrocarbon impurities in the toluene solvent which would normally be of no consequence and not detected except that the instrumental sensitivity required for methanol detection is such that they are also detected. Panel labeled Toluene illustrates the results of the GC/MS analysis of the toluene solvent used in this study. The mass spectrum recorded at the retention time for methanol does not present ions that are prominent in the mass spectrum for dilute authentic methanol in toluene solution (1:1000 by volume, m/z 15, 29, 31 and 32, Panel labeled Methanol). Panel labeled dMTase + ME DNA illustrates the results obtained for the DNA demethylation reaction [with methylated CpG DNA (400 ng) and purified DNA demethylase (4.8 mg) in a volume of 50 ml at 37°C overnight] and demonstrates that a peak with the retention time and mass spectrum consistent with its identification as methanol is detected. An additional ion at m/z 18 is most likely due to residual water in the toluene extract. Panels labeled dMTase + NM DNA and dMTase (protease K treated) + ME DNA are the results of the analyses for demethylation reaction with CpG DNA with purified DNA demethylase and protease K treated DNA demethylase with methylated CpG DNA [these reaction were performed with 4.8 mg of purified DNA demethylase and 400 ng DNA in a reaction volume of 50 ml with overnight incubation at 37°C, 10 units of protease K was used for treatment of 4.8 mg of DNA demethylase in a reaction volume of 42 ml at 50°C for 3 hours], respectively, and display none of

the ions associated with methanol. The ion at m/z 18 is due to residual water in the extract. Prominent ions in the range m/z 39 to 43 are due to trace hydrocarbons in the toluene.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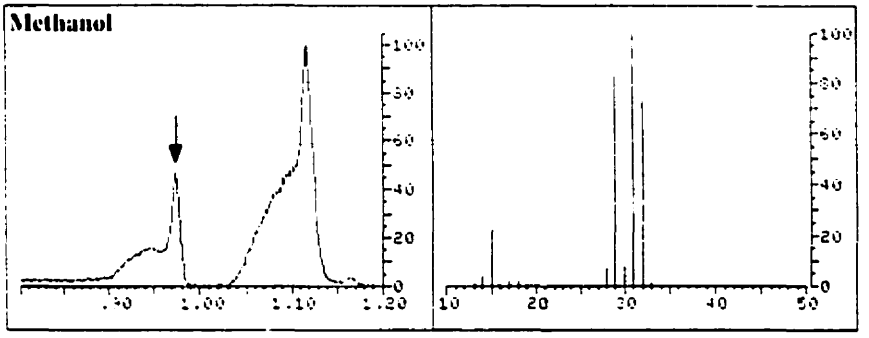
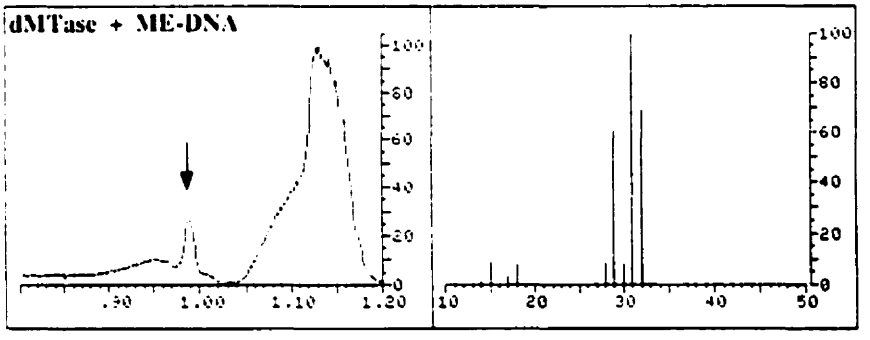
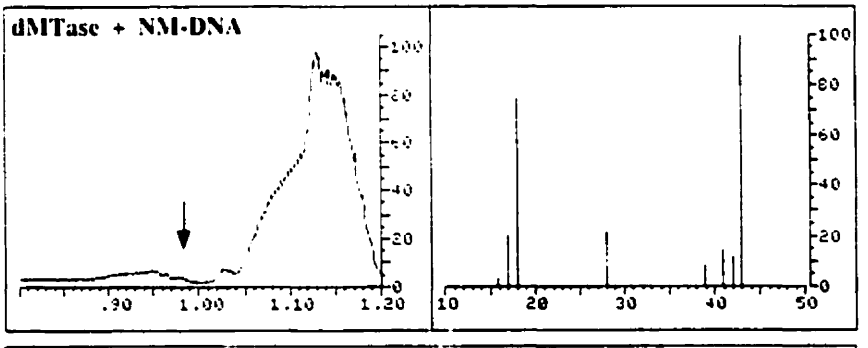

Methanol


dMTase + ME-DNA


dMTase + NM-DNA

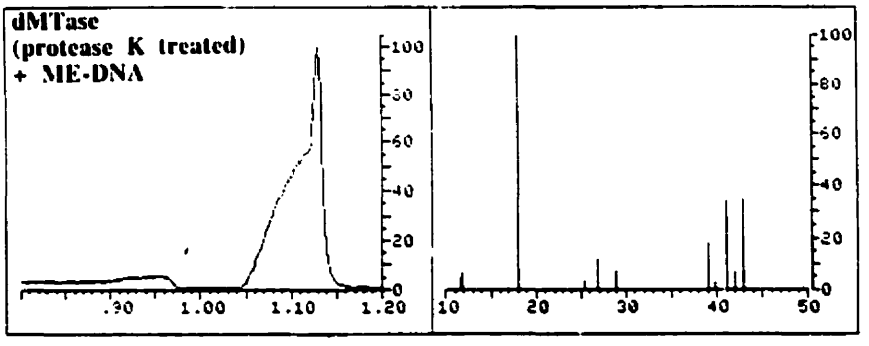
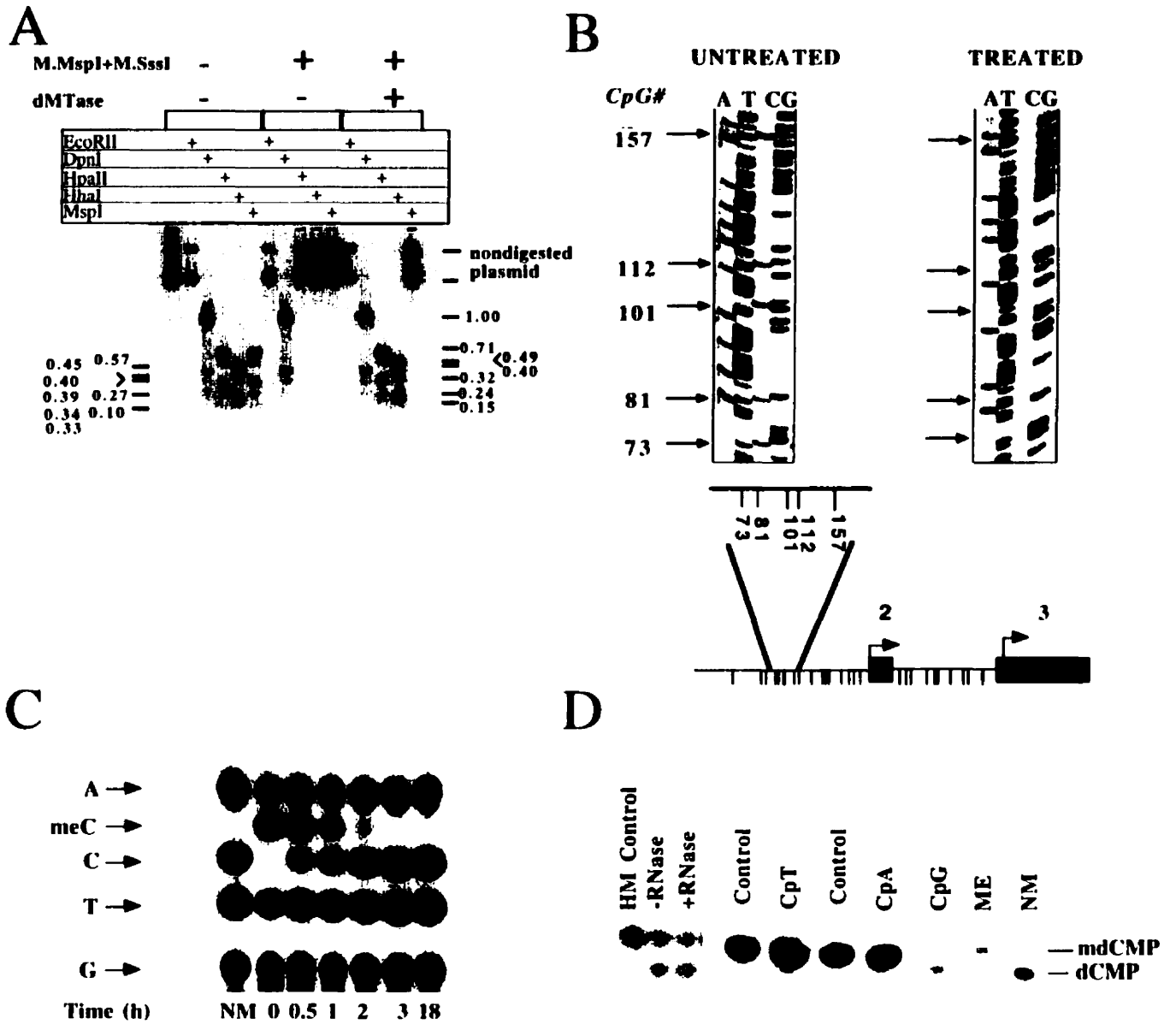

dMTase
(protease K treated)
+ ME-DNA

Fig. 3 Substrate Specificity of DNA demethylase

A. Demethylation of methylated SK. Plasmid pBluescript SK (5 ng) was methylated with M. *Sss* I methylase and M.*Msp* I methylase. DNA demethylase treated (1.2 mg of concentrated DEAE Sephacel fraction for 3h) (methylated SK+dMTase) and nontreated plasmid (methylated SK) as well as nonmethylated SK plasmid were digested with *Eco* RII, *Dpn* I, *Hha* I, *Msp* I and *Hpa* II. The digestion products were analyzed on a 2% agarose gel, blotted onto a Hybond N+ filter and hybridized with a ^{32}P labeled SK probe. The expected *Hpa* II/*Msp* I fragments are indicated on the right side of the figure, the expected *Hha* I fragments are indicated on the left side of the figure. Note that control plasmids are not digested with *Msp* I since the plasmid is methylated with *Msp* I MeTase) . **B.** Bisulfite methylation analysis of DNA demethylase treated pMetCAT plasmid. pMetCAT plasmid (Rouleau *et al.* 1992), methylated to completion with M.*Sss* I, was subjected to a DNA demethylase reaction as above. The DNA demethylase treated plasmid and nontreated control were subjected to bisulfite treatment as previously described (Clark *et al.*, 1994) and the genomic region bearing 5 CpG sites upstream of the second exon of DNA MeTase was amplified by PCR (Clark *et al.*, 1994) and sequenced. Twenty clones were sequenced per DNA sample, unmethylated cytosines are converted to thymidines by this protocol, whereas methylated cytosines are protected and are visualized as cytosines. One representative sequencing gel of bisulfite treated DNA is presented per condition. Arrows indicate the specific CpG sites by their position. The numbering is according to GenBank accession number M84387. Lower panel, the physical map of the genomic region residing upstream of exon 2 of DNA MeTase (indicated by filled boxes). Intronic sequence is indicated by a line. A blow up of the region amplified following bisulfite treatment is shown above the physical map, the different CpG sites in the fragment are presented as descending lines. **C.** Plasmid pBluescript SK (5 ng) was methylated with M.*Sss* I methylase and M.*Msp* I methylase. The methylated pBluescript SK was used as template to DNA synthesis using DNA polymerase and a^{32}pdGTP, mdCTP, dTTP, dATP

and hexanucleotide primers. Following two rounds of purification on NAP-5 column the substrate was subjected to demethylation using 0.12 mg of purified DNA demethylase for different time intervals as indicated. **D.** Demethylation of hemimethylated $[mdC^{32}pdG]_n.[dCpdG]_n$ (HM), $[mdC^{32}pdA]_n$ (CpA) and $[mdC^{32}pdT]_n$ (CpT) DNA substrates (Shemer *et al.*, 1991). One nanogram of the indicated substrates as well as a double methylated substrate $[mdC^{32}pdG]_n$ (CpG) were incubated either in the presence (0.3 mg) or absense of DNA demethylase (control for CpT and CpA and ME for CpG) and were then digested to 3' mononucleotides and analysed by TLC. Hemimethylated DNA was incubated with DNA demethylase in the presence or absence of RNase (100µg/ml). Non methylated $[dC^{32}pdG]_n$ served as a control for the position of dCMP.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M.MspI+M.SssI - + +
dMTase - - +
EcoRII
DpnI
HpaII
HhaI
MspI
nondigested plasmid
1.00
0.71
0.49
0.40
0.32
0.24
0.15
0.45 0.57
0.40
0.39 0.27
0.34 0.10
0.33
B
UNTREATED
TREATED
CpG#
A T CG
AT CG
157
112
101
81
73
73 81 101 112 157
2
3
C
A
meC
C
T
G
Time (h)
NM 0 0.5 1 2 3 18
D
HM Control
-RNase
+RNase
Control
CpT
Control
CpA
CpG
ME
NM
mdCMP
dCMP

Table 1: Purification of DNA demethylase from A549 cells.

Nuclear extract from A549 cells (1 ml , 6 mg) was applied sequentially onto a DEAE-Sephadex, SP-Sepharose, Q-Sepharose and DEAE-Sephacel columns as described in the methods. DNA demethylase was assayed using a 50 µl sample of each 0.5 ml fraction using the assay described in Fig.1 and (11). The specific activity of the active fractions after each purification step is represented as pmol CH_3 released/h mg of protein. The ratio of specific activity of DNA demethylase after each step to that of the nuclear extract is presented as fold purification.

Purification Step	**Total Protein** (μg)	**Specific Activity** (pMoles/h/μg)	**Fold Purification**
Nuclear Extract	6000	1.83×10^{-5}	-
DEAE-Sephadex	3.75	0.156	844.5
SP-Sepharose	0.77	0.663	35939.84
Q-Sepharose	0.46	1.13	62860
DEAE-Sephacel	0.018	10.19	552243

Table 2: Kinetic parameters for DNA demethylase

For determination of kinetic parameters, the DNA demethylase reactions were performed as in Fig.1 except that varying DNA concentrations, from 0.1 nM to 50 nM, were used in a total volume of 100 µl containing 1.2 mg of DEAE Sephacel DNA demethylase fraction. Since it has been established that the reaction proceeds for at least 3 hours (Fig.1B), the initial velocity of reaction was measured at one hour intervals. The velocity data was collected at each substrate DNA concentration. The K_m and V_{max} values for demethylase activity were determined from double reciprocal plots of velocity versus substrate concentration.

Substrate	**Km** (DNA)	**Vmax** (pMole/h)
methylated CpG	2.5 nM	340
hemi-methylated CpG	6.0 nM	402
methylated SK-DNA	3.3 nM	40.42

Discussion

Aberrations of DNA methylation patterns occur in cancer cells

Much evidence has established support for the hypothesis that uncontrolled changes of DNA methylation patterns occur in tumor cells (Baylin *et al.*, 1991). Originally most of the attention was focussed on the global hypomethylation seen in cancer cells (Feinburg and Vogelstein, 1983) as a possible mediator in the tumorigenic process. Recently, however, a large body of data suggests that many tumor suppressor genes, as well as genes involved in the prevention of tumor progression (cell adhesion molecules and angiogenesis inhibitors) are hypermethylated and inactivated in many cancer cells. This has led to the proposal that aberrant methylation is an epigenetic mechanism of inactivating tumor suppressors.

The mechanisms responsible for the altered DNA methylation patterns seen in tumor cells and the possible roles they play can be explained by two alternative hypotheses. First, an elementary explanation is that these changes reflect rare and random errors in DNA methylation, which are then selected for and made permanent during tumor progression. This explanation is consistent with the observation that both hypo- and hypermethylation are seen in tumor cells. It is possible that hypermethylation of some loci and hypomethylation of other loci provide a growth advantage to tumor cells. Random errors in methylation potentially involve events of both hyper- and hypomethylation and as predicted by this hypothesis, both are observed simultaneously in cancer cells.

The proposed alternative hypothesis is that changes in DNA methylation are essential components of oncogenic programs (Szyf, 1994; Szyf, 1996). This hypothesis is based on the progressively increasing knowledge concerning the mechanisms of action of a wide variety of oncogenes. Activation of a proto-oncogene to an oncogene by a genetic lesion releases a tumorigenic cellular program, rather than a random collection of events. Originally the main focus of attention was on the identity of cancer causing genes (oncogenes), where the

mutation of the proposed proto-oncogene can induce cancer, it is now accepted that it is as important to reveal the program that is induced by the activation of oncogenes. In the last decade it has become increasingly clear that a large number of oncogenes converge on a small number of nodal cellular signalling pathways that mediate the oncogenic signal. The gold standard example of this is the Ras-Jun signalling pathway (Angel and Karin, 1991). The Ras-Jun signalling pathway mediates a large number of cellular responses involved in developmental and physiological processes (Angel and Karin, 1991). Signals from many extracellular sources converge upon the small G-protein Ras converting it from its inactive form, in which it is bound to GDP, to its active form, where it is bound to GTP. Then the sequential activation of a number of protein kinases lead to the activation of the transcription factor Jun (Binetruy *et al.*, 1991). Aberrant activation of any of the proteins within this cascade can result in oncogenesis. Jun most probably transactivates genes that initiate the oncogenic state. Obviously, in order to understand this and possible other oncogenic pathways, it is essential to identify the downstream effectors of Jun ie. the genes that Jun transactivates. The downstream effectors of Jun are the main participants in triggering oncogenesis and are involved in a broad range of cancers induced by any of the oncogenes that feed into this pathway, therefore the downstream effectors of Jun are ideal targets for anticancer therapy. A large number of genes have AP-1 sites in their regulatory regions and hence can possibly be induced by aberrant Jun activation (Angel and Karin, 1991; Binetruy *et al.*, 1991), however it is not clear which of these genes play a critical role in oncogenesis.

An attractive speculation is that only a few of the proteins that are induced by Jun are vital and sufficient for oncogenesis and are located downstream to all oncogenic pathways. A possible explanation for the hypermethylation seen in cancer cells is that it is a consequence of an induction of DNA MeTase activity (Szyf, 1994). Several observations have demonstrated increased DNA MeTase activity and elevated *dnmt1* mRNA in transformed cells when compared to their normal counterparts in both cell lines and tumors (Kautianinen and Jones, 1986; el-

Deiry *et al.*, 1991), and that an increase in DNA MeTase is an early event in a mouse model of chemically induced lung carcinogenesis (Belinsky *et al.*, 1996). Chapter 2 of this thesis demonstrates that the regulatory regions of the human *dnmt1* gene contain a number of AP-1 transcription factor sites and are activated by the transcription factor Jun. Similarly, it has been shown that the identical mouse *dnmt1* regulatory region contains AP-1 sites and respond to Jun or Ras expression (Rouleau *et al.*, 1995) by increasing *dnmt1* mRNA levels, DNA MeTase activity and DNA methylation (MacLeod *et al.*, 1995). These results indicate that the Ras-Jun signalling pathway regulates the expression of *dnmt1* gene expression and places DNA MeTase as a downstream effector of a major oncogenic signalling pathway (Szyf *et al.*, 1994; MacLeod *et al.*, 1995; Yang *et al.*, 1997; Chapter 2).

Is the increase in DNA MeTase essential for the Ras-Jun mediated transformation program or is it just a consequence of cellular transformation? Fibroblast cells derived from *c-jun* knockout mice cannot be transformed by over expression of Ras (Johnson *et al.*, 1996). The identity of the AP-1 dependent gene(s) that is downstream to Jun and is required for cellular transformation, until recently, was not known. Previous data (Rouleau *et al.*, 1995; MacLeod *et al.*, 1995), and the data presented in Chapter 2 suggest that *dnmt1* may be a candidate. Many AP-1 dependent genes have been shown to have similar activity in *c-jun* knockout primary fibroblasts as in wild type fibroblasts (Johnson *et al.*, 1993), but recently our group has shown that, in the same knockout and wild type fibroblasts referred to above, *dnmt1* expression and DNA MeTase activity are dramatically reduced in the knockout line (Slack *et al.*, unpublished data). Furthermore the same study shows that transient ectopic expression of *dnmt1* in the *c-jun* knockout line rescues their tumorigenic potential (Slack *et al.*, unpublished data). These observations indicate that *dnmt1* may be the key mediator in promoting the cellular transformation program of the Ras-Jun signal transduction pathway. Another line of evidence supporting a causal role for DNA MeTase in Ras mediated cellular transformation comes from studies in an adrenal carcinoma cell

line Y1, which bears a 40 fold amplification of *c-ki-ras* gene (Schwab *et al.*, 1983). Previously, it had been demonstrated that the tumorigenic potential of this cell line can be inhibited by expression of a portion of the *dnmt1* cDNA in the antisense orientation (MacLeod and Szyf, 1995). Similarly it has been recently demonstrated that DNA MeTase is a downstream effector of the transformation pathway triggered by Fos (Bakin and Curran, 1999). Chapter 4 of this thesis demonstrates that implanting of Y1 cells into its syngeneic host mouse strain causes tumors and that treating inoculated mice with a modified antisense DNA oligonucleotide against *dnmt1* inhibited tumor growth *in vivo*. These observations indicate an essential role for *dnmt1* in tumorigenesis and also provide preclinical evidence that pharmacological inhibition of *dnmt1* expression is therapeutic in organisms with cancer.

If DNA MeTase is a fundamental downstream component of cellular transformation programs in general, then it should be involved in other defined transformation pathways. Cellular transformation induced by the SV40 T antigen is one of the most studied (Brinster *et al.*, 1984). Inactivation of the tumor suppressor protein Rb by protein-protein interactions with SV40 T is an important mechanism involved in cellular transformation (De Caprio *et al.*, 1988). This is a distinct, yet complimentary, transformation pathway from the Ras-Jun pathway. Ras and SV40 T antigen cooperate in the transformation of primary cells (Land *et al.*, 1983). Interestingly, SV40 transformed cells express higher levels of DNA MeTase (Chuang *et al.*, 1997; Kopp *et al.*, 1997). Recently, our group has shown that SV40 T antigen induces *dnmt1* mRNA levels by a posttranscriptional mechanism which requires the inactivation of the tumor suppressor protein Rb (Slack *et al.*, 1999). In non transformed cells it has been demonstrated that *dnmt1* expression is regulated in a growth dependent manner which is posttranscriptional (Szyf *et al.*, 1991). Chapter 3 of this thesis sheds light on the mechanism of this posttranscriptional regulation. It demonstrates that the 3' untranslated region of *dnmt1* can impart a cell cycle dependent regulation upon the heterologous rabbit β-globin gene, that it attracts a cell cycle specific mRNA binding complex, and that a

labile protein factor is responsible for rapid elimination of the *dnmt1* mRNA during the G_0-G_1 portion of the cell cycle. It is possible that cooperation between Ras and SV40 T antigen results in synergistic activation of DNA MeTase. The referred experiments and the data presented in this thesis indicate that DNA MeTase plays a causal role in multiple cellular transformation programs (for regulatory scheme see introduction Fig. 1-4), and that inhibition of *dnmt1* expression results in the reversal of cellular transformation.

Convincing evidence for the hypothesis that high activity of DNA MeTase is essential for cellular transformation is derived from experiments done using the *min* mouse strain. This strain of mouse bears a homologous mutation to the human adenomatous polyposis coli (APC) mutation and develops intestinal polyps (Laird *et al.*, 1995). A genetic cross between a *min* mouse and a mouse containing only one functional allele of *dnmt1* (*dnmt1 -/+*) develops a reduced number of polyps (Laird *et al.*, 1995). These data were interpreted to suggest that the reduced DNA MeTase results in reduction of methylated cytosines in the genome, thus reducing the probability of cytosine to thymidine transitions caused by deamination of 5-methyl-cytosine (Laird *et al.*, 1995). More recent data, generated by the same group, failed to support this hypothesis (Jackson-Grusby *et al.*, 1997). Instead mutation of *apc* may initiate an oncogenic pathway that induces, and requires, DNA MeTase. This increase in DNA MeTase activity and the resulting cellular transformation, is partially inhibited by the deletion of one copy of the *dnmt1* gene, as observed in Laird *et al.*, 1995. The wild-type APC protein binds the β-catenin protein, a protein that has been shown to be a member of Tcf and Lef transcription induction complex (Korinek, *et al.*, 1997). The description of the APC oncogenic signalling pathway indicates that, similar to the Ras-Jun signalling pathway, there is an involvement of a constitutively activated transcription factor complex, the β-catenin:hTcf-4 complex (Korinek, *et al.*, 1997). Expression of wild-type APC removes β-catenin from hTcf-4 and terminates transcriptional activation (Korinek, *et al.*, 1997). An attractive possibility is that expression of mutant APC results in constitutive activation of DNA MeTase through the β-catenin:hTcf-4 complex.

Interestingly, the distal promoter of human *dnmt1* , described in Chapter 2 of this thesis, contains a large number of Tcf binding sites. Preliminary reporter gene/co-transfection assays indicate that this promoter's activity can be enhanced by the APC pathway (Campbell *et al.*, unpublished data).

How does over expression of DNA MeTase cause cellular transformation?

Hypermethylation and inactivation of tumor suppressors is a result of DNA MeTase induction.

Over expression of DNA MeTase leads to the hypermethylation and inactivation of a number of genes that can either suppress tumorigenesis (Merlo *et al.*, 1995; Baylin *et al.*, 1998; Graff *et al.*, 1997), tumor invasion (Yoshiura, *et al.*,1995), angiogenesis (Ahuja, *et al.*, 1997), or genes involved in the maintenance of differentiated cellular identity (Szyf *et al.*, 1989). Over expression of DNA MeTase may be the signal to initiate the termination of a cognate program of gene expression and the induction of a gene expression program leading to cellular transformation. Ectopic expression of *dnmt1* leads towards hypermethylation and cells that express high levels of DNA MeTase have hypermethylated genes in comparison to their non transformed parental tissue (Vertino *et al.*, 1996). Much data has been accumulated that supports the hypermethylation and inactivation of tumor suppressors as having an important role in oncogenesis (Baylin *et al.*, 1998). A major caveat in this hypothesis is reconciling how a general induction in DNA MeTase activity can result in specific hypermethylation of a single subset of genes but not any others. The observation that tumor cells are globally hypomethylated (Feinburg and Vogelstein, 1983) adds an additional level of complexity to this issue. It may be that specific trans acting factors target the DNA MeTase to these sites (Szyf *et al.*, 1989; Vertino *et al.*, 1996; Szyf, 1991). Alternatively, it may be possible that an increase in DNA MeTase leads to random hypermethylation. Advantageous hypermethylation events are then selected for during a multilevel transformation process. If random methylation followed by selection is the true

mechanism of transformation, then forced expression of DNA MeTase should result in a gradual, time dependent progression to transformation, which allows for selection to take its course. Recently, our group has demonstrated that transient expression of *dnmt1* can induce cellular transformation (Slack *et al.*, unpublished data) suggesting that this notion may not accurately describe the situation.

Aberrant activation of dormant origins of DNA replication by elevated DNA MeTase

An increase in DNA MeTase may be specifically required to activate dormant origins of DNA replication, and thus eliciting the cellular replication program of cancer cells. Previous findings suggest that a larger number of origins of DNA replication are active in SV40 transformed cells, than in non transformed cells (Martin and Oppenheim, 1977). It is known that the DNA MeTase enzyme is present in the DNA replication fork (Leonhardt *et al.*, 1992; Chuang *et al.*, 1997), and that newly replicated DNA at origins of DNA replication is simultaneously methylated as it is synthesized (Araujo *et al.*, 1998). It is possible that the state of methylation of these origins can dictate their activity since it has been shown that some origins have a cluster of hypermethylated CGs and that their methylation may be required for the origin to function (Rein *et al.*, 1997). It is possible that the mammalian genome has different classes of origins of DNA replication which are differentially activated, based on their state of methylation. The presence of DNA MeTase in the DNA replication fork of a sub-class of DNA replication origins may be required for activating dormant methylation sensitive origins of DNA replication. Activation of these normally inactive origins may be associated with bypassing growth inhibitory signals that normal cells respond to.

Induction of cellular transformation by protein-protein interactions of the DNA MeTase protein and components of the replication machinery

DNA MeTase has a peptide sequence in its N-terminal domain that

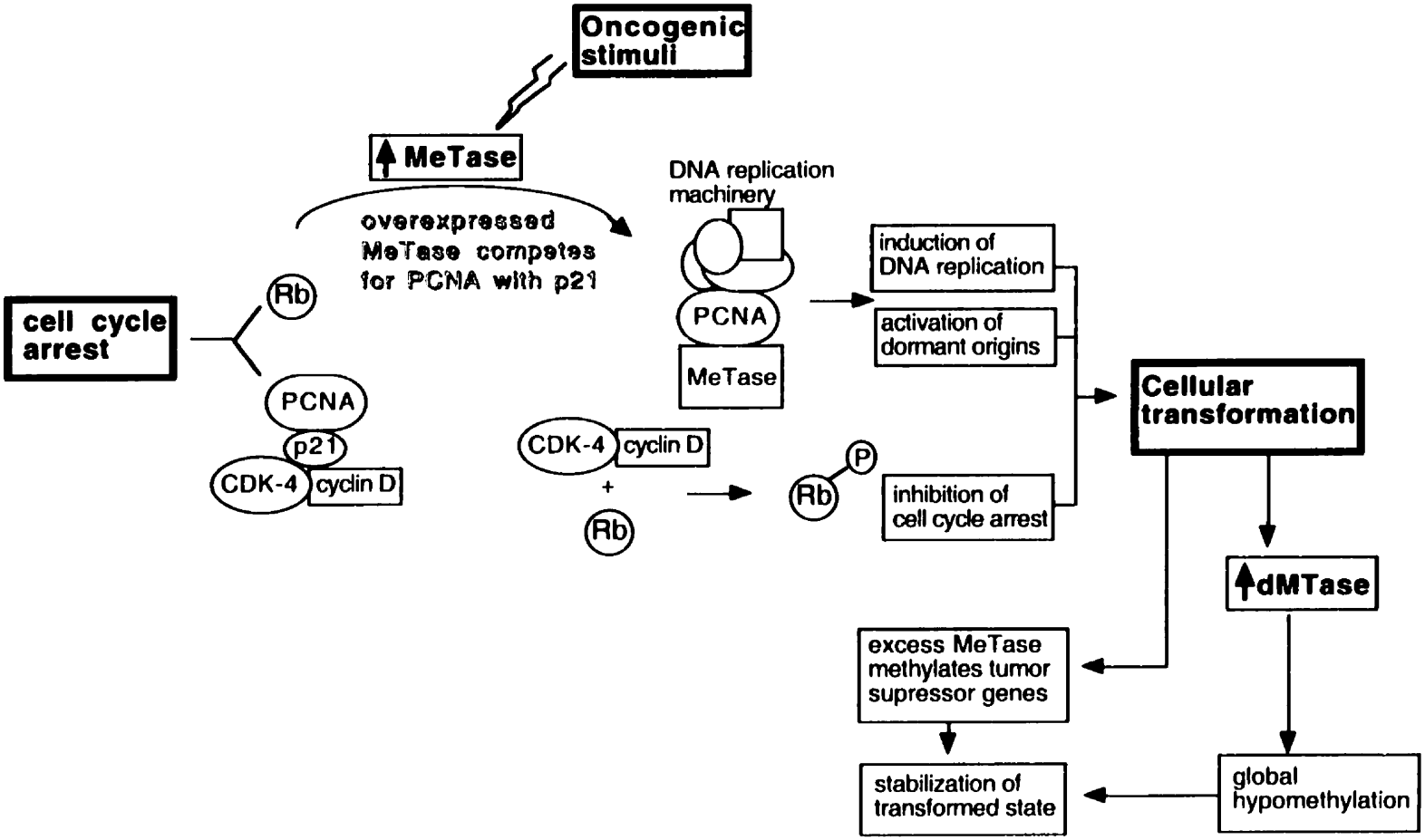
recognizes and binds to the proliferating cell nuclear antigen (PCNA) (Chuang *et al.*, 1997). Binding of PCNA to DNA MeTase competes out the binding of p21 to PCNA (Chuang *et al.*, 1997). p21 is a universal inhibitor of cyclin kinases (Xiong *et al.*, 1993a), a mediator of the p53 tumor suppressor function (el-Deiry *et al.*, 1993), and mediator of other growth suppressor signals such as TGFβ (Datto *et al.*, 1995). It has also been demonstrated that p21 can inhibit DNA replication directly by interacting with PCNA and inhibiting its activity (Waga *et al.*, 1994). Earlier observations have shown that the quaternary complex of p21-PCNA-cyclinD1-CDK4 regulates the G_1-S transition of the cell cycle through inhibition of the phosphorylation of Rb (Xiong *et al.*, 1993b) and by other Rb independent mechanisms (Dimri *et al.*, 1996).

Normal and tumor cells can potentially grow, replicate, and cycle through the cell cycle. Normal cells respond to growth inhibitory signals such as terminal differentiation or contact inhibition by arresting and preventing entry into the cell cycle, while tumor cells appear to ignore these signals. p21 is induced upon loss of cell anchorage (a measure of tumorigenicity in culture [Freedman and Shin, 1974]), which is consistent with its role as a common mediator of growth arrest signals (Wu and Schontal, 1997). It may be possible that p21 plays a central role in mediating the growth inhibitory signals that are associated with terminal differentiation. In support of this notion, it has been observed that ectopic expression of p21 has a strong anti tumorigenic effect (Yang *et al.*, 1995) and that the expression of p21 in normal non transformed cells is restricted to the G_1 phase of the cell cycle (Li *et al.*, 1996) while it is highly expressed in terminally differentiated cells (Halevy *et al.*, 1995). In the transformed state, elevated levels of DNA MeTase may disrupt the p21-PCNA complex, and thus override the growth inhibitory signals exerted by p21, leading to improper entry into cell cycle. Transformation as described by this hypothesis (Fig. 2-1) predicts that the alterations in DNA methylation are secondary events in the transformation process.

If induced DNA MeTase is required for overriding growth inhibitory signals but not for interpreting regular growth signals, the inhibition of DNA MeTase will

Figure 2-1: A model of the role of the DNA methylation machinery in cellular transformation.

In non transformed, non-cycling cells, the quaternary complex p21-PCNA-cyclinD1-CDK4 inhibits the cell's transition into the cell cycle by inhibiting phosphorylation of Rb, and by other Rb independent mechanisms. Oncogenic stimuli converge to induce expression of DNA methyltransferase (MeTase). The increased DNA MeTase interacts with PCNA by competing out p21, disrupting the growth inhibitory quaternary complex. The free cyclinD1-CDK4 complex can now phosphorylate (P) and thereby inactivate Rb relieving suppression of the cell cycle. The DNA MeTase loaded DNA replication machinery activates dormant origins of DNA replication, increasing the rate of DNA replication. These processes lead to the initial transformation of the cell. In order to maintain or stabilize the transformed state, the increased levels of DNA MeTase results in specific hypermethylation of tumor suppressor genes and other genes that slow cell growth. In order to cope with the elevated DNA MeTase levels, the transformed cell also induces the expression of DNA demethylase (dMTase), which results in the global hypomethylation observed in transformed cells.


Oncogenic stimuli
MeTase
overexpressed MeTase competes for PCNA with p21
DNA replication machinery
PCNA
MeTase
induction of DNA replication
activation of dormant origins
cell cycle arrest
Rb
PCNA
p21
CDK-4
cyclin D
CDK-4
cyclin D
+
Rb
P
Rb
inhibition of cell cycle arrest
Cellular transformation
dMTase
excess MeTase methylates tumor supressor genes
stabilization of transformed state
global hypomethylation

only affect tumor cell growth, which can by assayed by cell growth in soft agar or growth in a syngeneic host, but not normal cell growth. Chapter4 of this thesis (Ramchandani *et al.*, 1997) and Laird *et al.*, 1995, demonstrate that inhibition of DNA MeTase by different means does not lead to overt toxicity to normal tissue while there is a significant brake put on tumor cell growth. An explanation may be that p21 is not expressed in normal growing cells during S phase of the cell cycle (Li *et al.*, 1996), therefore inhibition of DNA MeTase will not have an effect on normal cells through p21. Transformed cells represent an aberration of a somatic cell, in which the normal cognate program is not to replicate. It is possible that these transformed cells express or can induce the expression of p21, but ignore its growth inhibitory effect through the elevated levels of DNA MeTase. In support of this hypothesis is the fact that p21 is expressed in most cancer cells and that inactivating mutations of p21 in cancer cells have not yet been identified (Shiohara *et al.*, 1997).

This protein-protein interaction model is also consistent with the observation that the overall level of DNA methylation is not higher, but lower, in transformed cells in spite of elevated levels of DNA MeTase. Since the major transforming event requires an increase of the DNA MeTase protein *per se*, and not increased DNA methylation, the overall level of DNA methylation is not important for the transformation process. The reason that an increase in overall DNA methylation is not seen can be explained by DNA MeTase's limited capacity to *de novo* methylate DNA (Gruenbaum *et al.*, 1983). It is possible that there are only a limited number of DNA sequences that contain the *cis* acting signals that attract the *trans* acting factors that are required for targeting DNA MeTase for *de novo* methylation (Szyf, 1991; Graff *et al.*, 1997). In conjunction with the biochemical limitations of DNA MeTase there appears to be a specific DNA demethylase activity induced in transformed cells (Chapters 5 and 6; Szyf *et al.*, 1995) which contributes to the observation (and will be discussed in more detail later).

Studies in animals that were given methyl deficient diets indicate that the livers from these animals have hypomethylated DNA and develop neoplasia at a

high frequency (Wainfan *et al.*, 1989). These observations were interpreted to indicate that hypomethylation of DNA plays a causal role in the development of cancer (Wainfan *et al.*, 1989). However a later study by the same group demonstrates that animals exposed to a methyl deficient diet also expressed elevated levels of DNA MeTase enzyme (Christman *et al.*, 1993). As this model predicts (Fig. 2-1), it is the elevated levels of DNA MeTase protein, and not the methylation change that is responsible for the transformation event. The increase in DNA MeTase may originally have been brought about as a response to the lack of methylated DNA caused by the methyl deficient diet and then initiated a cellular transformation program. In support of this explanation is the observation that DNA MeTase becomes elevated in a feedback response to the demethylating agent 5-aza-C (Yang *et al.*, 1997). More recently our group has shown that the proximal promoter region of *dnmt1* contains a heavily methylated region that becomes demethylated when DNA MeTase is inhibited which results in the induction of this promoter (Slack *et al.*, 1999b). Therefore it appears that hypomethylation can lead to cellular transformation by causing a feedback increase in the level of DNA MeTase enzyme.

DNA MeTase and cellular transformation

DNA MeTase is a candidate to be a fundamental component in the process of cellular transformation because it can act at several independent levels to organize the multiple changes needed to move a cell into the transformed state. The three scenarios for the role of DNA MeTase presented above are not necessarily mutually exclusive and can be unified into one hypothesis (Fig. 2-1). p21 and p21-like proteins function to bring about cellular arrest and differentiation of normal cells. In non cycling cells DNA MeTase is expressed at very low levels (Szyf *et al.*, 1991). Aberrant activation of any of the oncogenic signalling pathways in non cycling cells leads to an induction of DNA MeTase. The induced levels DNA MeTase displace p21 from the quaternary complex it forms with PCNA-cyclinD1-

CDK4, and by doing so relieves p21's growth inhibitory effect (Chuang *et al.*, 1997). The replication machinery that is now loaded with DNA MeTase will then activate dormant methylation dependent origins of replication resulting in an faster progression through S-phase of the cell cycle. The excess DNA MeTase activity will then result in aberrant *de novo* methylation and subsequent permanent inactivation of tumor suppressor genes (Vertino *et al.*, 1996), which will stabilize the transformed state.

DNA demethylase is induced in cancer cells

Another observation that needs to be accounted for is the global hypomethylation seen in cancer cells (Feinburg and Vogelstein, 1983) in spite of the presence of elevated DNA MeTase levels (Kautiainen and Jones, 1986; El-Deiry *et al.*, 1991). As was previously eluded to, the DNA MeTase has a diminished capacity for carrying out *de novo* methylation, and that factors which assist in targeting DNA MeTase for site specific *de novo* methylation may be limiting. A second explanation is that cancer cells express high levels of a DNA demethylase that actively removes methyl groups from DNA as has been suggested before (Szyf, 1994). Interestingly, ectopic expression of the *ras* oncogene not only induces DNA MeTase (Rouleau *et al.*, 1995; MacLeod *et al.*, 1995), but it also induces a DNA demethylating activity (Szyf *et al.*, 1995). Chapter 5 and 6 of this thesis describe the purification, characterization, and cloning of a *bona fide* DNA demethylase activity from human cancer cells. The assays for dMTase activity described in chapters 5 and 6 and the cloning of DNA dMTase cDNA enables a study of its expression at different cellular states. As observed in Fig. 2-2, DNA dMTase activity is readily observed in all carcinoma cell lines, but it is undetectable in non transformed human cells. The absence of dMTase activity in human primary cells reflects the situation *in vivo* since dMTase activity is undetectable in preparations from different murine tissues whereas dMTase activity

is present in a murine carcinoma cell line P19 that was transfected with the H-Ras protooncogene (Szyf *et al.*, 1995), or human tumors carried as xenografts in the same strain of mouse (Fig. 2-2 COLO 205, A549. Hela). These conclusions were verified using the radioactive-trapping volatilisation assay shown in Fig.2-2c. Briefly, this assay utilized methylated DNA where the methyl group is labelled with ^{3}H, and since one of the products of the demethylation reaction is methanol (Chapter 6), a volatile moiety, demethylation can be assayed by migration of volatile counts into the scintillation phase of a double tube set up (Fig. 2-2d).

Since dMTase mRNA has been detected using a sensitive poly A+ Northern blot in all normal human tissues (Chapter 5), a possible hypothesis is that the absence of detected dMTase activity in normal tissues reflects a quantitative difference in DNA dMTase mRNA between normal tissues and cancer lines. A Northern blot analysis and quantification of DNA dMTase mRNA by a slot blot analysis shown in Fig. 2-3 using total RNA supports this hypothesis. Minute levels of DNA dMTase mRNA are detected in normal tissues, but high levels of dMTase are expressed in a murine carcinoma cell line Y1 that bears a 30 fold amplification of *Ki-ras* (Schwab, *et al.*, 1983).

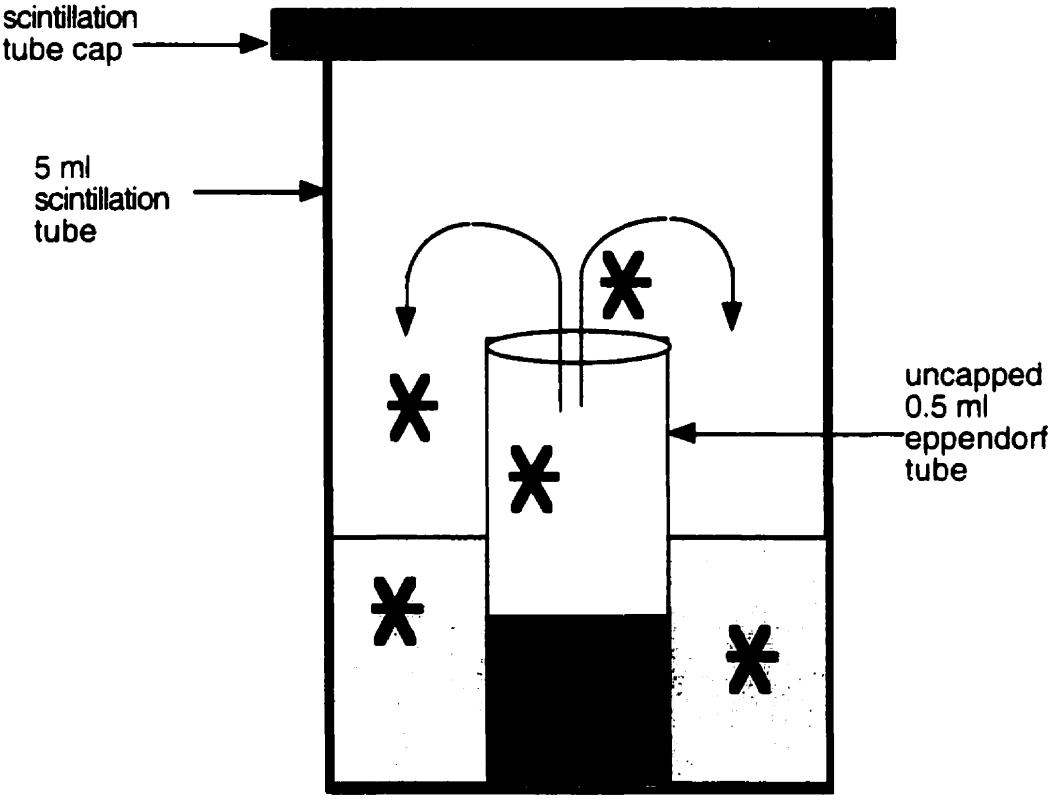
What is the role for a DNA demethylase activity in a transformed cell? It may be that enhanced DNA MeTase will lead to *de novo* methylation of, and subsequent suppression of, genes that are essential for general cellular viability. Prolonged ectopic expression of DNA MeTase is difficult to accomplish in cultured cells which lends weight to this hypothesis (Tucker *et al.*, 1996). In order to accommodate the high levels of DNA MeTase that are required to override the growth inhibitory signals in the process of cellular transformation, the newly transformed cell may induce a DNA demethylase activity as a protective mechanism to prevent inactivation of essential genes (see model Fig. 2-1). This model predicts that inhibition of DNA dMTase should display some sort of toxicity to transformed cells in a specific fashion. In accordance with this prediction transformed cell growth is inhibited when cells are transfected with a constitutively active vector expressing the DNA dMTase in the antisense orientation (Fig. 2-4a).

Figure 2-2 DNA dMTase activity *in vivo* is induced in transformed tissues.

a, dMTase activity in equal concentrations of DEAE Sephadex fractions 9 and 10 purified from; A549 (non small cell lung carcinoma), H446 (small cell lung carcinoma) MHRF (primary human fibroblasts), COLO 205 and Hela cells was determined at initial rate conditions using a $[mdC^{32}pdG]_n$ (1ng) double stranded oligomer substrate. MRHF* lane indicates an "over loaded" sample to ensure there is no residually detectable activity. The treated DNA samples were digested to 3' mononucleotides and subjected to TLC analysis. NM, non methylated substrate control; Control, methylated untreated substrate. **b**, dMTase activity in DEAE fractions 8, 9 and 10 purified from P19ras cells, and different mouse tissues (Spleen, Liver, Muscle, Heart, Brain). mdCMP, methylated cytosine; dCMP, non methylated cytosine **c**, The volatilisation of $[^3H]$-CH_3OH from methylated SK DNA by DEAE fractions 10 prepared from different mouse tissues (Muscle, Kidney, Brain, Heart, Spleen, Liver), P19 cells, P19ras cells, Y1 cells and A549 cells. **d.** Radioactive trapping volatilisation assay. $[^3H]$- CH_3-SK DNA (methylated with M.SssI at a specific activity of 4 $x10^6$dpm/mg) was incubated with DNA dMTase in a reaction volume of 50 µl in a 0.5 ml eppendorf tube. The tube was uncapped and placed floating in a sealed 5 ml scintillation vial containing 1.5 ml of scintillation liquid and incubated overnight at 37°C. In the absence of DNA demethylase activity the CH_3 groups are covalently attached to DNA, since there is no contact between the DNA and the scintillation fluid, no counts are registered in the scintillation counter even after long incubation. In the presence of demethylase the $[^3H]$-CH_3 groups are volatilised as methanol and are transferred to volatile phase and mix in the liquid scintillation cocktail emitting radioactive counts.

a
NM
Control
A549
A549
H446
H446
MRHF
MRHF*
dCMP
mdCMP
COLO 205
A549
Hela
dCMP
mdCMP
b
P19ras
Spleen
Liver
Muscle
Heart
Brain
dCMP
medCMP
c
volatilized methyl residues (dpm)
0
500
1000
1500
Muscle
Kidney
Brain
Heart
Spleen
Liver
P19
P19 ras
Y1
A549
Control

d



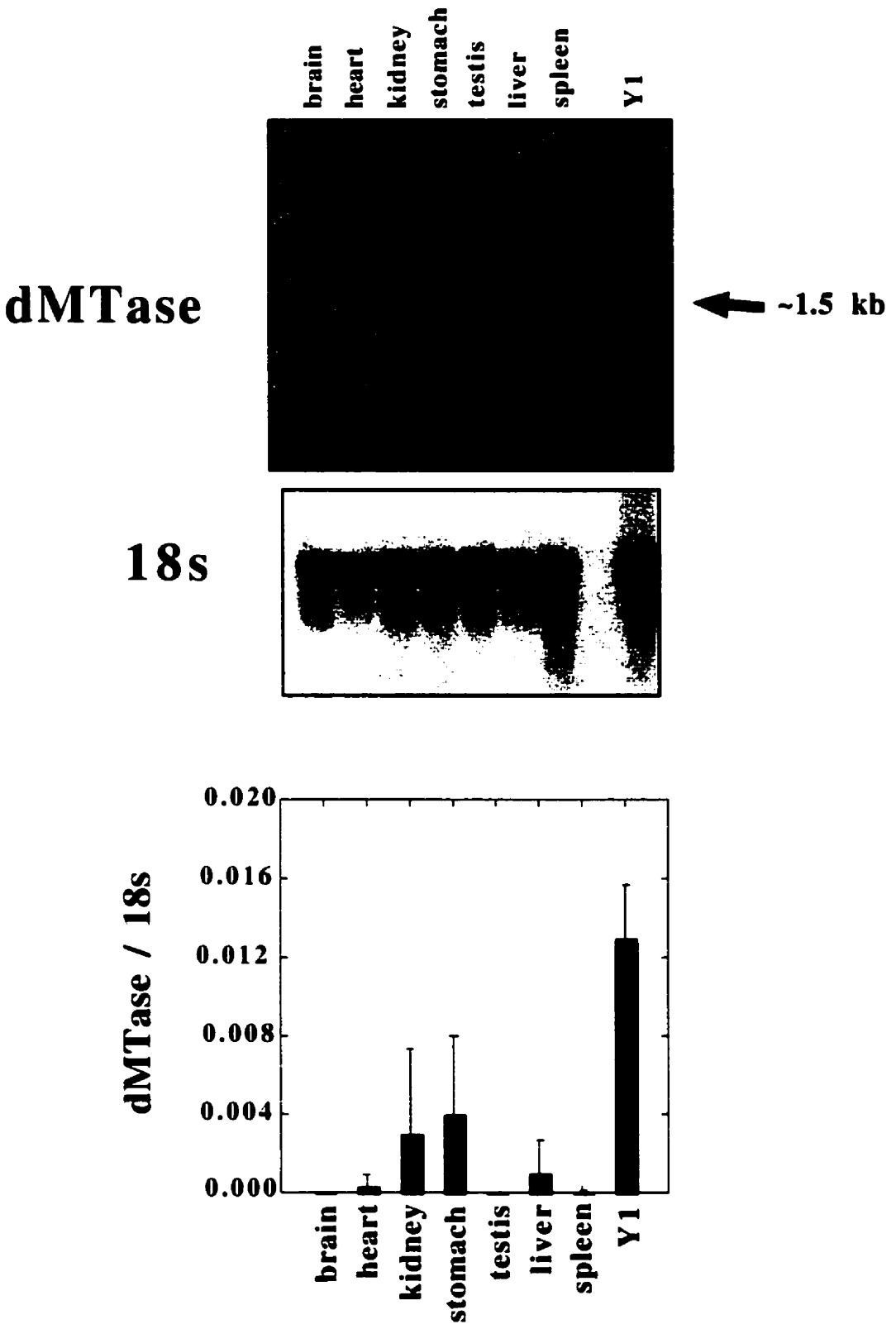
Tritiated methanol

Scintillation fluid

Demethylation reaction mixture

Figure 2-3: DNA dMTase expression *in vivo* is induced in transformed cells.

Northern and slot blot analysis of dMTase expression. Northern blot of 20mg of total RNA from different mouse tissues (brain, heart, kidney, stomach, testis, liver, spleen) and Y1 cells. Top panel: hybridization with ^{32}P labelled dMTase cDNA, middle panel: same blot, stripped and rehybridized with a ^{32}P labelled 18s rRNA oligonucleotide, bottom panel: Slot blot analysis in triplicate of 50mg of total RNA from the same tissues as above. The slot blot was first hybridized with a dMTase cDNA probe and the intensity of signal was determined by a phosphorimager the membrane was stripped and rehybridized to an 18s rRNA probe. The intensity of dMTase signal was normalized to the 18s rRNA signal.


brain
heart
kidney
stomach
testis
liver
spleen
Y1
dMTase
~1.5 kb
18s
dMTase / 18s
0.020
0.016
0.012
0.008
0.004
0.000
brain
heart
kidney
stomach
testis
liver
spleen
Y1

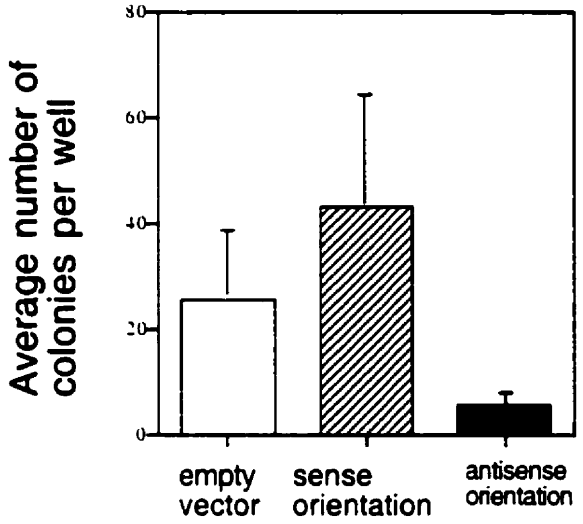
Similarly, using a vector where transcription from the vector can be induced using the tetracycline analogue doxycycline, transformed cell growth is inhibited by expression of the DNA dMTase in the anti sense orientation (Fig. 2-4b). These observations indicate that DNA dMTase is a candidate to develop anti-cancer therapeutics against.

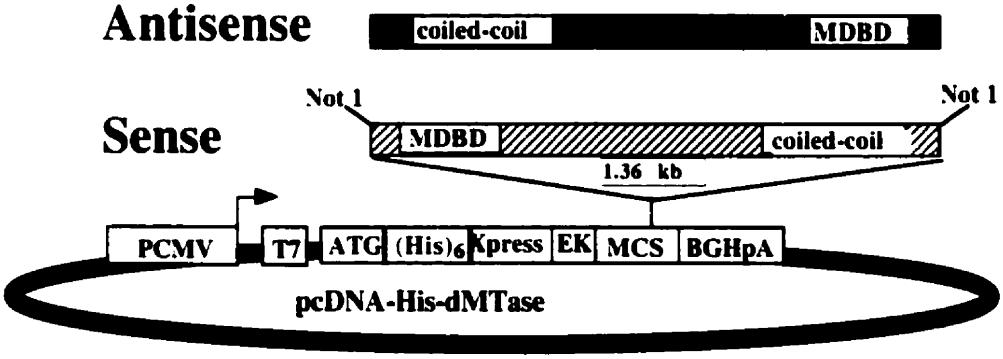
Figure 2-4 Expression of DNA demethylase in the Antisense orientation inhibits tumorigenesis ex vivo.

a. The constitutively active vectors, pcDNA 3.1His, carrying the human dMTase in either the sense or antisense orientation were constructed and transiently transfected into 1 million HEK 293 cells, selected for 24 hours with puromycin, 10 μg/ ml, and allowed to grow for seven days. After seven days colonies were scored. Expression of the DNA dMTase cDNA in the antisense orientation reduced colony formation.

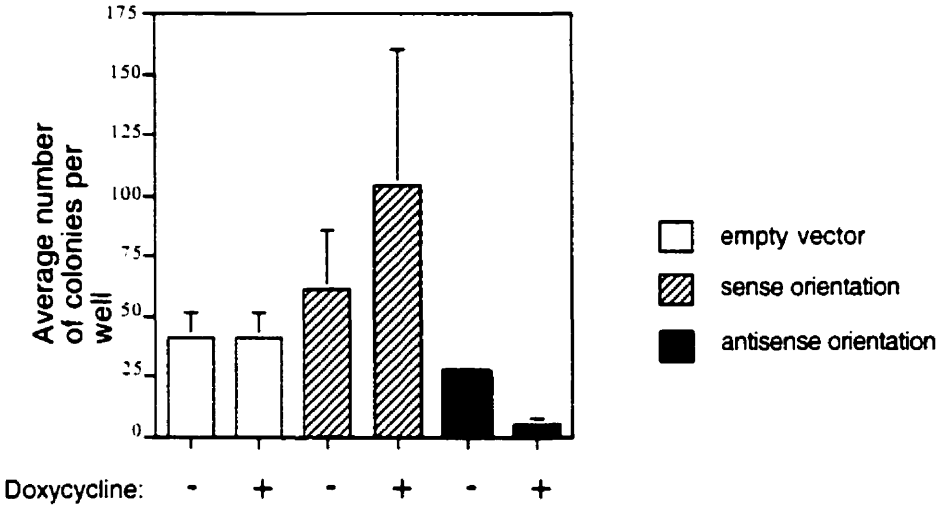
b. The tetracycline inducible vectors, pRetro-TetON, carrying the human dMTase cDNA in either the sense or antisense orientation were constructed and transiently transfected into 1 million HEK 293 cells, treated for 48 hours either in the presence or absence of doxcycycline (a tetracycline analogue), 10 μg/ ml, selected for the last 24 hours with puromycin, 10μg/ml, and then plated on soft agar and allowed to grow for seven days. After seven days colonies were scored. The doxycycline induced expression of the DNA dMTase cDNA in the antisense orientation reduced colony formation.

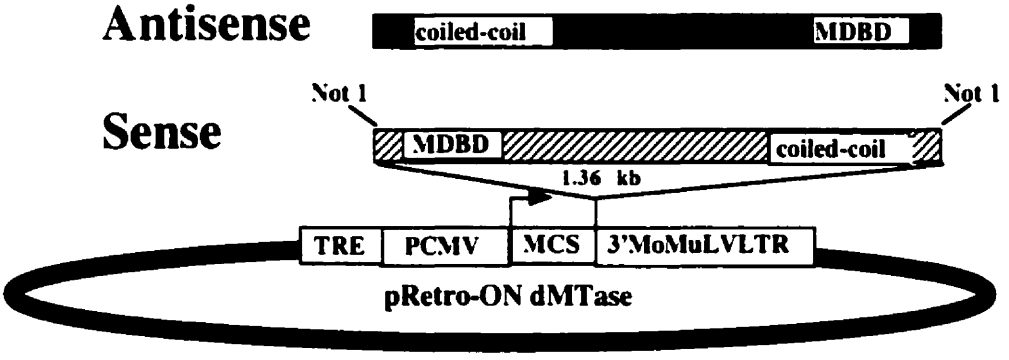
a


Average number of colonies per well
80
60
40
20
0
empty vector
sense orientation
antisense orientation


Antisense
coiled-coil
MDBD
Sense
Not 1
Not 1
MDBD
coiled-coil
1.36 kb
PCMV
T7
ATG
(His)6
Xpress
EK
MCS
BGHpA
pcDNA-His-dMTase

b


175
150
125
100
75
50
25
0
Average number of colonies per well
Doxycycline: - + - + - +
empty vector
sense orientation
antisense orientation


Antisense
coiled-coil
MDBD
Sense
Not 1
Not 1
MDBD
coiled-coil
1.36 kb
TRE
PCMV
MCS
3'MoMuLVLTR
pRetro-ON dMTase

Summary

The goals of this thesis were **a)** to identify the enzymatic machinery responsible for producing the DNA methylation patterns seen in mammalian cells, **b)** to identify how this machinery may be misregulated in cancer, and **c)** to test whether the methylation machinery is a viable target for the development of anti-cancer therapeutics.

a) The vast majority of the literature pertaining to DNA methylation in eukaryotes has identified the protein product of the *dnmt1* gene as the major enzymatic player responsible for methylating DNA. However, the process by which DNA is demethylated was still very controversial, mainly due to the longstanding notion that true demethylation of DNA was an energetically unlikely event to occur in an intracellular context, so the true identity of the enzyme(s) responsible for DNA demethylation was not known. Chapters 5 and 6 of this thesis demonstrate the existence of a true or *bona fide* DNA demethylation activity from cancer cells and a cDNA which encodes a peptide that can demethylate DNA. The specificity of the reaction for methylated CG dinucleotides was demonstrated and the reaction products of the demethylation reaction were identified to be methanol and nonmethylated DNA.

b) Much of the literature of the last 16 years that pertains to mammalian DNA methylation has focused on aberrant patterns of methylation found in cancer cells, hypermethylated and inactivated tumor suppressor genes, and elevated levels of DNA MeTase activity in cancer cells. Clearly, the processes that regulate the expression of DNA MeTase have gone awry in the cancerous state, yet the mechanisms which regulate the expression of DNA MeTase were relatively unknown or debatable due to several conflicting reports. Chapter 1 of this thesis lays the foundation for a comprehensive investigation of the transcriptional control of the expression of the *dnmt1* gene by elucidating the complete genomic structure of the *dnmt1* gene. The first cloning of a mammalian gene responsible for encoding DNA methylation activity (Bestor *et al.*, 1988) provided the starting point for identifying a promoter sequence which could initiate a *dnmt1* mRNA transcript

(Rouleau *et al.*, 1992) and respond to oncogenic signalling pathways (Rouleau *et al.*, 1995; MacLeod *et al.*, 1995). However, the actual relevance of these findings was later questioned because of cDNA sequence that was found to exist further 5' to the previously reported sequence (Yoder *et al.*, 1996; Tucker *et al.*, 1996). Chapter 2 uses the genomic DNA cloned in Chapter 1 to dissect the regions of transcriptional initiation that are used by the *dnmt1* gene. Using three independent methods, Chapter 2 describes multiple regions of transcriptional initiation in the *dnmt1* locus which are consistent with all of the previous reports. Furthermore, Chapter 2, demonstrates that both oncogenic (Ras-Jun) and tumor suppressor (Rb) pathways can regulate the transcriptional expression of *dnmt1*, suggesting that misregulation of *dnmt1* is an essential component of cellular transformation since several different cellular control points that are misregulated in oncogenesis converge at *dnmt1*.

In non transformed cells, expression of *dnmt1* is restricted to S-phase of the cell cycle and the mechanism of this restriction was shown to be a posttranscriptional phenomenon (Szyf *et al.*, 1991). Recently, cellular transformation mediated by the SV 40 large T antigen has been shown to require an upregulation of *dnmt1* expression by a posttranscriptional mechanism (Slack *et al.*, 1999a). Identifying the mechanism through which *dnmt1* expression is regulated is essential for understanding how cellular transformation occurs. Chapter 3 identifies the 3' untranslated region of *dnmt1* mRNA as being involved in posttranscriptional regulation of *dnmt1*.

c) Inhibition of *dnmt1* expression in cancer cells inhibits their tumorigenic potential (MacLeod and Szyf, 1995) and genetic manipulation of mice resulting in offspring with reduced relative DNA MeTase levels also reduces their propensity to develop potentially cancerous lesions (Laird *et al.*, 1995). However, to be a useful target in cancer therapy, specific pharmacological inhibition of *dnmt1* expression in an *in vivo* tumor model must be demonstrated. Chapter 4 accomplishes this using modified antisense oligonucleotides directed against *dnmt1* mRNA.

The discovery of DNA dMTase in chapters 5 and 6 of this thesis fills in the missing information in the literature regarding the general state of methylation of the genome of cancer cells and the general levels of DNA MeTase activity. Cancer cells were shown to by hypomethylated when compared to their parental non cancerous cells (Feinberg and Vogelstein, 1983; Feinberg *et al.*, 1988) yet they have increased DNA MeTase activity (Kautiainen and Jones, 1986; El-Deiry *et al.*, 1991). The paradoxical nature of these observation is resolved by the existence of a DNA dMTase activity, which is over expressed in cancer cells (Fig. 2-2, 2-3). Preliminary evidence presented in the discussion section of this thesis (Fig. 2-4) indicates that inhibition of the expression of the cDNA cloned in Chapter 5 in transformed cells reverses their transformation potential which indicates that therapeutics against DNA dMTase may have positive pharmacological outcomes in the treatment of cancer.

Taken together the data in this thesis indicate that loss of proper control over the enzymes that establish and maintain the DNA methylation pattern of the mammalian genome is a critical component of cellular transformation. The fact that multiple signalling pathways that are implicated in cancer converge to affect the multi-level regulation of the expression of *dnmt1*, suggests that this notion is correct. Also the fact that inhibition of both the DNA MeTase and DNA dMTase inhibits tumorigenicity strengthens this notion and lays the foundation for a rigorous effort to develop inhibitors of DNA MeTase and DNA dMTase as therapeutics for the treatment of cancer.

References

Abramovich, C., Yakobson, B., Chebath, J. and Revel, M. (1997): A protein-arginine methyltransferase binds to the intracytoplasmic domain cf the INFAR1 chain in the type 1 interferon receptor. *EMBO J.* **16**: 260-266

Adams R.L., McKay E.L., Craig L.M., Burdon R.H. (1979): Mouse DNA methylase: methylation of native DNA. *Biochim. Biophy. Acta.* **561**: 345-357.

Ahuja N., Mohan A.L., Li Q., Stolker J.M., Herman J.G., Hamilton S.R., Baylin S.B., Issa J.P. (1997): Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res.* **57**:3370-3374.

Akhtar, S., and Agrawal, S. (1997) In vivo studies with antisense oligonucleotides. *Trends Pharmacol. Sci.* **18**:12-18.

Alix, J. (1986): Relationship between methylation and maturation of ribosomal RNA in prokaryotic and eukaryotic cells. *In*:: Biological methylation and drug design (Borchardt, R.T., Creveling, C.R., and Ueland P.M., eds), pp 175-187, Humana Press, N.J.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990): Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Amacher, D. E. and Turner, G. N. (1987) The mutagenicity of 5-azacytidine and other inhibitors of replicative DNA synthesis in the L5178Y mouse lymphoma cell. *Mutation Res.* **176**:123-131.

Angel P., Karin M. (1991): The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta.* **1072**:129-157.

Antequera, F., Boyes, J. and Bird A. (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62**:503-514.

Araujo, F.D., Knox, J.D., Szyf, M., Price, G.B., and Zannis-Hadjopoulos, M. (1998): Concurrent replication and methylation at mammalian origins of replication. *Mol. Cell Biol.* **18**(6): 3475-3482.

Arondel, V., Benning, C. and Somerville, C.R. (1993): Isolation and functional expression in Escherichia coli ofa gene encoding phosphatidylethanolamine methyltransferase. *J. Biol. Chem.* **268**:16002-16008.

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

Axelrod, J. and Tomchick, R.J. (1958): Enzymatic O-methylation of adrenaline and other catechols. *J. Biol. Chem.* **233**: 702-705.

Bagchi, S., Weinman, R. and Raychaudhuri, P. (1991): The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell*, **65**, 1063-1072.

Bairoch, A., Bucher, P. & Hofman, K. The PROSITE database, its status in 1997. *Nucleic Acids. Res.* **25**, 217-221 (1997).

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

Baylin, S. B., Hoppener, J. W., de Bustros, A., Steenbergh, P. H., Lips, C. J. and Nelkin, B. D. (1986) DNA methylation patterns of the calcitonin gene in human

lung cancers and lymphomas. *Cancer Res.* **46**:2917-2922.

Baylin SB. Makos M. Wu JJ. Yen RW. de Bustros A. Vertino P. Nelkin BD. (1991) Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumor progression. *Cancer Cells* **3**:383-390.

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

Beard, C., Li, E. and Jaenisch, R. (1995). Loss of methylation activates Xist in somatic but not in embryonic cells. *Genes Dev.* **9**: 2325-2334.

Becker, P. B., Ruppert, S. and Schutz, G. (1987) Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. *Cell* **51**:435-443.

Belinsky SA, Nikula KJ, Baylin SB, Issa J-P: Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. Proc. Natl. Acad. Sci. USA 93, 4045-4050, 1996.

Benoit, G. and Trasler, J. M. (1994) Developmental expression of DNA methyltransferase messenger ribonucleic acid, protein, and enzyme activity in the mouse testis. *Biol. Reprod.* **50**:1312-1319.

Bestor, T. H., Laudano, A., Mattaliano, R. and Ingram, V. (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.* **203**:971-983.

Bestor, T. H. & Verdine, G. L. (1994) DNA methyltransferases: *Curr. Opin. Cell*

Biol. 6, 380-389.

Bhattacharya, S.K., Ramchandani, S., Cervoni, N. and Szyf, M. (1999): A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* **397**(6720): 579-583.

Bindoli, A., Rigobello, M.P. and Deeble, D.J. (1992): Biochemical and toxicological properties of the oxidative products of catecholamines. *Free Radic. Biol. Med.* **13**: 391-405.

Binetruy, B. , Smeal, T. and Karin, M. (1991) Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature* **351**:122-127.

Bird, A., Taggart, M., Frommer, M., Miller, O. J. and Macleod D. (1985) A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. *Cell* **40**:91-99.

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

Boguski, M. S., Lowe, T. M., & Tolstoshev, C. M. (1995): dbEST-database for expressed sequence tags. *Nat. Genet.* **4**,332-373.

Both, G.W., Banergee, A.K. and Shatkin, A.J. (1975): Methylation-dependent translation of viral messenger RNA in vitro. *Proc. Natl. Acad. Sci. (USA).* **72**: 1189-1193.

Bouchard, J., Walker, M. C., Leclerc, J. M., Lapointe, N., Beaulieu, R.and Thibodeau, L. (1990) 5-azacytidine and 5-azadeoxycytidine inhibit human immunodeficiency virus type 1 replication in vitro. *Antimicrob. Agents Chemother.*

34:206-209.

Brandeis, M., Ariel, M. and Cedar, H. (1993) Dynamics of DNA methylation during development. *Bioessays.* **15**:709-713.

Brinster R.L., Chen H.Y., Messing A., van Dyke T., Levine A.J., Palmiter R.D. (1984): Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. *Cell* **37**: 367-379.

Bryans, M., Kass, S., Seivwright, C. and Adams, R. L. (1992) Vector methylation inhibits transcription from the SV40 early promoter. *FEBS Lett.* **309**:97-102.

Buschhausen, G., Wittig, B., Graessmann, M., and Graessmann, A. (1987): Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. (USA)* **84**: 1177-1181.

Call, K. M., Jensen, J. C., Liber, H. L. and Thilly, W. G. (1986) Studies of mutagenicity and clastogenicity of 5-azacytidine in human lymphoblasts and Salmonella typhimurium. *Mutation Res.* **160**:249-57.

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

Carrel, L., Clemson, C.M., Dunn, J.M., Miller, A.P., Hunt, P.A., Lawrence, J.B., and Willard, H.F. (1996a). X-inactivation analysis and DNA methylation studies of the ubiquitin activating enzyme E1 and PCTAIRE-1 genes in human and mouse. *Human Mol. Genet.* **5**::391-401.

Carrel, L., and Willard, H.F. (1996b). An assay for X-inactivation based on

differential at the fragile X locus, FMR1. *Am. J. Med. Genet.* **64**: 27-30.

Cawthon, R. M., Weiss, R., Xu, G. F., Viskochill, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P and White, R. (1990) A major segment of the Neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* **61**:193-201.

Challita, P. M., Skelton, D., el-Khoueiry, A., Yu, X. J., Weinberg, K. and Kohn, D. B. (1995) Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. *J. Virol.* **69**:748-755.

Chapman, V., Forrester, L., Sanford, J., Hastie, N. and Rossant, J. (1984) Cell lineage-specific undermethylation of mouse repetitive DNA. *Nature* **307**:284-286.

Chen, C-Y. A., and Shyu, A-B. (1994) Interplay of two functionally and structurally distinct domains of the c-fos AU-rich element specifies it's mRNA destabilizing function *Mol. Cell. Biol.* **14**,8471-8482.

Chen, C-Y. A., and Shyu, A-B., (1995) AU-rich elements, characterization and importance in mRNA degradation *Trends. Biochem. Sci.* **20**, 465-470.

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

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

Christman, J. K., Sheikhnejad, G., Dizik, M., Abileah, S. and Wainfan, E. (1993) Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency. *Carcinogenesi*.s **14**:551-557.

Chu, G. and Mayne, L (1996): Xeroderma pigmentosum, Cockeyne syndrome and trichothidystrophy: do these genes explain the disease? *Trend. Genet.* **12**: 187-192.

Chuang L. S-H., Ian H-I., Koh T-W., Ng H-H., Xu G., Li B.F.L. (1997): Human DNA-(cytosine-5) Methyltransferase-PCNA complex as a target for p21waf1. *Science* **277**: 1996-2000.

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

Clark, S., Harrison, J. and Frommer, M. (1995) CpNpG methylation in mammalian cells. *Nature Genet.* **10**:20-27.

Clarke, S., Vogel, J., Deschesne, R. and Stick, J. (1988): Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc. Natl. Acad. Sci. (USA).* **85**: 4643-4647.

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

Constantinides, P. G., Jones, P. A. and Gevers W. (1977) Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. *Nature* **267**: 364-366.

Copeland, W. C. & Wang, T. S. (1991): Catalytic subunit of human DNA polymerase alpha overproduced from baculovirus-infected insect cells. Structural and enzymological characterization. *J. Biol. Chem.* **266,** 22739-22748.

Corpet, F., Gouzy, J. & Kahn, D.(1998): The ProDom database of protein domain families. *Nucleic Acids Res.* **26**, 323-326.

Covey, J. M., D'Incalci, M., Tilchen, E. J., Zaharko, D. S. and Kohn K. W. (1986) Differences in DNA damage produced by incorporation of 5-aza-2'-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells. *Cancer Res.* **46**:5511-5517.

Creusot, F., Acs, G. and Christman, J. K. (1982) Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J. Biol. Chem.* **257**:2041-2048.

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

Datto M.B., Li Y., Panus J.F., Howe D.J., Xiong Y., Wang X. (1995): Transforming growth factor b induces the cyclin-dependent kinase inhibitor of p21 through a p53-independent mechanism. *Proc. Natl. Acad. Sci. USA* **92**:5545-5549.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**:987-1000.

Dean, N. M. & McKay, R. (1994): Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **91**, 11762-11766.

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

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

de Groot, R.P., Kruyt, F.A., van der Saag, P.T. and Kruijer, W. (1990): Ectopic expression of c-jun leads to differentiation of P19 embryonal carcinoma cells. *EMBO J.* **9**, 1831-1837.

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

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

Dimri G.B., Nakanishi M., Desprez P.Y., Smith J.R., Campisi J. (1996): Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. *Mol. Cell. Biol.* **16**:2987-2997.

Dixon, R.A. and Pavia, N.L. (1995): Stress induced phenylpropanoid metabolism. *Plant Cell.* **7**: 1085-1097.

Djalali, M., Adolph, S., Steinbach, P., Winking, H. and Hameister, H. (1990) Fragile sites induced by 5-azacytidine and 5-azadeoxycytidine in the murine genome. *Hereditas* **112**:77-81.

Dony, C., Kessel, M., and Gruss, P., (1985) Post-transcriptional control of myc and p53 expression during differentiation of the embryonal carcinoma cell line F9 *Nature.* **317**, 636-639.

Eden, S. & Cedar, H. (1994): Role of DNA methylation in the regulation of transcription.*Curr. Opin. Gen. Dev.* **4,** 255-259.

Eden, S., Hashimshony, T., Keshet, I., Cedar, H. and Thorne, A.W. (1998): DNA methylation models histone acetylation. *Nature.* **394**(6696): 842.

Efstratiadis, A. (1994) Parental imprinting of autosomal mammalian genes. *Curr. Opin. Genet. Dev.* **4**:265-280.

Ehrlich, M., Gama-Sosa, M. A., Huang, L. H., Midgett, R. M., Kuo, K. C., McCune, R. A. and Gehrke, C. (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res.* **10**:2709-2721.

Elayan, H., Kennedy, B. and Ziegler, M.G. (1990): Epinephrine synthesis in rat skin by an N-methyltransferase. *Arch. Derm. Res.* **282**:194-197.

Elledge, S. J., and Harper, J. W. (1994) Cdk inhibitors: on the threshold of checkpoints and development. *Curr. Opin. Cell Biol.* **6,** 847-852.

el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S.

R. and Baylin, S. B. (1991) High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc. Natl. Acad. Sci. USA* **88**:3470-3474.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., *et al.*, WAF1 a potential mediator of p53 tumor suppression. *Cell* **75**:817-825.

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

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

Fisher,P.A., Wang,T.S., & Korn,D. (1979): Enzymological characterization of DNA polymerase alpha. Basic catalytic properties processivity, and gap utilization of the homogeneous enzyme. *J. Biol. Chem.* **254,** 6128-6137.

Frank, D., Keshet. I, Shani, M. Levine, A. Razin, A. and Cedar, H. (1991) Demethylation of CpG islands in embryonic cells. *Nature* **351**:239-241.

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

Fremont, M., Siegmann, M., Gaulis, S., Matthies, R., Hess, D., & Jost, J.P. (1997): Demethylation of DNA by purified chick embryo 5-methylcytosine-DNA Glycosylase requires both protein and RNA. *Nucleic Acids Res.* **25,** 2375-2380.

Friend, S.H., Horowitz, J.M., Gerber, M.R., Wang, X.F., Bogenmann, E., Li, F.P. and Weinberg, R.A. (1987). Deletions of a DNA sequence in retinoblastomas and

mesenchymal tumors: organisation of the sequence and its encoded protein. *Proc. Natl. Acad. Sci. USA* **84:** 9059-9063.

Frohman, M.A., Downs, T.R., Chomczynski, P. and Frohman, L.A.. (1989): Cloning and charaterization of mouse growth hormone-releasing hormone (GRH) complementary DNA: increased GRH messenger RNA levels in the growth hormone-deficient lit/lit mouse. *Molecular Endocrinology.*, **3**, 1529-1536.

Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L. and Paul C. L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A.* **89**:1827-1831.

Gama-Sosa, M. A., Midgett, R. M., Slagel, V. A., Githens, S., Kuo, K. C., Gehrke, C. W. and Ehrlich, M. (1983) Tissue-specific differences in DNA methylation in various mammals. *Biochim Biophys Acta.* **740**:212-219.

Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R. Kuo, K. C., Gehrke C. W. and Ehrlich M. (1983) The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res.* **11**:6883-6894.

Gartler, S. M. and Riggs, A. D. (1983) Mammalian X-chromosome inactivation. *Annu. Rev. Genet.* **17**:155-190.

Gilbert, W. (1978). Why genes in pieces? *Nature* **271**: 501.

Gilbert, W. (1985). Genes-in-pieces revisited. *Science* **228**: 823-824.

Gillies, S. D., Morrison, S. L., Oi, V. T. and Tonegawa, S. (1983) A tissue-specific transcription enhancer element is located in the major intron of a rearranged

immunoglobulin heavy chain gene. *Cell* **33**:717-728.

Gjerset, R. A. and Martin, D. W Jr. (1982) Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. *J. Biol. Chem.* **257**:8581-8583.

Glomset, J., Gelb, M. and Farnsworth, C. (1990): Prenyl proteins in eukaryotic cells: a new type of membrane anchor. *Trend. Biochem. Sci.* **15**: 139-142.

Goelz, S. E., Vogelstein, B., Hamilton, S. R. and Feinberg, A. P. (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* **228**:187-190.

Graff, J.R., Herman, J.G., Myohanen, S., Baylin, S.B., Vertino, P.M. (1997): Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation.*J. Biol. Chem.* **272**:22322-22329.

Gruenbaum, Y. Stein R. Cedar, H. and Razin, A. (1981) Methylation of CpG sequences in eukaryotic DNA. *FEBS Lett.* **124**: 67-71.

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

Hahlbrock, K. and Scheel, D. (1989): Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**: 347-369.

Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., *et al.*,: Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21

by MyoD *Science* **267**: 1018-1021.

Harbers, K., Schnieke, A., Stuhlmann, H., Jahner, D. and Jaenisch, R. (1981) DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. *Proc Natl Acad Sci U S A* **78**:7609-7613.

Hastie, N. D. (1994) The genetics of Wilms' tumor- a case of disrupted development. *Annu. Rev. Genet.* **28**:523-558.

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

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

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., Baylin, S.B. (1996): Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**:9821-9826.

Hermann, R. and Doerfler, W. (1991) Interference with protein binding at AP2 sites by sequence-specific methylation in the late E2A promoter of adenovirus type 2 DNA *FEBS Let.*. **281**:191-195.

Herschman, R. H. (1991) Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60**: 281-319.

Hinds, P. W. and Weinberg RA. (1994) Tumor suppressor genes. *Curr. Opin. Genet. Dev.* **4**:135-141.

Holliday, R. Pugh, J. E. (1975) DNA modification mechanisms and gene activity during development. *Science* **187**:226-232.

Holliday, R. (1990) DNA methylation and epigenetic inheritance. *Philos Trans .R .Soc .Lond B Biol .Sci.* **326**:329-338.

Hotchkiss, R. D. (1948) The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J. Biol. Chem.* **175**:315-332.

Hrycyna, C.A. and Clarke, S. (1993): Modification of eukaryotic signaling proteins by C-terminal methylation reactions. *Pharmacol. Ther.* **59**: 281-300.

Ibrahim, R.K., Bruneau, A. and Bantignies, B. (1997): Plant O-methyltransferases: molecular analysis, common signature and classification. *Plant Mol. Biol.* **36**: 1-10.

Issa, J. P., Zehnbauer, B. A., Civin, C. I., Collector, M. I., Sharkis, S. J., Davidson, N. E., Kaufmann, S. H. and Baylin, S. B. (1996): The estrogen receptor CpG island is methylated in most hematopoietic neoplasms. *Cancer Res.* **56**, 973-977.

Jackson-Grusby, L., Laird, P.W., Magge, S.N., Moeller, B.J., Jaenisch, R. (1997): Mutagenicity of 5-aza-2'-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl. Acad. Sci. USA* **94**: 4681-4685.

Jenuwein, T., Forrester, W. C., Fernandez-Herrero, L. A., Laible, G., Dull, M., and Grosschedl, R. (1997) Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature* **385:**269-272.

Johnson, R., van Lingen, B., Papaioannou, V.E., Spiegelman, B.M. (1993): A null

mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* **7**: 1309-1317.

Johnson, R., Spiegelman, B., Hanahan, D., Wisdom, R. (1996): Cellular transformation and malignancy induced by ras require c-jun. *Mol. Cell. Biol.* **16**: 4504-4511.

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

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

Jones, P. A., Wolkowicz, M. J., Harrington, M. A. and Gonzales, F. (1990) Methylation and expression of the Myo D1 determination gene. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **326**:277-284.

Jones, P. A., Wolkowicz, M. J., Rideout, W. M. 3d., Gonzales, F. A., Marziasz, C. M., Coetzee, G. A. and Tapscott, S. J. (1990) De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc. Natl. Acad. Sci. USA.* **87**:6117-6121.

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

Jost, J. P. (1993) Nuclear extracts of chicken embryos promote an active demethylation of DNA by excision repair of 5-methyldeoxycytidine. *Proc. Natl. Acad. Sci. USA.* **90**:4684-4688.

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

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

Juttermann, R., Li, E. and Jaenisch, R. (1994) Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. USA* **91**: 11797-11801.

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

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

Kaneda, N., Ichinose, H., Kobayashi, K., Oka, K., Kishi, F., Nakazawa, A., Kurosawa, Y., Fujita, K. and Nagatsu, T. (1988): Molecular cloning of cDNA and chromosomal assignment of the gene for human phenylethanolamine N-methyltransferase, the enzyme for epinephrine biosynthesis. *J. Biol. Chem.* **263**: 7672-7677.

Kass, S. U., Goddard, J. P. and Adams, R. L. (1993) Inactive chromatin spreads from a focus of methylation. *Mol Cell Biol.* **13**:7372-7379.

Kass, S.U., Landsberger, N., Wolffe, A.P. (1997): DNA methylation directs a time-dependent repression of transcription initiation. *Curr. Biol.* **7**: 157-165.

Katz, A. J. (1985) Genotoxicity of 5-azacytidine in somatic cells of Drosophila. (1985) *Mutation Res.* **143**:195-199.

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

Keshet, I., Lieman-Hurwitz, J. and Cedar H. (1986) DNA methylation affects the formation of active chromatin. *Cell* **44**:535-543.

Keshet, E. & Cedar, H. (1983): Effect of CpG methylation on Msp I. *Nucleic Acids Res.* **11**: 3571-3580.

Knight, S. J., Flannery, A. V., Hirst, M. C., Campbell, L., Christodoulou, Z., Phelps,
S. R., Pointon, J., Middleton-Price, H. R., Barnicoat, A., Pembrey, M. E., et al. (1993)
Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. *Cell* **74**:127-134.

Kodaki, T., Hosaka, K, Nikawa, J. and Yamashita, S. (1991): Identification of the upstream sequences responsible for the expression and regulation of the PEM1 and PEM2 genes encoding the enzymes of the methylation pathway in Saccharomyces cerevisiae. *J. Biochem.* **109**: 276-287.

Komura, J.. Okada, T. and Ono, T. (1995) Repression of transient expression by DNA methylation in transcribed regions of reporter genesa introduced into cultured

human cells. *Biochim. Biophys. Acta.* **1260**:73-78.

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

Kopp, M.U., Winterhalter, K.H., Trueb, B. (1997): DNA methylation accounts for the inhibition of collagen VI expression in transformed fibroblasts. *Eur. J. Biochem.* **249**: 489-496.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., Clevers, H. (1997): Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**: 1784-1787.

Kreig, P.A. and Melton, D.A. (1987): In vitro RNA synthesis with SP6 RNA polymerase *Methods Enzymol.*, **155**, 397-415.

Krieg, A.M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. & Klinman, D. M. (1995): CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374,** 546-549.

Kruczek I. Doerfler W. (1983) Expression of the chloramphenicol acetyltransferase gene in mammalian cells under the control of adenovirus type 12 promoters: effect of promoter methylation on gene expression. *Proc Natl Acad Sci U S A.* **80**:7586-7590.

Kuge, H. and Richter, J.D. (1995): Cytoplasmic 3' poly(A) addition induces 5' cap ribose methylation: implication for translational control of maternal mRNA. *EMBO J.* **14**: 6301-6310.

Kuge, H., Brownlee, G.G., Gershon, P.D. and Richter, J.D. (1998): Cap ribose

methylation of c-fos mRNA stimulates translation and oocyte maturation in Xenopus lavis. *Nucleic Acids Res.* **26**: 3208-3214.

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

Ladino, C.A. and O'Connor, C.M. (1992): Methylation of atypical protein aspartyl residues during the stress response of HeLa cells. *J. Cel. Physiol.* **153:**297-304.

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

Lal, D., Som, S. and Friedman, S. Survival and mutagenic effects of 5-azacytidine in Escherichia coli. (1988) *Mutation Res.* **193**:229-236.

Lalande, M. (1996). Parental Imprinting and human disease. *Ann. Rev. Genet.* **30:** 173-195.

Lamas, M., Monaco, L., Zazopoulos, E., Lalli, E., Tamai, K., Penna, L., Mazzucchelli, C., Nantel, F., Foulkes, N. S., and Sassone-Corsi, P. (1996) CREM: a master-switch in the trasncriptional response to cAMP. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **351**:561-567.

Land, H., Parada, L.F., Weinberg, R.A. (1983): Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**: 596-602.

Lane, B.G., Ofengand, J. and Gray, M.W. (1995): Psuedouridine and 2' O-

methylated nucleotides. Significance of their selective occurence in rRNA domains that function in ribosome-catalysed synthesis of the peptide bonds in proteins. *Biochimie.* **77**: 7-15.

Langner, K. D., Vardimon, L., Renz, D., and Doerfler, W. (1984) DNA methylation of three 5' C-C-G-G 3' sites in the promoter and 5' region inactivate the E2a gene of adenovirus type 2. *Proc. Natl. Acad. Sci. USA* **81**:2950-2954.

Lassar, A. B., Paterson, B. M. and Weintraub H. (1986) Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell* **47**:649-656.

Lavia, P., Ferraro, M., Micheli, A. and Olivieri G. (1985) Effect of 5-azacytidine (5-azaC) on the induction of chromatid aberrations (CA) and sister-chromatid exchanges (SCE). *Mutation Res.* **149**:463-467.

Lazaris-Karatzas, A., Montine, K, S. and Sonenberg, N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* **345**:544-547.

Lee PJ. Washer LL. Law DJ. Boland CR. Horon IL. Feinberg AP (1996) Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proc. Natl. Acad. Sci. USA* **93**:10366-10370.

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

Levine, A. J. (1993) The tumor suppressor genes. *Annu. Rev. Biochem.* **62**:623-651.

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

Li, R., Hannon, G.J., Beach, D., Stillman, B. (1996): Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Curr. Biol.* **6**:189-199.

Lichtenstein, M., Keini, G. Cedar, H. and Bergman, Y. (1994) B cell-specific demethylation: a novel role for the intronic kappa chain enhancer sequence. *Cell* **76**:913-923.

Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S. and Herschman H.R. (1996): The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. J. Biol. Chem. 271: 15034-15044.

Linton, M.F., Farese, R.V.,Jr., Chiesa, G., Grass, D.S., Chin, P., Hammer, R.E., Hobbs, H.H. and Young S.G.(1993). Transgenic mice expressing high plasma concentrations of human apolipoprotein B-100 and lipoprotein (a). *J. Clin. Invest.* **92**: 3029-3037.

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

Lowy, D. and Wilumsen, B. M. (1993) Function and regulation of Ras. *Annu. Rev. Biochem.* **62**, 851-891.

Luo, S., Robinson, J.C., Reiss, A.L., and Migeon, B.R. (1993). DNA methylation

of the fragile X locus in somatic and germ cells during fetal development: relevance to the fragile X syndrome and X-inactivation. *Somatic Cell and Mol. Genet.* **19**: 393-404.

Lupas, A. Van Dyke, M. & Stock, J.(1991): Predicting coiled coils from protein sequences. *Science* **252**, 1162-1164.

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

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

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

Malter, J. S. (1989) Identification of an AUUUA specific messanger RNA binding protein*Science* **246**, 664-666.

Martin, R.G., Oppenheim, A. (1977): Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. *Cell.* **11**:859-869.

Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B. and Baylin, S. B. (1992) Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer. *Proc. Natl. Acad. Sci. USA* **89**:1929-1933.

Makos-Wales, M., Biel, M. A., El Deiry, W., Nelkin, B. D., Issa, J-P., Cavenee, W. K., Kuerbitz, S. J. and Baylin, S. B. (1995) p53 activates expression of HIC-1, a

new candidate tumor suppressor gene on 17p13.3 *Nature Med.* **1**, 570-577.

Matsuda, M. (1990) Comparison of the incidence of 5-azacytidine-induced exencephaly between MT/HokIdr and Slc:ICR mice. *Teratology* **41**:147-54.

Mayer, R. J. & Walker, J. H. (1987) *Immunochemical methods in cell and molecular biology*. Academic Press, London.

Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P., Baylin, S. B. and Sidransky, D. (1995) 5' CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. *Nature Med.* **1**, 686-692.

Mertineit, C., Yoder, J.A., Taketo, T., Laird, D.W., Trasler, J.M., and Bestor, T.H. (1998): Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development*, **125**, 889-897.

Migeon, B. R. (1994) X-chromosome inactivation: molecular mechanisms and genetic consequences. *Trends. Genet.* **10** :230-235.

Miller, J.W., Selhub, J. and Joseph, J.A. (1996): Oxidative damage caused by free radicals produced during catacholamine autoxidation: protection effects of O-methylation and melatonin. *Free Radic. Biol. Med.* **21**: 241-249.

Mohandas, T. Sparkes, R. S. and Shapiro, L. J. (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science* **211**:393-396.

Monk, M., Boubelik, M. and Lehnert, S. (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages

during mouse embryo development. *Development* **99**:371-382.

Monk, M., Adams, R. L. and Rinaldi, A. (1991) Decrease in DNA methylase activity during preimplantation development in the mouse. *Development* **112**:189-192.

Monk, M. (1995). Epigenetic programming of the differential gene expression in development and evolution. *Dev. Genet.* **17**: 188-197.

Moore, J.P., Johannsson, A., Hesketh, T.R., Smith, G.A. and Metcalfe, J. (1984): Calcium signals and phospholipid methylation in eukaryotic cells. *Biochem. J.* **221**: 675-684.

Najbauer, J. and Aswad, D. (1990): Diversity of methyl acceptor proteins in rat pheochromocytoma (PC12) cells revealed after treatment with adenosine dialdehyde. *J. Biol. Chem.* **265**: 12717-12721.

Najbauer, J., Johnson, B., Youg, A. and Aswad, D. (1993): Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. *J. Biol. Chem.* **268**:10501-10509.

Najbauer, J., Orpiszewski, J. and Aswad, D. (1996): Molecular aging of tubulin: accumulation of isoaspartyl sites in vitro and in vivo. *Biochemistry* **35**: 5183-5190.

Nan, X., Campoy, F.J., Bird, A. (1997): MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* **88**: 471-481.

Nan, X., Ng, H.-H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., and Bird, A. (1998): Transcriptional repression by the methyl CpG binding protein

MeCP2 involves a histone deacetylase complex. *Nature* **393**: 386-389.

Nelkin, B. D., Przepiorka, D., Burke, P. J., Thomas, E. D., Baylin, S. B. (1991) Abnormal methylation of the calcitonin gene marks progression of chronic myelogenous leukemia. *Blood* **77**:2431-2434.

Nur, I., Szyf, M., Razin, A., Glaser, G., Rottem, S. and Razin, S. (1985) Procaryotic and eucaryotic traits of DNA methylation in spiroplasmas (mycoplasmas). *J. Bacteriol.* **164**:19-24.

Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N. E., Robbins, P. D. and Sakai, T.
(1993) CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* **8**:1063-1067.

Osgood, C. J. and Seward, S. M.(1989) 5-Azacytidine induces sex chromosome loss and interchange in immature germ cells of Drosophila mei-9 males. *Environ. Mol. Mut.* **14**:135-145.

O'Shea, E. K. Rutkowski, R. & Kim, P. S.(1989): Evidence that the leucine zipper is a coiled coil. *Science* **243**, 538-542.

Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B. and Davidson, N. E. (1994) Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res.* **54**:2552-2555.

Paroush, Z., Keshet, I., Yisraeli, J. and Cedar, H. (1990) Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. *Cell* **63**:1229-1237.

Parish, C., Smrcka, A. and Rando, R. (1995): Functional significance of beta gamma-subunit carboxymethylation for the activation of phospholipase C and phosphoinositide 3-kinase. *Biochemistry* **34**: 7722-7727.

Park, D.H., Baetge, E.E., Kaplan, B.B., Albert, V.R., Reis, D.J. and Joh, T.H. (1982): Different forms of phenylethanolamine N-methyltransferase: species specific posttranslational modification. *J. Neurochem.* **38**: 410-414.

Pazin, M.J. and Kadonaga, J.T. (1997): What's up and down with histone deacetylation and transcription? *Cell* **89**: 315-328.

Peterson, K. and Sapienza, C. (1993) Imprinting the genome: imprinted genes, imprinting genes, and a hypothesis. *Annu. Rev. Genet.* **27**:7-31.

Pfeifer, G. P., Steigerwald, S. D., Mueller, P. R., Wold, B. and Riggs, A. D. (1989) Genomic sequencing and methylation analysis by ligation mediated PCR. *Science* **246**:810-813.

Pfeifer, G. P., Steigerwald, S. D., Hansen, R. S., Gartler, S. M. and Riggs, A. D. (1990) Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. *Proc. Natl. Acad. Sci. USA* **87**:8252-8256.

Pfeifer, G. P., Tanguay, R. L., Steigerwald, S. D. and Riggs AD. (1990) In vivo footprint and methylation analysis by PCR-aided genomic sequencing: comparison of active and inactive X chromosomal DNA at the CpG island and promoter of human PGK-1. *Genes Dev.* **4**:1277-1287.

Philips, M.R. (1993) Carboxyl methylation of Ras-related proteins during signal transduction in neutrophils. *Science* **259**: 977-980.

Plumb, J. A., Wishart, C., Setanoians, A., Morrison, J. G., Hamilton, T., Bicknell, S. R. and Kaye, S. B. (1994): Identification of a multidrug resistance modulator with clinical potential by analysis of synergistic activity in vitro, toxicity in vivo and growth decay in a solid human tumor xenograft. *Biochem. Pharmacol.* **47**, 257-266.

Prendergast, G. C. and Ziff, E. B. (1991) Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* **251**:186-189.

Prendergast,G. C., Lawe, D. and Ziff, E. B. (1991) Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell* **65**: 395-407.

Ramchandani, S., MacLeod, A.R., Pinard, M., von Hofe, E., Szyf, M. (1997): Inhibition of tumorigenesis by a cytosine-DNA methyltransferase antisense oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA* **94**, 684-689.

Ramchandani, S., Bigey, P., and Szyf, M. (1998): Genomic structure of the human DNA methyltransferase gene. *Biol. Chem.* **379**(4-5): 535-540.

Ramchandani, S., Bhattacharya, S.K., Cervoni, N. and Szyf, M. (1999): DNA methylation is a reversible biological signal. *Proc. Natl. Acad. Sci. (USA).* **96**(11): 6107-6112.

Rando, R.R. (1996): Chemical biology of protein isoprenylation/methylation. *Biochim. Biophys.Act.* **1300**: 5-16.

Razin, A. and Cedar, H. (1977) Distribution of 5-methylcytosine in chromatin. *Proc.*

Nat. Acad. Sci USA **74**:2725-2728.

Razin, A. and Riggs A. D. (1980) DNA methylation and gene function. *Science* **210**:604-610.

Razin, A. and Szyf, M. (1984) DNA methylation patterns. Formation and function. *Biochim. Biophys. Acta.* **782** :331-342.

Razin, A., Webb. C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N. and Cedar, H. (1984) Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. *Proc. Nat. Acad. Sci USA* **81**:2275-2279.

Razin, A., Feldmesser, E., Kafri, T. and Szyf, M. (1985) Cell specific DNA methylation patterns; formation and a nucleosome locking model for their function. *Prog. Clinic. Biol. Res.* **198**:239-53

Razin, A., Szyf, M., Kafri, T., Roll, M., Giloh, H., Scarpa, S., Carotti, D. and Cantoni G. L. (1986) Replacement of 5-methylcytosine by cytosine: a possible mechanism for transient DNA demethylation during differentiation. *Proc. Nat. Acad. Sci USA* **83**:2827-2831.

Razin, A., Cedar, H. (1991): DNA methylation and gene expression. *Microbiol. Rev.* **55**:451-458.

Razin, A. and Kafri, T. (1994) DNA methylation from embryo to adult. *Prog. Nucleic Acid Res. Mol. Biol.* **48**:53-81.

Razin, A. (1998): CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J.* **17**(17): 4905-4908.

Ribieras, S., Song-Wang, X.G., Martin, V., Lointier, P., Frappart L. and Dante R. (1994): Human breast and colon cancers exhibit alterations of DNA methylation patterns at several DNA segments on chromosomes 11p and 17p. *J. Cel. Biochem.* **56**:86-96.

Rideout, W. M. 3d., Coetzee, G. A., Olumi, A. F. and Jones, P. A. (1990) 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* **249**:1288-1290.

Rideout, W. M 3rd., Eversole-Cire, P., Spruck, C. H 3rd., Hustad, C. M., Coetzee, G. A., Gonzales, F. A. and Jones, P. A. (1994) Progressive increases in the methylation status and heterochromatinization of the myoD CpG island during oncogenic transformation. *Mol. Cell. Biol.* **14**:6143-6152.

Rein, T., Zorbas, H., DePamphilis, M. (1997): Active mammalian replication origins are associated with a high-density cluster of mCpG dinucleotides. *Mol. Cell. Biol.* **17**: 416-426.

Riley, D. J., Lee, E. Y. -H. and Lee, W-H. (1994) The retinoblastoma gene. *Annu. Rev. Cell. Biol.* **10**:1-29.

Rosen, M. B., House, H. S., Francis, B. M. and Chernoff, N. (1990) Teratogenicity of 5-azacytidine in the Sprague-Dawley rat. *J. Toxicol. Environ. Health.* **29**:201-210.

Rost, B (1996). PHD: predicting one-dimensional protein structure by profile based neural networks. *Met. Enzym.* **266**, 525-539.

Rouleau, J., Tanigawa, G. and Szyf, M. (1992) The mouse DNA

methyltransferase 5'-region. A unique housekeeping gene promoter. *J . Biol. Chem.* **267**:7368-7377.

Rouleau, J., MacLeod, A. R. and Szyf, M. (1995) Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. *J. Biol. Chem.* **270**:1595-1601.

Royer-Pokora, B. and Schneider, S. (1992) Wilms' tumor-specific methylation pattern in 11p13 detected by PFGE. *Genes Chromosom. Cancer* **5**:132-140.

Sapienza, C., Peterson, A. C., Rossant, J., Balling, R., (1987) Degree of methylation of transgenes is dependent on gamete of origin. *Nature* **328**:251-254.

Schlessinger, J. and Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron.* **9**:383-391.

Scrable, H., Cavenee, W., Ghavimi, F., Lovell, M., Morgan, K. and Sapienza, C. (1989) A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting.
Proc. Natl. Acad. Sci. U S A. **86**:7480-7484.

Schluckebier, G., O'Gara, M, Saenger, W. and Cheng, X. (1995): Universal catalytic domain structure of Ado-met dependent methyltransferase. *J. Mol. Biol.* **247**: 16-20.

Schwab, M., Alitalo, K., Varmus, H. E. & Bishop, M. (1983): A cellular oncogene (c-Ki-ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumor cells. *Nature* **303**, 497-501.

Seiser, C., Knofler, M., Rudelstorfer, I., Haas, R., and Wintersherger, E. (1989): Mouse thymidine kinase: the promoter sequence and the gene and pseudogene structures in normal cells and in thymidine kinase deficient mutants. *Nucleic Acids Res.*, **17**, 185-195.

Shaw, G., and Kamen, R., (1986) A conserved AU sequence from 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation;*Cell* **46**, 659-667.

Shemer, R., Eisenberg, S., Breslow, J.L., and Razin, A. (1991). Methylation patterns of the human APOA-1/C-III/A-IV gene cluster in adult and embryonic tissues suggest dynamic changes in methylation during development. *J. Biol. Chem.* **266:** 23676-23681.

Shinar, D., Yoffe, O., Shani, M., & Yaffe, D. (1989): Regulated expression of muscle-specific genes introduced into mouse embryonal stem cells: inverse correlation with DNA methylation. *Differentiation* **41,** 116-126.

Shiohara, M., Koike, K., Komiyama, A., Koeffler, H.P. (1997): p21WAF1 mutations and human malignancies. *Leukemia Lymphoma* **26**:35-41.

Siegfried, Z. & Cedar, H. (1997): DNA methylation: a molecular lock. *Curr. Biol.* **7,** 305-307.

Sierakowska, H., Sambade, M.J., Agrawal, S. and Kole, R. (1996). Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* **23:** 12840-12844.

Simon, D., Stuhlmann, H., Jahner, D., Wagner, H., Werner, E., and Jaenisch, R. (1983) Retrovirus genomes methylated by mammalian but not bacterial

methylase are non-infectious. *Nature* **304**:275-277.

Singer, J., Roberts-Ems, J. and Riggs, A. D. (1979) Methylation of mouse liver DNA studied by means of the restriction enzymes msp I and hpa II. *Science* **203**: 1019-1021.

Singer-Sam, J., Robinson, M. O. Bellve, A. R., Simon, M. I. and Riggs, A. D. (1990) Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase, and Zfy gene transcripts during mouse spermatogenesis. *Nucleic Acids Res.* **18**:1255-1259.

Slack, R.S., Hamel, P.A., Bladon, T.S., Gill, R.M. and McBurney, M.W. (1993): Regulated expression of the retinoblastoma gene in differentiating embryonal carcinoma cells. *Oncogene*, **8**, 1585-1591.

Slack, A., Cervoni, N., Pinard, M. and Szyf, M. (1999): DNA methyltransferase is a downstream effector of cellular transformation triggered by SV 40 large T antigen. *J. Biol Chem.* **274**: 10105-10112.

Slack, A., Cervoni, N., Pinard, M. and Szyf, M. unpublished data

Smith, J. A., and Pari, G. S. (1995) Expression of human cytomegalovirus UL36 and UL37 genes is required for viral DNA replication. *J. Virol.* **69**:1925-1931.

Snyder, R. D. and Lachmann, P. J. (1989) Differential effects of 5-azacytidine and 5-azadeoxycytidine on cytotoxicity, DNA-strand breaking and repair of X-ray-induced DNA damage in HeLa cells. *Mutation Res.* **226**:185-190.

Sonenberg, N., Morgan, M.A., Merrick, W.C. and Shaktin, A.J. (1975): A polypeptide in eukaryotic initiation factors that crosslinks to the 5'-terminal cap in

mRNA. *Proc. Natl. Acad. Sci. (USA).* **75**: 4843-4847.

Springer, M., Goy, M. and Adler, J. (1979): Protein methylation in behavioral control mechanisms and in signal transduction. *Nature.* **280**: 279-284.

Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A., & Cedar, H. (1982): Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc. Natl. Acad. Sci. USA* **79**, 61-65.

Surdej, P., Riedl, A., and Jacobs-Lorena, J. (1994) Regulation of mRNA stability in development; *Annu. Rev. Genet.* **28**, 263-282.

Sutcliffe, J. S., Nelson, D. L., Zhang, F., Pieretti, M., Caskey, C. T., Saxe, D. and Warren, S. T. (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum. Mol. Genet.* **1**:397-400.

Sutter, D. and Doerfler W. (1980) Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. *Proc. Natl. Acad. Sci. USA* **77**:253-256.

Swain, J. L., Stewart, T. A. and Leder, P. (1987) Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell* **50**:719-727.

Szyf, M., Avraham-Haetzni, K., Reifman, A., Shlomai, J., Kaplan, F., Oppenheim, A. and Razin A. (1984) DNA methylation pattern is determined by the intracellular level of the methylase. *Proc Natl Acad Sci USA* **81**:3278-3282.

Szyf, M., Eliasson, L., Mann, V., Klein, G. and Razin, A. (1985) Cellular and viral DNA hypomethylation associated with induction of Epstein-Barr virus lytic cycle.

Proc Natl Acad Sci USA **82**:8090-8094.

Szyf, M., Schimmer, B. P. and Seidman, J. G. (1989) Nucleotide-sequence-specific de novo methylation in a somatic murine cell line. *Proc Natl Acad Sci U S A.* **86**:6853-6857.

Szyf, M., Milstone, D. S., Schimmer, B. P., Parker, K. L. and Seidman, J. G. (1990) cis modification of the steroid 21-hydroxylase gene prevents its expression in the Y1 mouse adrenocortical tumor cell line. *Mol. Endocrin.* **4**:1144-1152.

Szyf, M. Tanigawa, G. and McCarthy, P. L Jr. (1990) A DNA signal from the Thy-1 gene defines de novo methylation patterns in embryonic stem cells. *Mol. Cell. Biol.* **10**:4396-4340.

Szyf, M. (1991) DNA methylation patterns: an additional level of information?. *Biochem. Cell Biol.* **69**:764-767.

Szyf, M., Bozovic, V. and Tanigawa, G. (1991) Growth regulation of mouse DNA methyltransferase gene expression. *J. Biol. Chem.* **266**:10027-10030.

Szyf, M., Rouleau, J., Theberge, J. and Bozovic V. (1992) Induction of myogenic differentiation by an expression vector encoding the DNA methyltransferase cDNA sequence in the antisense orientation. *J Biol Chem.* **267**:12831-12836..

Szyf, M. (1994) DNA methylation properties: consequences for pharmacology.*Trends Pharmacol Sci.* **15**:233-238.

Szyf, M. Theberge, J. and Bozovic, V. (1995) Ras induces a general DNA

demethylation activity in mouse embryonal P19 cells. *J. Biol. Chem.* **270**:12690-12696.

Szyf, M. (1996): The DNA methylation machinery as a target for anticancer therapy. *Pharmacol. Ther.* **70**:1-37.

Szyf, M. (1998): Targeting DNA methyltransferase in cancer. *Cancer Metastasis Rev.*, **17**, 219-231.

Takeuchi, I. K. and Takeuchi, Y. K. (1985) 5-Azacytidine-induced exencephaly in mice. (1985) *J. Anat.* **140** :403-412.

Tamame, M., Antequera, F., Villanueva, J. R. and Santos T. (1983) High-frequency conversion to a "fluffy" developmental phenotype in Aspergillus spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. *Mol. Cell. Biol.* **3**:2287-2297.

Tasheva, E. S. and Roufa DJ. (1994) Densely methylated DNA islands in mammalian chromosomal replication origins. *Mol. Cell. Biol.* **14**: 5636-5644.

Tate, P. H. & Bird, A. P. (1993): Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Gen. Dev.* **3**, 226-231.

Taylor, S. M. and Jones, P. A. (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **17**:771-779.

Tessitore, L, Dianzani, I., Cui, Z. and Vance, D.E. (1999): Dimished expression of phosphatidylethanloamine N-methyltransferase 2 during hepatocarcinogenesis. *Biochem. J.* **337**(Pt 1): 23-27.

Tollefsbo, T. O. and Hutchison, C. A. (1995) Mammalian DNA (Cytosine-5-)-methyltransferase expressed in *Escherichia coli*, purified and characterized. *J. Biol. Chem.* **270**, 18543-1550.

Toth, M., Muller, U. and Doerfler, W. (1990) Establishment of de novo DNA methylation patterns. Transcription factor binding and deoxycytidine methylation at CpG and non-CpG sequences in an integrated adenovirus promoter. *J. Mol. Biol.* **214**:673-683.

Trasler, J. M., Alcivar, A. A., Hake, L. E., Bestor, T. and Hecht, N. B. (1992) DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells. *Nucleic Acids Res.* **25**:2541-2545.

Treisman, R. (1985) Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences;*Cell* **42**, 889-902.

Tuck, M.T. (1992): The formation of internal 6-methyladenine residues in eukaryotic messenger RNA. *Int. J. Biochem.* **24**: 379-386.

Tucker, K.L., Talbot, D., Lee, M.A., Leonhardt, H., and Jaenisch, R. (1996): Complementation of methylation deficiency in embryonic stem cells by a DNA methyltransferase minigene. *Proc. Natl Acad. Sci. USA*, **93**, 12920-12925.

Ura, K., Kurumizaka, H., Dimitov, S., Almouzni, G., and Wolffe, A.P. (1997): Histone acetylation: influence on transcription, nucleosome mobility and positioning, and linker histone-dependent transcriptional repression. *EMBO J.* **16**: 2096-2107.

Vairapandi, M. and Duker, N. J. (1993) Enzymic removal of 5-methylcytosine from DNA by a human glycosylase. *Nucleic Acids Res.* **21**:5323-5327.

Van Buul, C., Damm, J. and van Knippenberg, P. (1983): Kasugamycin resistant mutants of Bacillus strearothermophilus lacking the enzyme for the methylation of two adjacent adenosines in 16S ribosomal RNA. *Mol. Gen. Genet.* **189**: 475-478.

Vance, D.E, Walkey, C.J. and Cui, Z. (1997): Phosphatidylethanolamine N-methyltransferase from liver. *Biochim. Biophys. Acta.* **1348**: 142-150.

Vertino, P. M. Issa, J. P., Pereirasmith, O. M. and Baylin, S. B. (1994) Stabilization of DNA methyltransferase level and CpG island hypermethylation precede SV40-induced immortilization of human fibroblasts. *Cell Growth Diff.* **5**:1395-1402.

Vertino, P.M., Yen, R.W., Gao, J., Baylin, S.B. (1996): De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. *Mol. Cell. Biol.* **16**:4555-4565.

Waalwijk, C. and Flavell, R. A. DNA methylation at a CCGG sequence in the large intron of the rabbit beta-globin gene: tissue-specific variations. (1978) *Nucleic Acids Res.* **5**: 4631-4634.

Waalwijk, C. and Flavell, R. A. (1978) MspI, an isoschizomer of hpaII which cleaves both unmethylated and methylated hpaII sites. *Nucleic Acids Res.* **5**:3231-3236.

Waga, S., Hannon, G.J., Beach, D., Stilman, B. (1994): The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**:574-578.

Wahlfors, J., Hiltunen, H., Heinonen, K., Hamalainen, E., Alhonen, L. and Janne,

J. (1992) Genomic hypomethylation in human chronic lymphocytic leukemia. *Blood* **80**:2074-2080.

Wainfan, E., Dizik, M., Stender, M., Christman, J.K. (1989): Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. *Cancer Res.* **49**:4094-4097.

Walder, R. Y. & Walder, J. A. (1988): Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc. Natl. Acad.Sci.* USA **85**, 5011-5015.

Walkey, C.J., Donohue, L.R., Bronson, R., Agellon, L.B. and Vance, D.E. (1997): Disruption of the murine gene encoding phosphatidylethanolamine N-methyltransferase. *Proc. Natl. Acad. Sci. (USA).* **94**: 12880-12885.

Walkey, C.J., Shieds, D.J. and Vance, D.E. (1999): Identification of three novel cDNAs for human phosphatidylethanolamine N-methyltransferase and localization of the human gene on 17p11.2. *Biochim Biophys. Acta.* **1436**: 405-412.

Wang, C., Lin, J. and Lazarides, E. (1992): Methylation of 70,000-Da heat shock proteins in 3T3 cells: alterations by arsenite treatment, by different stages of growth and by virus transformation. *Arch. Biochem. Biophys.* **297**: 169-175.

Watson, J.D. and Crick, F.H.C. (1953): Genetic implications of the structure of deoxyribonucleic acid. *Nature.* **171**: 964-967.

Weinberg, R. A. (1985) The action of oncogenes in the cytoplasm and nucleus. *Science* **230**:770-776.

Weiss, A., Keshet, I., Razin, A.,& Cedar, H.(1996): DNA demethylation in vitro:

Involvement of RNA. *Cell* **87,** 709-718.

Wilks, A., Seldran, M. and Jost, J. P. (1984) An estrogen-dependent demethylation at the 5' end of the chicken vitellogenin gene is independent of DNA synthesis. *Nucleic Acids Res.* **12**:1163-1177.

Wolffe, A.P. (1997): Sinful repression. *Nature.* **387**: 16-17.

Wollenweber, E and Dietz, V.H. (1981): Occurence and distribution of free flavonoid aglycones in plants. *Phytochemistry.* **20**: 869-932.

Woodcock, D. M., Crowther, P. J. and Diver, W. P. (1987) The majority of methylated deoxycytidines in human DNA are not in the CpG dinucleotide. *Biochem. Biophys. Res. Com.* **145**:888-894.

Woodcock, D. M., Crowther, P. J., Jefferson, S. and Diver, W. P. (1988) Methylation at dinucleotides other than CpG: implications for human maintenance methylation.
Gene **74**: 151-152.

Wu, J. C. and Santi, D. V. (1985) On the mechanism and inhibition of DNA cytosine methyltransferases. *Prog. Clinic. Biol. Res.* **198**:119-129.

Wu, J., Issa, J. P., Herman, J., Bassett, D. E. Jr., Nelkin, B. D. and Baylin, S. B. (1993) Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* **90**:8891-8895.

Wu, R.C., Schontal, A.H. (1997): Activation of P53-P21(WAF1) pathway in response to disruption of cell-matrix interactions. J. Biol. Chem. **272**: 29091-

29098.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., Beach, D. (1993): p21 is a universal inhibitor of cyclin kinases. *Nature* **366**:701-704.

Xiong, Y., Zhang, H., Beach, D. (1993): Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev.* **7**:1572-1583.

Yamane, H.K. and Fung, B.K. (1993): Covalent modifications of G-proteins. *Annu. Rev. Pharmacol. Toxicol.* **32**: 201-241.

Yang, Z.Y., Perkins, N.D., Ohno, T., Nabel, E.G., Nabel, G.J. (1995): The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. *Nat. Med.* **1**:1052-1056.

Yang, J., Deng, C., Hemati, N., Hanash, S.M., Richardson, B.C. (1997): Effect of mitogenic stimulation and DNA methylation on human T cell methyltransferase expression and activity. *J. Immunol.* **159**:1303-1309.

Yasumura, Y., Buonsassisi, V. & Sato, G. (1966): Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. *Cancer Res.* **26**, 529-535.

Yeivin, A. and Razin, A. (1993): Gene methylation patterns and expression. *In* DNA methylation: Molecular biology and biological Significance. (J.P. Jost and H.P. Saluz eds.). Basel, Switzerland: Berkhauser Verlag, pp. 523-568

Yen, R. W., Vertino, P. M., Nelkin, B. D., Yu, J. J., el-Deiry , W., Cumaraswamy, A., Lennon , G. G., Trask, B. J., Celano, P. and Baylin, S. B.

(1992) Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucleic Acids Res.* **20**:2287-2291.

Yisraeli, J. and Szyf, M. The pattern of methylation of eukaryotic genes. (1984) in "*DNA Methylation and its Biological Significance.*"Springer Verlag Inc. N. Y. (Razin, A. Cedar, H. and Riggs, A. D. eds.) pp 353-378.

Yisraeli, J., Frank, D., Razin, A. and Cedar, H. (1988) Effect of in vitro DNA methylation on beta-globin gene expression. *Proc Natl Acad Sci U S A.* **85**:4638-4642.

Yoder, J.A., Yen, R.W.C., Vertino, P.M., Bestor, T.H. and Baylin, S.B. (1996). New 5' regions of the murine and human genes for (cytosine-5)-methyltransferase. *J. Biol. Chem.* **271**: 31092-31097.

Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., Hirohashi, S. (1995): Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc. Natl. Acad. Sci. USA* **92**:7416-7419.

Yu, P.H. (1978): Phenylethanolamine N-methyltransferase from the brain and adrenal medulla of the rat: a comparison of their properties. *Neurochemical Research.* **3**: 755-762.

Zahler, A. M., Williamson, J. R., Cech, T. R. & Prescott DM (1991): Inhibition of telomerase by G-quartet DNA structures. *Nature* **350,** 718-720.

Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A.W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L., and Brewer, G ., (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF-1 *Mol. Cell. Biol.* **13**, 7652-7665.

Zimmermann, F. K. and Scheel, I. (1984) Genetic effects of 5-azacytidine in Saccharomyces cerevisiae. *Mutation Res.* **139**:21-24.

Zuccotti, M. and Monk, M. (1995) Methylation of the mouse Xist gene in sperm and eggs correlates with imprinted Xist expression and paternal X-inactivation. *Nature Genet.* **9**:316-320.