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CHARACTERIZATION OF DNA METHYLATION PATTERNS
IN TUMORIGENESIS AND THE USE OF DNA
METHYLATION ANALYSIS TO GAUGE TOXIC POTENTIAL

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CHARACTERIZATION OF DNA METHYLATION PATTERNS IN TUMORIGENESIS AND THE USE OF DNA METHYLATION ANALYSIS TO GAUGE TOXIC POTENTIAL

By

Rebecca Erin Watson

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ABSTRACT

CHARACTERIZATION OF DNA METHYLATION PATTERNS IN TUMORIGENESIS AND THE USE OF DNA METHYLATION ANALYSIS TO GAUGE TOXIC POTENTIAL

By

Rebecca Erin Watson

DNA methylation is an important epigenetic mechanism controlling patterns of genetic expression. In promoter regions of several genes, methylcytosine binding proteins decrease gene expression by inhibiting the binding of transcription factors and/or by facilitating chromatin condensation. Additionally, DNA methylation silences transposable elements, contributing to genomic stability. Aberrant patterns of methylation have been implicated in cancer and in certain neurological, immunological, and developmental disorders. To test the hypothesis that the ability to maintain normal patterns of methylation is inversely related to susceptibility to cancer and perhaps other toxic outcomes, I have characterized patterns of DNA methylation associated with carcinogenesis, as well as those elicited by treatment with cytolethal and noncytolethal concentrations of model compounds. I demonstrate that differences in the ability to maintain GC-rich patterns of methylation might, in part, underlie the differences in tumor susceptibility between the relatively tumor-sensitive C3H/He and B6C3F1 (C57/BL6 X C3H/He) mice compared to the relatively tumor resistant C57/BL6 strain. I also describe alterations in global, GC-rich and gene-specific methylation status in the promotion stage of carcinogenesis using an initiation-promotion SENCAR mouse skin model in which mice were initiated with dimethylbenz(*a*)anthracene and promoted with various doses

of cigarette smoke condensate for different amounts of time. Notably, increases in GC-rich methylation were observed in a dose-and time-dependent, reversible manner during the promotion stage. Threshold levels of CSC necessary to detect changes in GC-rich methylation patterns following 9 wks promotion were predictive of those required for a marked increase in tumor number following 29 wks of promotion. Increased methylation in the promoter region of the tumor suppressors *MGMT* and *p16* was observed only in tumor tissue, and this is the first report describing how reversible alterations in methylation correlate inversely with the expression of the *HoxA5* tumor suppressor gene. Overall, these alterations in methylation status could contribute to the clonal expansion of increasingly abnormal cells in the promotion stage. Additionally, since alterations in gene expression due to changes in DNA methylation could potentially contribute to a number of toxic outcomes, I examined the global and GC-rich methylation status of rat hepatoma cells treated with cytolethal and non-cytolethal concentrations of model compounds not previously known to alter methylation. The observation that 2/4 of these compounds affected methylation indicates that chemically-mediated changes in DNA methylation might be more prevalent than commonly assumed. When used in conjunction with cytolethality and genotoxicity data, DNA methylation analysis of these compounds provided a basis for the more rational ranking of these compounds based on estimated toxic potential. Overall, the results of these studies support the view that DNA methylation may be viewed as a secondary mechanism underlying carcinogenesis and perhaps other toxic outcomes, and that DNA methylation assessment can enhance the ability to gauge the toxic potential of chemicals.

This dissertation is dedicated to Aleksandr Watson

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
A	adenine
ATP	adenosine triphosphate
BHMT	betaine homocysteine methyltransferase
bp	base pair
BWS	Beckwith-Widemann syndrome
C	cytosine
CL	cytolethal
CMT3	CHROMOMETHYLTRANSFERASE 3
CpG	cytidine-guanosine dinucleotide in which cytosine is 5'to guanine and a phosphate (p) group separates them.
CpCpG	cytidine-cytidine-guanosine trinucleotide
CpNpG	cytidine-any base-guanosine trinucleotide
cpm	counts per minute
CTCF	vertebrate enhancer blocking protein; a transcription factor
CSC	cigarette smoke condensate
dAzaC	5-deoxy-2'-azacytidine
DHF	dihydrofolate
DMBA	7, 12-dimethylbenz[*a*]anthracene
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
dNTP	deoxy ATP, deoxy TTP, deoxy CTP, deoxy GTP

dpm	disintegrations per minute
E	embryonic day
GFAP	glial fibrillary acidic protein
GJIC	gap junctional intercellular communication
GST	glutathione S-transferase
G	guanine
H3, H4	histone 3, histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HP1	heterochromatin protein 1
ICF	immunodeficiency, centromeric instability, and facial anomalies syndrome
ICR	imprint control region
Igf-2	insulin like growth factor 2
IL-2	interleukin-2 (T-cell growth hormone)
K	lysine
MeCP2	methylcytosine binding protein 2
MGMT	O^6 methylguanine methyltransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTR	methionine synthase
NCL	noncytolethal
N^5-methyl THF	N^5-methylenetetrahydrofolate
PB	phenobarbital

PCR	polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosyl-L-methionine
T	thymine
TSA	trichostatin A
U	units
UV	ultraviolet
Zn	zinc

INTRODUCTION

Epigenetics

The modern use of the term "epigenetics" refers to the study of heritable control of gene expression occuring without a change in DNA coding sequence (Wolffe and Matzke, 1999). Epigenetics involves the selective use of genomic information through the activation and inactivation of particular genes in order to fine-tune genetic expression (Nakao, 2001). Epigenetic changes are heritable, and for this reason, when one considers the inheritance of genetic information, one must take into account both the transmission of sequence information as well as the transmission of alternative states of gene activity (Watson and Goodman, 2002a).

Epigenetic mechanisms

Often, epigenetic mechanisms involve a conformational change in chromatin or influence the ability of transcription factors to bind to the DNA. This can be accomplished through modification of histones and/or nucleosomes, as well as through DNA methylation. It is important to recognize that these epigenetic modifications are not mutually exclusive; often several epigenetic mechanisms cooperate to regulate transcriptional activity.

Acetylation and methylation of histones influence the conformation of chromatin. "Active" chromatin, or euchromatin, is characterized by a more open structure accessible to proteins involved in DNA transcription, while "inactive" chromatin, or heterochromatin, is characterized by a closed conformation that inhibits accessibility of these proteins to the chromatin. Histone acetylation, catalyzed by histone

acetyltransferases (HAT), facilitates the formation of euchromatin. This is readily reversed by histone deacetylases (HDACs), which condense chromatin (Nakao, 2001). Methylation of particular lysine (K) residues on histones is associated with different states of transcriptional activity. For instance, methylation of lysine at the ninth residue of histone 3 (H3-K9) is associated with heterochromatin (Lachner et al., 2001). H3-K9 methylation also inhibits acetylation of H4-K16, which is associated with active chromatin (Nishioka et al., 2002). Conversely, methylation of H3-K4 facilitates transcription by inhibiting H3-K9 methylation, as well as by promoting acetylation of H3 (Fischle et al., 2003).

Furthermore, ATP-dependent nucleosomal remodeling proteins such as the SWI/SNF-related complexes use ATP hydrolysis to introduce superhelical torsion into nucleosomal DNA. This leads to a nucleosome conformation that contains exposed DNA bulges or loops, altering the accessibility of the chromatin to various proteins governing transcription (Li, 2002).

In general, DNA methylation, i.e. methylation of the 5' sites of cytosine residues, decreases gene activity (Attwood, 2002). Methylcytosine binding proteins inhibit binding of transcription factors and enhancer elements by steric hindrance and/or by interfering with the ability of these factors to recognize their cognate *cis* elements (Attwood, 2002). For instance, DNA methylation of the AP2 consensus site has been shown to inhibit binding of the AP2 transcription factor in adenovirus DNA (Hermann et al., 1991). Similarly, methylation prevents c-Myc from binding to its regulatory element (Lindahl, 1982). In some cases, however, binding of the transcription factor might inhibit methylation. Though DNA methylation is associated with a lack of Sp1 binding

(Lindsay and Bird, 1987), the presence of a methylated CpG site within its recognition sequence did not prevent Sp1 binding (Tazi and Bird, 1990). A suggested explanation for this finding is that binding of Sp1 actually protects the DNA from methylation such that methylation only occurs secondarily to the absence of Sp1 and a decrease in gene activity (Höller et al., 1988). Alternatively, it is possible that methylation in regions surrounding the Sp1 consensus site is required for chromatin condensation and subsequent inhibition of Sp1 binding.

DNA methylation frequently interacts with other epigenetic mechanisms in synergistic fashion to regulate gene transcription. There are a variety of methylcytosine binding proteins which recruit complexes that facilitate histone deacetylation. For instance, methylcytosine CpG binding domain protein 2 (MBD2), and methylcytosine binding protein 2 (MeCP2) both interact with the Sin3/HDAC complex and recruit histone deacetylases to the methylated area (Nan et al., 1998). Additionally, H3-K9 methylation is rapidly reversed by the demethylating agent 5-aza-2'deoxycytidine (dAzaC), suggesting a mechanistic link between DNA and histone methylation (Nguyen, et al., 2002).

There has been some discord about whether DNA methylation initiates gene silencing, or typically occurs as a consequence of chromatin remodeling or other factors (Clark and Melki, 2002). In some cases, it is known that although DNA methylation appears to correlate with decreased genetic expression, it does not initiate gene silencing. This is observed in X-chromosome inactivation where CpG islands are methylated subsequent to gene silencing mediated by *Xist* RNA (Csankovszki et al., 2001). Also, although methylation of *p16* has been shown to be tightly linked to *p16* expression levels

(Patel et al., 2000; Herman et al., 1996), in human mammary cells cell lines, chromatin condensation and *p16* inactivation is sometimes observed prior to *p16* methylation (Mermoud et al., 2002). In *Arabidopsis* and *Neurospora*, DNA methylation is thought to follow chromatin modification (Jackson et al., 2002; Tamaru and Selker, 2001). Specifically, in *Arabidopsis*, mutation of the KRYPTONITE H3-K9 histone methyltransferase gene leads to a reduction in CHROMOMETHYLTRANSFERASE 3 (CMT3)- mediated CpNpG methylation, suggesting that histone methylation is a prerequisite for the activity of CMT3 (Jackson et al., 2002). This view is supported by the finding that CMT3 binds to the *Arabidopsis* homologue of the heterochromatin protein 1(HP1), which is typically bound to methylated H3-K9 (Jackson et al., 2002).

On the other hand, in many cases, and particularly in mammalian DNA, methylation appears to be a more dominant force in the inactivation of genes. Transcriptionally silenced genes exhibiting both CpG island methylation and histone deacetylation cannot be reactivated by the histone deacetylation inhibitor trichostatin A (TSA) without prior treatment with dAzaC (Cameron et al., 1999; Kress et al., 2001; Fahrner et al., 2002). Thus, gene silencing can probably occur through a variety of mechanisms and the order of epigenetic events leading to gene silencing might vary in a species- and perhaps gene-specific fashion.

Additionally, even in cases in which DNA methylation does not initiate gene silencing, it appears to be important for the maintenance of gene silencing, e.g. methylated genes on the X chromosome can be partially re-activated following dAzaC treatment (Csankovski et al., 2001). Plus, whether or not DNA methylation initiates gene

silencing, it correlates strongly with transcriptional repression (Attwood et al., 2002), and represents a potentially useful marker of possible expression changes.

DNA methylation

DNA methylation plays a critical role in maintaining normal genetic expression in eutherian mammals. Approximately 4% of mammalian cytosine residues, or about 1% of total DNA bases, are methylated (Ehrlich et al., 1982). DNA methylation is involved in the maintenance of normal patterns of transcriptional regulation involved in several crucial biological processes. Aberrant patterns of DNA methylation are commonly implicated in altering expression of genes that have the potential to play a role in the carcinogenic process if they are aberrantly expressed (Laird, 1997), and an increasing body of research indicates that aberrant DNA methylation might play a role in additional human disorders (as reviewed in Robertson and Wolffe, 2000; and Watson and Goodman, 2002a).

Much of the promoter-specific methylation occurs at CpG islands, 200 bp or longer stretches of DNA with a 50% or greater GC content and a higher-than-expected CpG content (Gardiner-Garden and Frommer, 1987). CpG islands are particuarly prevalent in 5' flanking promoter regions and are present in the promoter regions of approximately 56% of mammalian genes (Antequera and Bird, 1993), and methylation at these sites generally silences gene expression. Additionally, a large amount of methylation occurs outside of the CpG islands, particularly at transposable elements throughout the genome (Attwood et al., 2002; Ehrlich, 2002). Methylation silences the expression of these transposable elements and thus helps maintain genomic integrity

(Carnell and Goodman, 2003). Also, methylation at particular imprint control regions (ICRs) located in non-CpG island regions is important for the proper expression of imprinted genes, i.e., genes expressed in a maternal or paternal specific manner (Bell and Felsenfeld, 2000).

The bulk of DNA methylation in mammals occurs at the CpG dinucleotide, (most reviews on DNA methylation do not even mention non-CpG methylation; Laird, 1997; Robertson and Jones, 2000), and the vast majority of DNA methylation research is focused at the CpG dinucleotide, often using techniques that selectively detect CpG sites so that methylation at other cytosine sites would not be detected. However, there have been sporadic reports indicating CpNpG methylation is present in mammalian cells (Ray et al., 1995; Clark et al., 1995; Clark et al., 1997). Additionally, my studies indicated that phenobarbital and cigarette smoke condensate induced changes at both CpG and CpCpG sites (Watson and Goodman, 2002b; Watson et al., 2003). It is possible that mechanisms responsible for the methylation status of CpG sites and CpNpG sites vary, and a likely explanation might be some site specificity in methyltransferases. As previously mentioned, CpNpG methylation in *Arabidopsis* is accomplished by a specific methyltransferase gene (Jackson et al., 2002). Also, in mammalian embryonic stem cells, the level of *de novo* methyltransferase Dnmt3a has been correlated with the presence of non-CpG methylation (Ramsahoye et al., 2000).

Methylation status can be altered by 3 general mechanisms: *de novo* methylation at previously unmethylated CpG sites, failure to maintain DNA methylation following DNA replication (passive demethylation), or loss of DNA methylation at methylated CpG sites by an active demethylation process not linked to cell division (Figure 1) (Laird,

1997). Maintenance methylation is accomplished by DNA methyltransferase 1 (Dnmt1), which preferentially acts at hemimethylated DNA molecules (Bestor et al., 1988), and *de novo* methylation is accomplished by Dnmts 3a and 3b (Okano et al., 1999). Alterations in the levels and/or activities of any of these methyltransferases, or a change in the rate of active or passive demethylation might alter methylation patterns. In addition to methyltransferases and demethylation processes, DNA methylation is controlled by cellular proliferation and differentiation (Figure 2). DNA of rapidly proliferating cells must be methylated quickly and properly to maintain methylation, and an increase or decrease in the rate of proliferation might influence methylation levels (Kanduc and Prisco, 1992). Cellular differentiation controls and can be controlled by changes in DNA methylation. Also, methylation change in cell populations might lead to tissue differentiation, and a specific type of differentiated cell might elicit changes in DNA methylation (Holliday, 1987; Razin and Cedar, 1994). Furthermore, alterations in the level of S-adenosyl methionine (SAM), the proximate methyl group required for DNA methylation, could affect DNA methylation.

The amount of SAM available to methylate DNA is regulated by components of the 1-carbon choline/methionine/folate metabolic pathway (Figure 3). DNA methyltransferases catalyze the reaction in which SAM donates a methyl group to DNA or another methyl group acceptor (e.g. RNA or protein) such that the end products

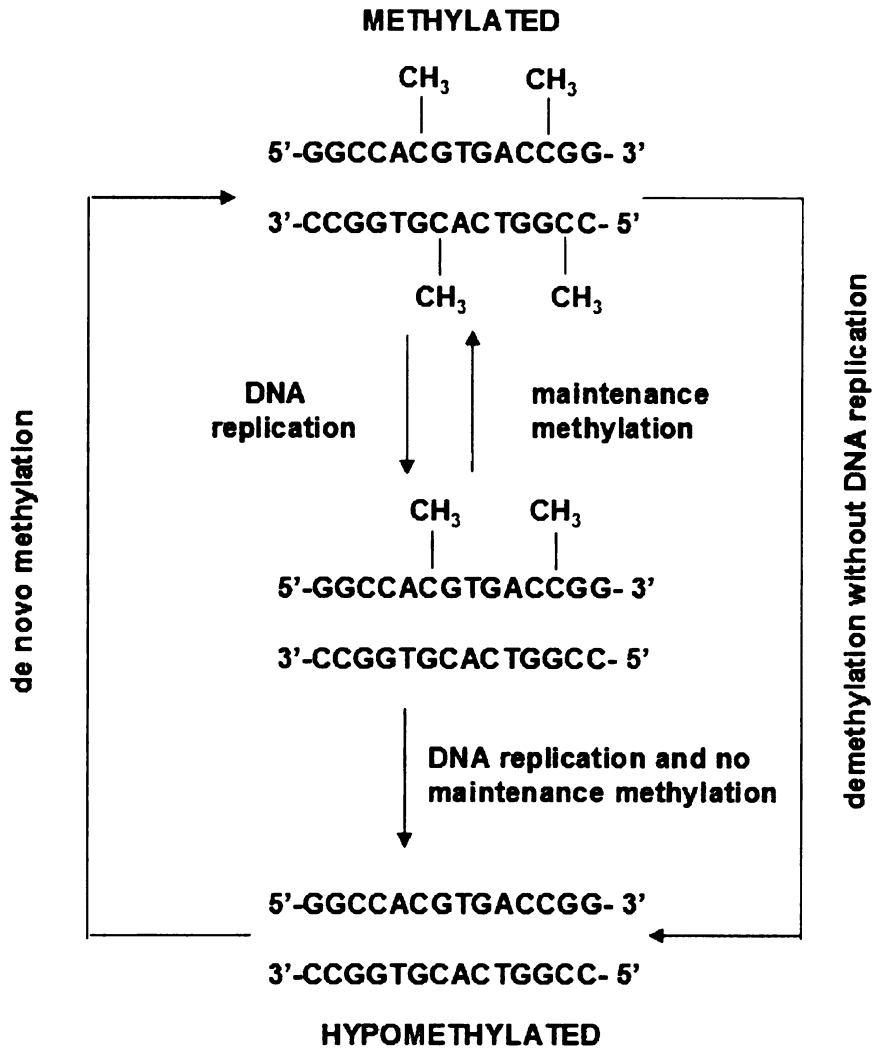


Figure 1. Maintenance of DNA methylation. Newly synthesized DNA is hemimethylated. Shortly after DNA replication, an S-adenosylmethionine (SAM)-requiring maintenance methylase recognizes hemimethylated sites and methylates cytosine at the 5' position to reestablish the original methylation pattern. A failure to maintenance methylate (e.g., due to decreased levels of SAM and/or inhibition of maintenance methylase during periods of cell proliferation) can result in daughter cells that contain hemimethylated DNA sites. The next round of replication would then lead to cells containing hypomethylated DNA, the hypomethylated state would be inherited by subsequent generations. Furthermore, there are opportunities for demethylation that are not linked to DNA replication and de novo methylation, which does not require a hemimethylated signal.
-Adapted from Hergersberg, *Experientia* 1991.
-Methylcytosine residues are represented as C-CH_3.

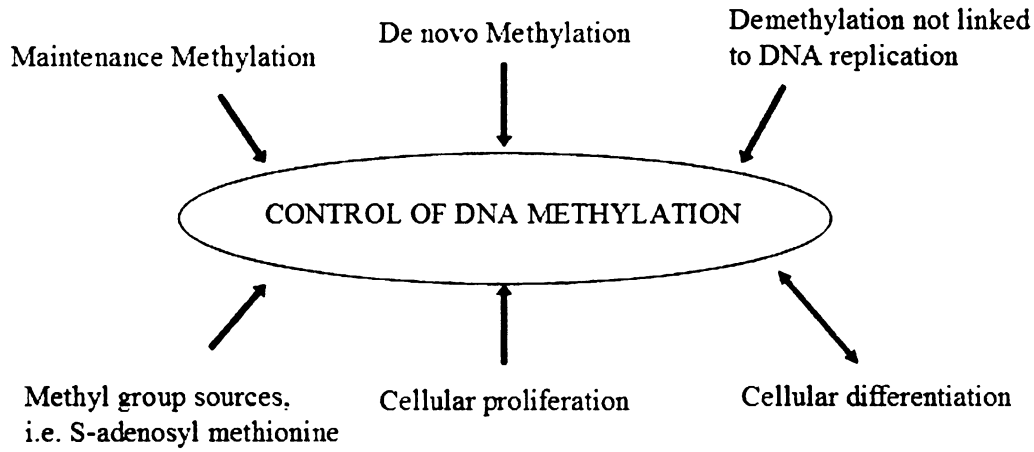


Figure 2. Multiple factors controlling DNA methylation. A particular pattern of DNA melthylation is the product of multiple, interdependent factors. Alteration of one or more of these can lead to major changes in methylation status. The state of differentiation can affect methylation, and methylation status can influence the state of differentiation; thus, the double-headed arrow between methylation and differentiation.

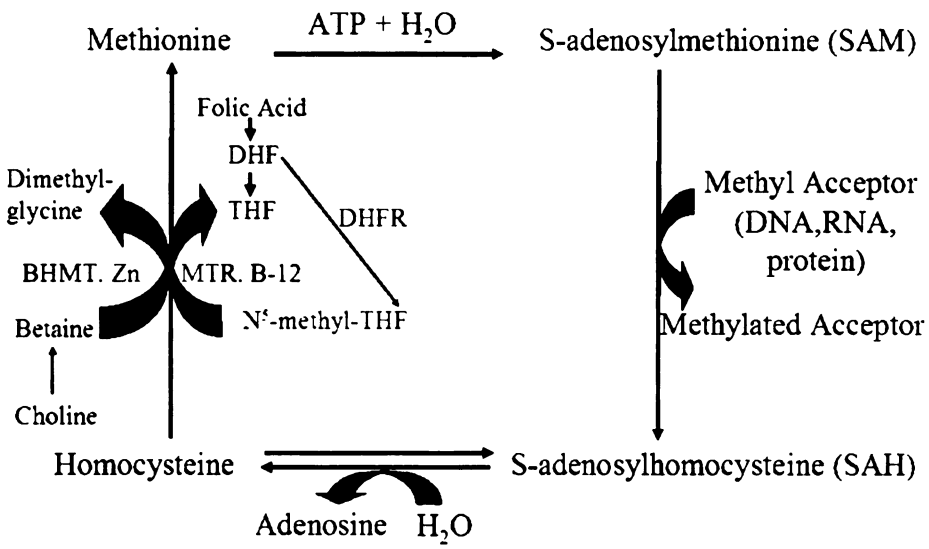


Figure 3. The 1-carbon choline/folate/methionine metabolic pathway responsible for DNA methylation. S-adenosylmethionine serves as the methyl group donor for methyl acceptors such as DNA and arsenic. Following methylation, S-adenosylmethionine (SAM) is converted to S-adeonsylhomocysteine (SAH). SAH is hydrolyzed to homocysteine, in a reversible reaction. A methyl group is then added to homocysteine in one of two ways. In one pathway, a methyl group from N^5 methyltetrahydrofolate (N^5-methyl-THF) is added to form methionine in a reaction catalyzed by methionine synthase (MTR). In this reaction, B-12 is needed as a co-factor. Folic acid is converted to dihydrofolate, which is converted to N^5-methyl-THF. Alternatively, choline serves as a precursor for the synthesis of betaine, which donates a methyl group to homocysteine in a reaction requiring betaine methyltransferases (BHMT) and zinc (Zn)

include a methylated acceptor and S-adenosylhomocysteine (SAH) (Bottiglieri, 2000). In a reversible reaction, SAH is hydrolyzed to homocysteine, a precursor for methionine. There are two pathways by which a methyl group is added to homocysteine to synthesize methionine. Synthesis of methionine in a B-12 dependent reaction catalyzed by methionine synthase (MTR) involves the transfer of a methyl group from N^5-methyene tetrahydrofolate (N^5-methyl THF) (Van den Veyver et al., 2002). This pathway requires folic acid for recycling of N^5-methyl THF. Alternatively, a methyl group from betaine can be coupled to homocysteine in a reaction catalyzed by betaine homocysteine methyltransferase (BHMT), which requires zinc as a cofactor. This pathway does not occur in all cells; BHMT is only available in mammalian liver and kidney (Finkelstein et al., 1983). In this reaction, choline is a precursor for betaine (Ziesel and Blusztajn, 1994). Finally, methionine is hydrolyzed to SAM (Van den Veyver et al., 2002)

To some extent, all the factors involved in the maintenance of DNA methylation are interdependent, such that alteration in one factor may be made up for by altering another factor. For instance, a decrease in *de novo* methylation or SAM levels may be compensated for by a decrease in the rate of active or passive demethylation. However, a severe alteration in any one of these factors, such as a large decrease in the availability of SAM due to abnormal functioning of the 1-carbon metabolic pathway, can influence DNA methylation.

Roles of DNA methylation in a variety of biological processes

DNA methylation plays a critical role in the regulation of several biological processes, including development, tissue-specific gene expression, X-inactivation, the expression of imprinted genes, and the silencing of transposable elements. Additionally, recent studies have indicated that particular patterns of DNA methylation are necessary for T-cell activation and astrocyte differentiation, both very specific, vital mechanisms. The prevalence and importance of the processes regulated in part by DNA methylation underscores the importance of this mechanism in maintaining human health.

Dynamic changes in DNA methylation during embryogenesis allow for normal development (Brandeis et al., 1993), and murine embryos lacking *Dnmt1*, *Dnmt3a* or *Dnmt3b* will die *in utero* or soon afterwards (Okano et al., 1999). Prior to gastrulation, most DNA sequences undergo extensive DNA methylation, followed by extensive demethylation during pre-implantation development, and finally, selective gene-specific demethylation, which might contribute to tissue-specific patterns of methylation allowing for organ-appropriate genetic expression (Brandeis et al., 1993). DNA methylation modulates the expression of many genes during development allowing for both major changes in or important fine-tuning of expression (Ehrlich, 2003).

Interestingly, normal patterns of methylation appear to be more reliably established when an animal is conceived with an egg and sperm compared to using nuclear transfer techniques (Cezar et al., 2003), and subsequently the expression of genes regulated by methylation, particularly the expression of imprinted genes, is altered in cloned animals (Pennisi 2001). It has been proposed that global methylation losses and a lack of the normal waves of methylation and demethylation during development might

contribute to the developmental failure of cloned bovine fetuses (Cezar et al., 2003). Perhaps there is a functional reason for the observation that sperm typically exhibit a higher level of methylation compared to the egg and the paternal and maternal genomes vary in global methylation levels in the early stages of development (Mayer et al., 2000). This disparity in methylation patterns of the gametes might somehow be necessary for the establishment of normal methylation patterns in the progeny.

It has been well established that maternal and paternal specific DNA methylation patterns play a key role in the regulation of imprinted genes, which are genes that are monoallelically expressed in a paternal- or maternal-specific fashion (Bell and Felsenfeld, 2000). *Igf2* (Insulin-like growth factor 2) is an example of a paternally-expressed imprinted gene that relies on methylation patterns for monoallelic expression (Cui et al., 2001). Methylation of a particular imprint control region (ICR) on the paternal allele blocks the binding of the vertebrate enhancer blocking protein CTCF, which would otherwise inhibit access of an enhancer protein to the *Igf2* promoter region (Bell and Felsenfeld, 2000). On the maternal allele, the lack of methylation in the ICR region allows for CTCF to bind, forming a block between the enhancers located distally to the CTCF binding region, and the *Igf2* promoter region (Bell and Felsenfeld, 2000).

The role for DNA methylation in tissue-specific gene expression has long been proposed, but few studies have been performed to verify this. As previously mentioned, it is thought that tissue-specific methylation occurs during development (Brandeis et al., 1993; Ehrlich, 2003). In the mouse, the terminal deoxynucleotidyl transferase gene (TdT) expressed in only in early B and T lymphoid precursors a few days after birth was shown to exhibit methylation patterns that correlate inversely with expression. (Nourrit et

al., 1999) Also, methylation patterns of *HoxA5* (also a tumor suppressor) and *HoxB5*, both members of the Hox family of genes that play a role in the establishment of the vertebrate axis, are differentially methylated in a tissue-specific fashion in the mouse (Hershko et al., 2003). Most notably, a recent study by Futscher et al. described that the tissue-specific expression of the maspin gene SERPINB5 is tightly and inversely related to methylation of this gene (2003).

Furthermore, DNA methylation is associated with X-inactivation in females. CpG dinucleotides on the inactive X-chromosome are mostly methylated (Avner and Heard, 2002). As previously mentioned, it is thought that while DNA methylation probably does not initiate X-inactivation, methylation contributes to the maintenance of genetic repression on the inactive X, as evidenced by partial de-repression of inactive X genes by dAzaC (Csankovszki et al., 2001).

DNA methylation of transposable elements is another example of non-promoter region methylation. These elements become progressively methylated upon integration into mammalian genomes, inhibiting their expression (Walsh and Bestor, 1999). Inappropriate expression of these elements might otherwise lead to aberrant genetic expression of normally silenced transcripts and/or transcriptional interference with other genes (Robertson and Jones, 2000). Thus, methylation of these elements protects genomic DNA from potentially harmful alterations, illustrating the importance of DNA methylation in the maintenance of genomic integrity (Canell and Goodman, 2003).

Additional roles for DNA methylation have been described in astrocyte differentiation and T-cell activation, both very specific, critical processes. DNA methylation is present in the promoter region of the glial fibrillary acidic protein (GFAP)

gene at embryonic day (E) 11.5 in the mouse, preventing the binding of the STAT3 transcription factor (Takizawa et al., 2001). By E14.5, methylation at this region is no longer present, allowing for the binding of STAT3, and the transcription of GFAP, a gene necessary for the differentiation of neuroepithelial cells into astrocytes (Takizawa et al., 2001). Additionally, demethylation of the promoter region of the T-cell growth hormone gene interleukin-2 (Il-2) occurs following T-cell activation, allowing for the enhancement of Il2 transcription, which in turn makes the T-cells competent to produce cytokines which then activate additional T-cells (Bruniquel and Schwartz, 2003).

These examples illustrate particular mechanisms dependent upon DNA methylation. They support the view of DNA methylation as a basic homeostatic mechanism, which, when altered, could lead to numerous untoward effects, including, but not limited to cancer.

Epigenetic changes vs. mutation in cancer

In the past, carcinogenesis was more or less equated with mutagenesis. Now that there is an increasing amount research indicating that epigenetic factors might also be involved in heritable alterations in phenotype, this view has changed (Jones and Laird, 1999; Jones and Baylin, 2002; Feinberg, 2001). It is true that mutation can contribute to carcinogenesis, however, not all mutagens are carcinogens (Zeiger, 2001). Additionally, since epigenetic mechanisms have been shown to contribute to cancer, not all carcinogens are mutagens (Momparler, 2003).

In some cases, an epigenetic change and a mutation might induce functionally equivalent outcomes. A schematic presented in Figure 4 indicates how this might take

place. If a cell loses an allele of the *p53* tumor suppressor gene, expression of *p53* from the remaining allele could be altered by either a mutation or an epigenetic event. Mutation of *p53* is a frequent event that leads to a decreased expression of this gene in neoplastic tissue (Hollstein et al., 1991). Increased methylation in the *p53* promoter region also correlates to a decrease in expression (Pogribny et al., 2000), and promoter region methylation of *p53* has been observed in certain neoplastic tissues (Vizmanos et al., 2003). The overall effect of both the *p53* mutation and the increase in methylation of the *p53* promoter region is to decrease the amount of functional *p53* gene product, which would decrease the rate of apoptosis in response to genetic damage, a factor likely contributing to carcinogenesis (Figure 4).

In addition to functioning in parallel to induce cancer, mutagenesis and DNA methylation can also affect one another. DNA methylation can indirectly contribute to the prevalence of mutation. 5-methylcytosine is much more likely than cytosine to be deaminated to thymine, and methylation is thought to be a driving force behind the high prevalence of C:G to T:A transitions (Magewu et al., 1994). Thus, the high rate of mutation at CpG dinucleotides might be due, in part, to methyltransferase-facilitated deamination (Laird and Jaenisch, 1994). Also, DNA methylation of promoter regions of genes that repair DNA such as the *MutL* mismatch repair homologue 1 gene *hMLH1* (Kim, 2003) *and* O^6-methylguanine-DNA methyltransferase (*MGMT*) (Viswanthan et al., 2003) reduces the expression of these genes, and also the ability to repair DNA, contributing to an increase in the prevalence of mutation.

Furthermore, the presence of DNA adducts such as O^6 methylguanine can interfere with the ability of DNA MTase to bind to hemimethylated DNA and restore

DNA methylation content following replication (Weitzman et al., 1994). Interestingly, the *MGMT* gene that would remove this adduct is frequently inactivated by promoter-region methylation in neoplastic tissue (Ma et al, 2003). Thus, hypermethylation of a *MGMT* can lead to an increase in unrepaired DNA adducts, leading to inhibition of maintenance methylation, which would be expected to lead to hypomethylation. This finding is particularly interesting because it represents a possible mechanism whereby DNA could be both hyper and hypomethylated, a common observation in tumor tissue (Ehrlich, 2002).

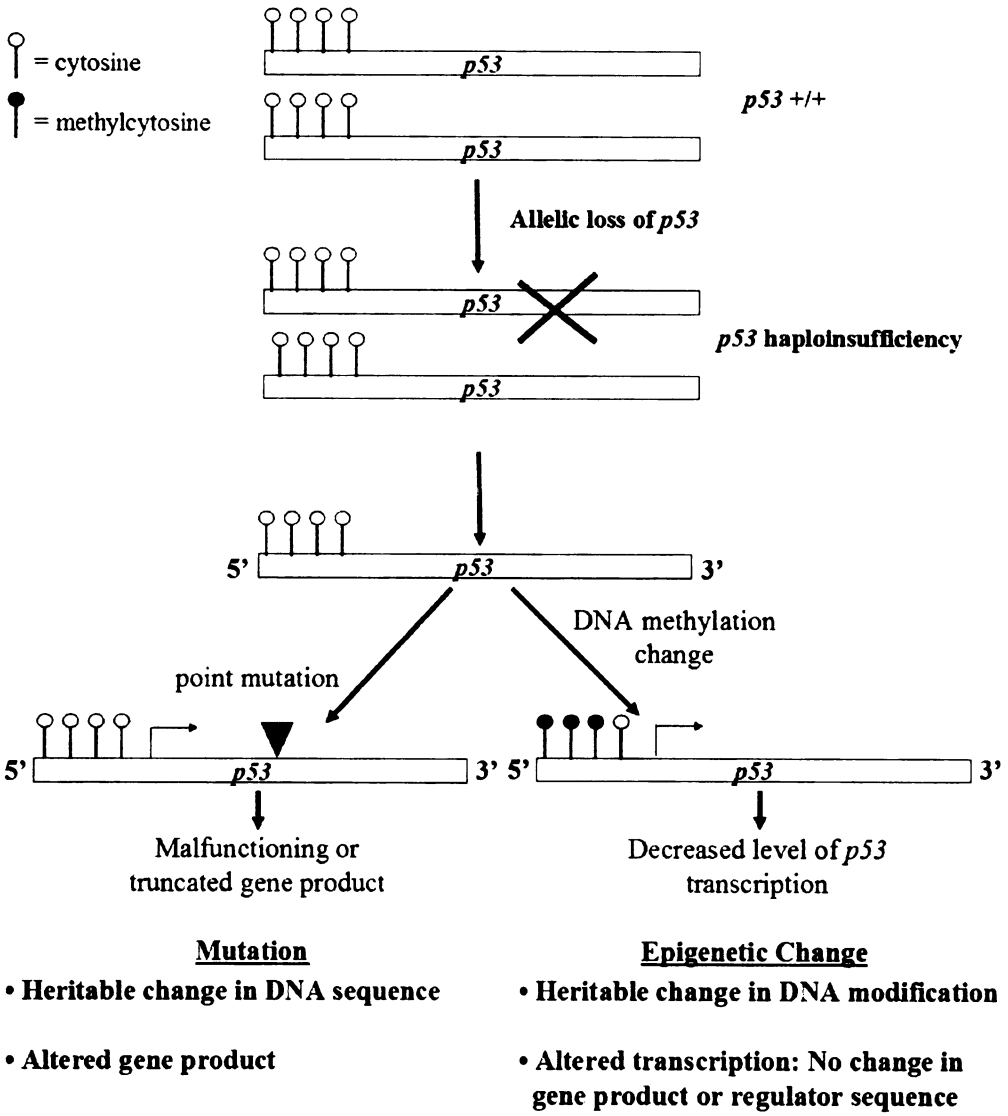


Figure 4. Mutation vs. epigenetic change. A loss of a *p53* allele leads to haploinsufficiency. A point mutation in the *p53* gene can lead to the production of a malfunctioning or truncated gene product. An epigenetic change such as DNA methylation can also contribute to decreasing the level of functional p53 gene product. In this example, DNA methylation of the promoter region of *p53* leads to a decrease in the level of *p53* transcription. A decrease in functional *p53* would reduce the rate of apoptosis in response to DNA damage, thus increasing proliferation of damaged cells, and potentially leading to cancer. This scheme illustrates an instance of how both mutation and epigenetic effects can contribute to carcinogenesis.

The involvement of DNA methylation in cancer

In the United States, 1 of 4 American deaths is attributed to cancer, making this disease the second leading cause of mortality (American Cancer Society, 2003). Thus, from a public health perspective, the study of mechanisms underlying carcinogenesis is important because it provides a basis by which cancer prevention and treatment could be enhanced. Furthermore, characterization of the factors involved in carcinogenesis provides insight into basic biological mechanisms such as the regulation of cell growth and differentiation.

Cancer involves the uncontrolled growth and spread of abnormal cells. Cancer can be caused by both external factors such as tobacco and UV light and/or by internal factors such as obesity, hormone imbalance, and inherited mutations. The six major hallmarks necessary for carcinogenesis are: self-sufficiency in growth signals, evasion of apoptosis, sustained angiogenesis, immortality, limitless replicative potential, and metastasis (Hanahan and Weinberg, 2000). Cancer is viewed as a multistep process which occurs via three experimentally- defined major stages: initiation, promotion, and progression (Pitot and Dragan, 1994). Initiation involves an irreversible, heritable alteration in a cell such that it has a growth advantage over surrounding cells in the promotion stage, which provides an environment for the initiated cell to clonally expand (Pitot and Dragan, 1994). Promoting agents such as tobacco and phenobarbital facilitate the clonal expansion of initiated cells (Schulte-Hermann et al., 1990). Clonal expansion eventually results in the production of cells with additional alterations which give them further growth advantages over the surrounding cells (Figure 5). An important feature of the promotion stage is that it is

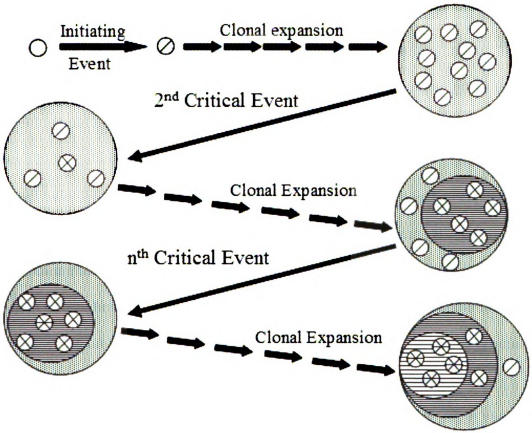


Figure 5. Initiation and cell proliferation in multistage carcinogenesis. The critical events referred to involve heritable changes in the genome caused by mutation or an epigenetic mechanism. This diagram has been adapted from Swenberg et al. (1987) to illustrate that epigenetic changes such as altered DNA methylation, in addition to mutation, may play a key role in carcinogenesis. Each line through a cell represents a critical event. Altered DNA methylation may be a mechanism underlying selective clonal expansion, i.e., hypomethylation may facilitate an aberrant increase in expression of oncogenes and/or hypermethylation may silence tumor suppressor genes. Either of these events could provide a cell with a selective growth advantage over the surrounding cells.

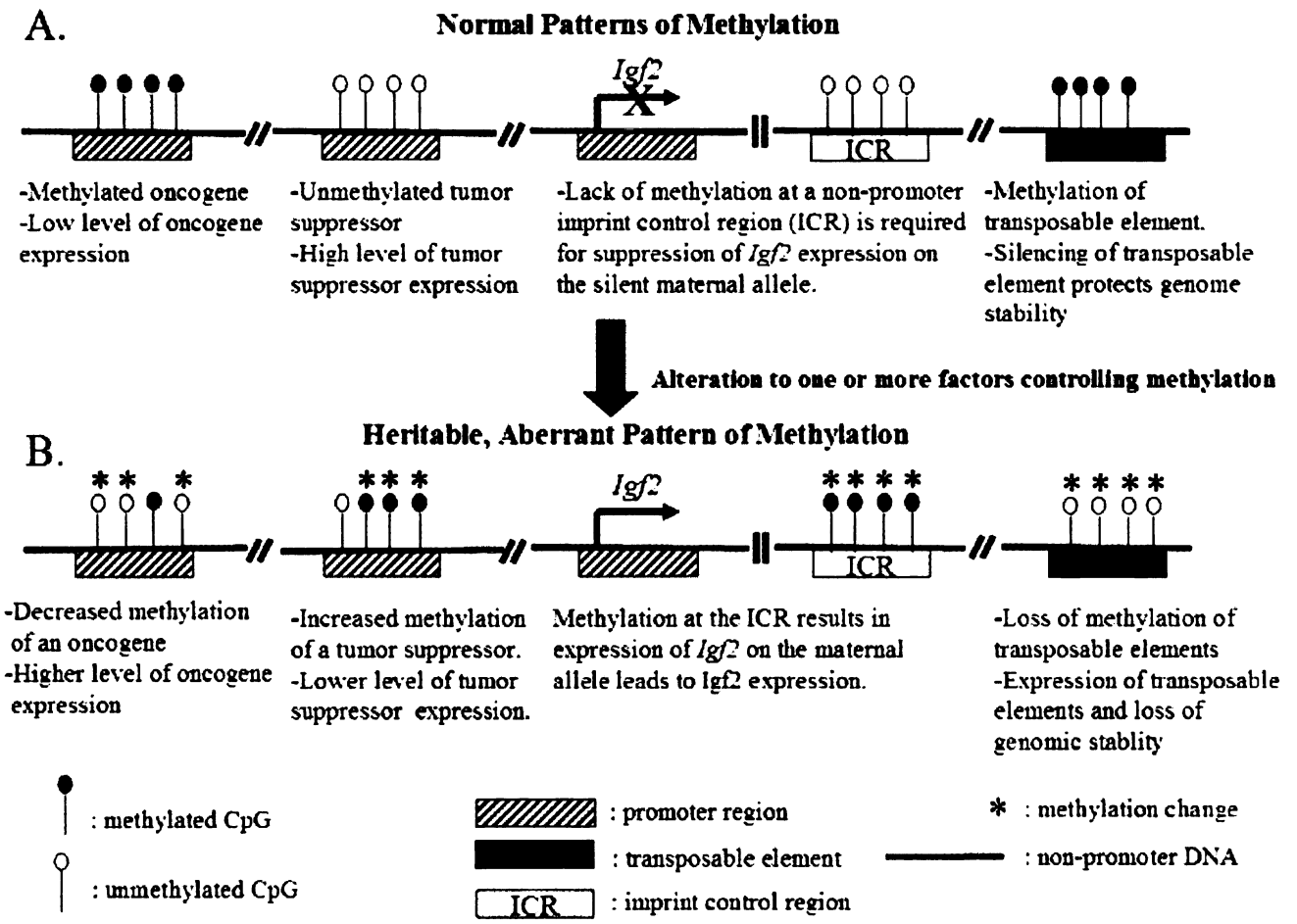
reversible, and the dose-response relationship for promoters exhibits a threshold (Pitot, 1982). Otherwise, if promotion and clonal expansion continues, a subset of cells might enter the progression stage. Cells in the progression stage are characterized by promoter independent clonal expansion and changes in ploidy (Dragan et al., 1993). The specific heritable alterations that occur during the initiation/promotion/progression model of carcinogenesis could include mutations and/or epigenetic changes (Goodman and Watson, 2002), as will be discussed in greater depth in Chapters 2 and 3, along with the characterization of methylation changes during the promotion stage and an explanation of how these changes might play a role in the clonal expansion of abnormal cells.

When examining the role DNA methylation might play in cancer, it is instructive to keep in mind where these changes are occurring. Methylation of CpG islands within the promoter region of several tumor suppressors is linked to the decreased expression of these genes and is a common observation in cancer cell lines and primary tumors (Gonzalez-Zuleta et al., 1995). Also, age-progressive methylation of promoter regions of tumor suppressors as well as hypomethylation of growth associated genes is thought to be involved in the age-dependent increase in cancer incidence (De Pinko et al., 2000; Yenbutr, P. et al., 1998). However, most methylation does not occur at CpG islands; the bulk of methylation is present at non-promoter regions, including transposable elements throughout the genome (Ehrlich, 2002). Thus, if one were to merely examine the average change in methylation across the genome, a conclusion about methylation changes at the CpG islands could not be made. A frequent finding in cancer is a decrease in global levels of methylation, aberrant patterns of methylation at ICR regions regulating the

transcription of imprinted genes, as well as an increased amount of methylation at particular sites, typically in the promoter regions of tumor suppressors (Ehrlich, 2002).

Altered DNA methylation might lead to carcinogenesis in several ways including: 1) hypomethylation of promoter regions leading to overexpression of oncogenes, 2) hypermethylation of promoter regions leading to suppression of tumor suppressors, 3) aberrant methylation in imprint control regions (ICRs) leading to the abnormal expression of imprinted genes, which can contribute to carcinogenesis if under- or over-expressed, and 4) hypomethylation at transposable elements leading to transcriptional interference and genomic instability (Figure 6). In this context, methylation might contribute to the cellular alterations which occur in the initiation/promotion/progression models of carcinogenesis, particularly within the promotion stage in which initiated cells clonally expand in the environment provided by the promoting agent. Several changes in methylation status would be expected to provide cells with a growth advantage. For instance, methylation in the promoter region of a tumor suppressor such as *p16,* would decrease *p16* expression, increasing the cellular proliferation rate. Also, hypomethylation leading to the increased expression of oncogenes or the abnormal expression of transposable elements would be likely to confer a growth advantage.

Figure 6. Illustration of four possible alterations in normal patterns of DNA methylation that might facilitate tumorigenesis. (A) Normal methylation patterns. (B) Aberrant methylation. A decrease in methylation of the promoter region of an oncogene can result in increased expression, whereas an increased methylation of the promoter region of a tumor suppressor gene can silence its expression. Altered methylation can affect imprinting. An increase in methylation of the imprint control region (ICR) of the imprinted oncogene *Igf2* may lead to expression of *Igf2* from the normally silenced maternal allele in addition to the expression that occurs normally from the paternal allele. Furthermore, a decrease in methylation of transposable elements can lead to their expression and, thus, contribute to genetic instability. Additionally, 5-methylcytosine may deaminate spontaneously to thymine, resulting in a C:G to T:A point mutation.


A.
Normal Patterns of Methylation
Igf2
ICR
-Methylated oncogene
-Low level of oncogene expression
-Unmethylated tumor suppressor
-High level of tumor suppressor expression
-Lack of methylation at a non-promoter imprint control region (ICR) is required for suppression of Igf2 expression on the silent maternal allele.
-Methylation of transposable element.
-Silencing of transposable element protects genome stability
Alteration to one or more factors controlling methylation
B.
Heritable, Aberrant Pattern of Methylation
Igf2
ICR
-Decreased methylation of an oncogene
-Higher level of oncogene expression
-Increased methylation of a tumor suppressor.
-Lower level of tumor suppressor expression.
Methylation at the ICR results in expression of Igf2 on the maternal allele leads to Igf2 expression.
-Loss of methylation of transposable elements
-Expression of transposable elements and loss of genomic stablity
: methylated CpG
: unmethylated CpG
: promoter region
: transposable element
ICR : imprint control region
* : methylation change
: non-promoter DNA

DNA methylation is a secondary mechanism underlying carcinogenesis

Carcinogens may be classified as acting via a genotoxic or nongenotoxic mode of action. Genotoxic agents interact directly with DNA, while nongenotoxic chemicals do not. Instead, nongenotoxic carcinogens often elicit their effects via a secondary mechanism. It is proposed that DNA methylation acts as a secondary mechanism in carcinogenesis (Goodman and Watson, 2002).

DNA methylation fulfills the requirements set forth for a secondary mechanism involved in carcinogenesis, including: 1) a biologically plausible mechanism, 2) a nongenotoxic mechanism, 3) ability to experimentally measure the marker, 4) a threshold-exhibiting mechanism, 5) data supporting human relevance, and 6) data indicating that carcinogenesis can be blocked by inhibiting the mechanism (Goodman and Watson, 2002). The role of DNA methylation in carcinogenesis has been well established (Counts and Baylin, 1995; Laird, 1997). DNA methylation is an epigenetic, nongenotoxic mechanism and DNA can be measured by a variety of methods (Shiraishi et al., 2002). Alterations in methylation induced by treatment with PB and cigarette smoke condensate (CSC) are dose-dependent (Ray et al., 1994; Watson et al., 2003). DNA methylation alterations are not unique to rodents; numerous studies have described methylation alterations in neoplastic tissues in humans, and genes found to be regulated by methylation (i.e. *p16*) in rodents are frequently altered by methylation in the human as well (Patel et al., 2000; Mateos et al., 2002). Additionally, patterns of methylation in rodent and human tumors are similar; there is typical a decrease in global levels of methylation as well as hypermethylation at selected regions (Ehrlich et al., 2002). Supplementation of the diet with components necessary for 1-carbon metabolism

required for DNA methylation such as choline, methionine, folic acid have been found to be chemotherapeutic (Simile et al., 1996; Kim et al., 1996; Van den Veyver, 2002).

Models used to assess the role of DNA methylation in cancer

Two of the most widely used models for the assessment of carcinogenic potential as well as for the study of mechanisms underlying carcinogenesis are the mouse liver tumor model and the SENCAR initiation-promotion mouse skin model. In my studies, these models allowed for the characterization of methylation changes induced by treatment with rodent tumor promoting agents within the context of the multistage model of carcinogenesis. Additionally, the classic non-genotoxic rodent tumor promoter phenobarbital (PB), often used in conjunction with the mouse liver tumor model, is described below.

Mouse liver tumor model

The relatively tumor-sensitive B6C3F1 mouse commonly used in this model is the result of a cross between the relatively tumor-resistant C57BL/6 strain and the relatively tumor-sensitive C3H/He strain. B6C3F1 mice exhibit a spontaneous hepatoma incidence of approximately 30% after 18 months of age (Becker, 1982), and are very sensitive to the induction of tumors by a number of chemicals, making this stock an excellent model for the analysis of mechanisms underlying carcinogenesis (Maronpot, 1987). Furthermore, the mouse liver tumor model exhibits sequential histological changes (Goodman et al., 1991). The appearance of altered hepatic foci correlates with the promotion stage of carcinogenesis and is thought to represent the clonal expansion of

an initiated cell within the context of the multistage model of carcinogenesis previously discussed (Klaunig et al., 1990). Specifically, treatment with the nongenotoxic promoting agent phenobarbital leads to a more marked increase in the cellular proliferation rate within the already existing lesions, supporting the notion that promoting agents provide a favorable environment for the clonal expansion of initiated cells (Pitot et al., 1987). Chemically-induced increases in the size, number and proliferation rate of these altered foci can be examined in a dose-response fashion.

During the course of hepatocarcinogenesis, altered foci develop into adenomas, then frank carcinomas. The source(s) of progenitor cells involved in the etiology of hepatocarcinoma include(s) oval cells, which are likely to function as facultative stem cells (Ruch and Trosko, 1999). Some reports propose that progenitor cells might also include duct cells and hepatocytes (Sell, 2003). Since stem cells share characteristics with tumor cells, including immortality, loss of gap junctional communication, and inability for contact inhibition (Trosko and Chang, 2001), it seems that these stem-like oval cells would be the most likely progenitor cells. However, based in part on recent findings that bone marrow-derived cells have been shown to replenish a number of cell populations including liver cells (Krause et al., 2001), the notion that dedifferentiation of less potent cells (Blau et al, 2001) seems to be a possibility, though the extent of dedifferentiation in the liver resulting in progenitor cells from the more determined hepatocytes and duct cells is likely to be minimal.

Additionally, to study mechanisms possibly underlying differences in tumor susceptibility, one can compare liver from the relatively tumor-sensitive B6C3F1 and/or C3H/He groups, with the relatively tumor-resistant C57BL/6 strain. The spontaneous

liver tumor incidence in B6C3F1, C3H/He, and C57BL/6 mice after approximately 2 years is 30%, 80% and less than 5%, respectively (Becker, 1982; Buchmann et al., 1991; Grasso and Ginsler, 1975). Tumor-sensitive mice also are more sensitive to chemically-induced tumorigenesis; C3H/He mice are 20-50X more susceptible than C57BL/6 mice to the induction of tumor formation by the carcinogens N-diethylnitrosamine (Drinkwater and Ginsler, 1986) and N-ethyl-N-nitrosourea (Hanigan et al., 1988).

Further characterization of any mechanistic differences between the strains related to the tumor susceptibility differences might provide better insight into the importance of processes involved in carcinogenesis. Additionally, since these strains are commonly-employed experimental models, a better understanding of the reason for the difference in tumor susceptibility would, in part, provide an improved basis for the use of these models. This is particularly relevant when the model used meant to assess the carcinogenic risk a chemical poses to humans, as is frequently done using the B6C3F1 mouse in the 2-year National Toxicity Program bioassay (Haseman and Elwell, 1996).

It has been shown that multiple susceptibility loci within an *Hcs* (hepatocarcinogen sensitivity) locus account for approximately 85% of the difference in sensitivity seen between the groups (Drinkwater et al., 1986). It is possible that genes within the *Hcs* locus might govern processes important to GJIC and/or DNA methylation, both mechanisms which have been shown to be differentially altered in a strain-specific manner in response to phenobarbital treatment (Klaunig and Ruch, 1987; Warner et al., 2003; Ray et al., 1994; Counts et al., 1996; Watson and Goodman, 2002b and others).

In response to phenobarbital treatment, GJIC communication is reduced in the relatively tumor-sensitive B6C3F1 but not in the relatively resistant C57BL/6 strain. Gap

junctions allow for the cell-to-cell transport of ions and molecules less than 1kDa in diameter (Bruzzone et al., 1996), including components necessary for cellular homeostasis, regulation, apoptosis and differentiation (Ruch, 2000).

Differences in strain susceptibility have also been linked to differences in the ability to maintain normal patterns of DNA methylation (Ray et al., 1994; Counts et al., 1996; Watson and Goodman, 2002b). Following partial hepatectomy or a 2 wk treatment with a tumor promoting (500 ppm) dose of PB, hypomethylation of the *raf* oncogene and up-regulation of *raf* and *H-ras* in spontaneous and PB-induced tumors was observed in the tumor-sensitive B6C3F1 stock, and not in the C57BL/6 strain (Ray et al., 1994). Additionally, a 2 wk treatment with PB led to a greater amount of global hypomethylation in the B6C3F1 mouse compared to the C57BL/6 strain (Counts et al., 1996). Interestingly, increased cellular proliferation in response to PB was more marked in the relatively tumor-resistant C57BL/6 strain compared to the tumor-sensitive B6C3F1 stock (Counts et al., 1996). This indicates that the more pronounced global hypomethylation in the B6C3F1 stock cannot be explained by a decreased ability for maintenance methylation secondary to an increased amount of proliferation induced by PB (Counts et al., 1996). Overall, these findings demonstrate that the ability to maintain global patterns of methylation as well as methylation at particular oncogenes was related inversely to tumor susceptibility. Ray et al., found that the hypomethylation of *H-raf* correlated to a decrease in expression, providing an example of how hypomethylation might contribute to oncogene activation (1994). Global hypomethylation could represent de-repression of transposable elements and/or activation of oncogenes, both plausible contributing factors in carcinogenesis (Counts et al., 1995). However, measurements of

global, average levels of methylation do not reveal how methylation patterns might change differently in specific regions, and frequently DNA is globally hypomethylated, with regional hypermethylated at specific GC-rich regions, including CpG islands in the promoter regions of genes. Thus, hypermethylation at GC-rich regions could contribute to the silencing of tumor suppressor genes, a frequent observation in neoplastic tissue (Esteller et al., 2002). Therefore, to further examine the role methylation might play in the differences in tumor susceptibility between these groups of mice, I examined the GC-specific methylation status of C57BL/6, C3H/He, and B6C3F1 given the same 2 wk, 0.05% PB promotion schedule described by Counts et al., 1996. This study is the focus of Chapter One and is also described in Watson and Goodman, 2002b.

Phenobarbital

Phenobarbital (PB) was pertinent to my studies due to its ability to alter patterns of methylation as described above. Thus, the use of PB permitted the assessment of differences in the capacity to maintain patterns in methylation between C57BL/6, C3H/He and B6C3F1 mice.

Phenobarbital (PB) is a sedative and anticonvulsant agent that is also a classic example of a nongenotoxic rodent liver tumor promoter (Peraino et al., 1973; Feldman et al., 1981). PB up-regulates mixed function oxidase enzymes, including CYP2B1, and increases cell proliferation during the initial 1-2 wks of administration at a promoting dose (Lee, 2000; Newberne et al., 1990). The effect of PB on the presence and size of altered hepatic foci exhibits a linear dose-response within the approximate 16-1200 ppm dose range following initiation with diethylnitrosamine and promotion with PB for 20

wks (Maekawa et al., 1992). A non-tumor promoting (20 ppm) dose of PB did not lead to changes in methylation status in either group, indicating that PB's effect on methylation status is threshold-exhibiting. Additionally, in a dose-response study performed with 0, 2, 15, and 500 ppm doses of PB for 33 wks, animals treated with the 2 ppm dose of PB exhibited a decreased prevalence in liver tumors, indicating that lower doses of PB might have a hormetic effect (Kinoshita et al., 2003).

It has been shown that PB blocks GJIC in the rodent liver (Ito et al., 1998; Moennikes et al., 2000; Warner et al., 2003) a significant finding because the proper functioning of gap junctions is closely related to cancer risk, and most, if not all cancer cells exhibit decreased levels of GJIC (Trosko, 2001). Connexins are subunits of gap junction channels, and Cx32 is the predominant connexin in rodent liver (Yamasaki and Naus, 1996). *Cx32* null mice with either a C3H/He or C57BL/6 background are 5-10 X more sensitive to tumor formation compared to mice with functional *Cx32* (Moennikes et al., 1999). Importantly, *Cx32* null mice are not susceptible to further promotion with PB, suggesting that PB might somehow reduce *Cx32* activity. When C57BL/6 and B6C3F1 mice were promoted with PB, while the tumor-sensitive B6C3F1 stock exhibited marked decrease in GJIC, neither group had a reduced *Cx32* expression (Moennikes et al., 2000). While this study demonstrates an interesting strain difference in PB-mediated GJIC inhibition, it also indicates that PB does not mediate this response through a decrease in *Cx32* expression (Warner et al., 2003). There is one study in the rat reporting PB-induced reduction in *Cx32* expression (Neveu et al., 1994). However, two more studies in the rat did not decrease *Cx32* mRNA or protein levels (Chaumontet et al., 1996; Krutsovskikh et al., 1995). Given the inconsistent reports of PB-induced changes in

Cx32 expression levels, it appears that PB might instead inhibit Cx32 through a post-translational mechanism.

SENCAR mouse skin model

The SENCAR (sensitive to carcinogenesis) mouse skin model is the result of mice selectively bred in the 1960's and 70's for sensitivity to papilloma formation in response to 7,12-dimethylbenz[a]anthracene (DMBA) initiation and the croton oil (containing TPA) promoting agent (Stern and Conti, 1996). These mice are extremely sensitive to carcinogenesis, and generally respond more rapidly and uniformly to the induction of skin tumors than other available strains or stocks. SENCAR mice respond more rapidly and uniformly to the induction of skin tumors than any other available strains and are extremely sensitive to carcinogenesis due to initiation by 7,12-deimethylbenz[*a*]anthracene (DMBA) and promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Hennings et al., 1997; Coghlan et al, 2000). Thus, this model is ideal for the assessment of the initiating and/or promoting potential of topically applied agents. Importantly, this model allows for the clear demarcation of different stages in cancer, allowing for one to examine the ability of compounds to act as initiating and/or promoting agents; the compounds of interest are simply topically applied to the skin (Slaga, 1996). This model provided an excellent way to examine progressive, dose and time-dependent changes in global, GC-rich, and gene-specific DNA methylation patterns, and I used this model in order to characterize alterations in methylation status during the promotion stage of carcinogenesis. I was particularly interested in determining if DNA

methylation changes were reversible, and characterizing how DNA methylation could contribute to the clonal expansion of increasingly abnormal cells in the promotion stage.

Using this model, I examined the effect of cigarette smoke condensate (CSC) on methylation patterns as well as its potential to act as a promoting agent within the multistage model of carcinogensis (Watson et al., 2003). Characteristics of a classic promoting agent include the need to repeatedly apply rather large doses over a given period of time, and the reversibility of clonal expansion of initiated cells when the promoting agent is withdrawn (Dragan et al., 1993). I was interested in determining possible roles for DNA methylation in clonal expansion in the promotion stage. I analyzed global, GC-rich and gene-specific methylation patterns in order to get a complete picture of the cascade of methylation changes that might be involved. Additionally, in order to test for reversibility, global and GC-rich methylation analysis was performed for animals which were treated with CSC and then untreated for a time before necropsy. Details of these studies are provided in Chapters Two and Three.

Chemically-induced changes in DNA methylation

A number of agents other than phenobarbital have been shown to elicit methylation alterations. The cytosine analogue 5-aza-2'deoxycytidine (dAzaC) causes hypomethylation of DNA by irreversibly binding to methyltransferases (Avramis et al., 1989), and administration of dAzaC to pregnant mice results in perturbation of embryonal DNA synthesis, low fetal weight, and death of rapidly proliferating cells (Rogers et al., 1994). Similarly, the antileukemic adenosine analogues 2-chloro-2'deoxyadenosine (cladribine) and 9- arabinosyl-2-fluoroadenine (fludarabine) inhibit

DNA methyltransferases (Wyczechowska and Fabianowska-Majewska, 2003). Administration of the phytoestrogen genisten in the diet of mice has been shown to lead to increases in methylation at CpG islands (Day et al., 2002). Nickel leads to increased DNA methylation levels as well as chromatin compaction (Lee, 1995), and it has been proposed that the carcinogenic actions of arsenite, dichloroacetic acid, and trichloroacetic acid are related to their ability to induce hypomethylation and upregulate the oncogene *c-myc* (Chen et al., 2001). Furthermore, cigarette smoke condensate leads to increases in GC-rich and gene specific methylation patterns in tumor and non-tumor tissue as well as global hypomethylation in papillomas (Watson et al., 2003). And most recently, valproate, a drug used for epilepsy and mood stabilization, has been found to trigger DNA methylation decrease and histone acetylation independently of DNA replication through the DNA-binding protein 2/DNA demethylase (MBD2/dMTase), and this represents a rare example of chemically-induced active demethylation (Detich et al., 2003)

The exact mechanism(s) by which a particular compound elicits change in DNA methylation is often unknown. However, chemically-induced changes in DNA sometimes occur through perturbation of components of DNA methylation 1-carbon choline/folate/methionine metabolic pathway responsible for DNA methylation. (Figure 3). Disruption of this cycle would have the capacity to affect DNA methylation. For example, *in vivo*, arsenic is methylated by SAM, and administration of arsenic is thought to hypomethylate DNA by decreasing the availability of SAM to DNA (Okoji et al., 2002). In mice, a choline and methionine deficient diet leads to a decrease in global methylation levels (Counts et al., 1996). Conversely, and excess of components utilized

in the SAM pathway might also contribute to methylation change. Pups of pregnant mice given sufficient vs. supplemented levels of B-12, folate, and choline exhibited different levels of methylation in a transposable element which governs expression of coat color. (Waterland and Jirtle, 2003). A decreased amount of components required for the functioning of the 1-carbon metabolic cycle also influences DNA methylation.

The role of DNA methylation in non-cancer related toxic outcomes

The link between mental disorders and methylation aberrations indicates that DNA methylation plays a vital role with regard to the normal functioning of the central nervous system (Robertson and Wolffe, 2000). A further indication for an important role of methylation in the brain is the observation of high levels of neuronal methyltransferase (Goto et al., 1993).

One of the first mental disorders to be linked to errors in methylation was fragile-X syndrome, a predominantly male form of mental retardation. Patients with this disorder display an increase in methylation at the CpG island upstream of the FMR1 (fragile-X mental retardation) gene coupled with a decrease in FMR1 expression (Robertson and Wolffe, 2000). An additional mental disorder linked to alterations in methylation is Rett syndrome, an X-linked disorder responsible for a predominantly female form of mental retardation that appears to stem from a mutation in the gene that encodes the methylcytosine-binding protein MeCP2 (Nan et al., 1997). Furthermore, Prader-Willi and Angelman syndromes, both characterized by severe mental deficits, are linked to alterations in the methylation patterns of a differentially methylated region

within the SNRPN promoter/exon1 region on the paternal and maternal alleles, respectively (Shemer et al., 2000).

Beckwith-Widemann syndrome (BWS) is an example of a developmental disorder due to alteration of methylation-regulated imprinting mechanisms. BWS is characterized by developmental growth disorders, which, in some cases, is accompanied by increased expression of *Igf2* (Issa and Baylin, 1996). *Igf2* is typically a paternally expressed gene, but loss of imprinting may be caused by abnormal patterns of methylation (Maher and Reik, 2000).

Additionally, ICF (immunodeficiency, centromeric instability, and facial anomalies syndrome) is both a mental, immunological, and developmental disorder linked to altered methylation. ICF is characterized by immunosuppression, mental retardation, and particular facial characteristics (Wijmenga et al., 1998). Patients exhibit mutations in Dnmt3a, a de novo methyltransferase gene, which lead to abnormal hypomethylation in constitutive and facultative (X-inactive chromosome) heterochromatin (Xu et al., 1999).

Use of methylation analysis as a tool to gauge toxic potential

Thus far, the bulk of research on the use of methylation analysis as a tool to identify potential toxic outcomes has focused on the early detection of cancer. Hypermethylation of *p16* in the sputum and/or plasma identified 92.0% (46/50) of the lung cancer patients studied (Liu et al., 2003) and methylation of the promoter regions of *p16* and *MGMT* tumor suppressor genes has been detected in the sputum DNA of all patients with squamous cell carcinoma of the lung up to 3 yrs before clinical diagnosis

(Palmisano et al., 2000). Recently, a blood test for methylation-mediated loss of imprinting has shown promise in the detection of colon cancer (Cui et al., 2001).

Given the significance of DNA methylation in a variety of processes, the number of possible *in vivo* targets that could potentially elicit methylation change, and an increasing amount of evidence for chemically-induced alterations in DNA methylation, it is probable that a number of compounds alter DNA methylation, and these alterations would have the capacity to elicit a number of untoward effects. DNA methylation can be viewed as a general homeostatic mechanism, and compounds that have the capacity to disrupt this might be more likely to elicit toxic effects compared to those that do not. Given the plethora or potential therapeutic agents and environmental compounds that require toxicity testing, the detection of potential toxic outcomes at early stages is crucial. It is important to recognize that DNA methylation in and of itself is not necessarily indicative of toxicity, and that DNA methylation change is reversible (Ramshandani et al., 1999). Furthermore, the ability to hypomethylate DNA underlies the anti-carcinogenic activities of dAzaC, cladribine and fludarabine (Wyczechowska and Fabianowska-Majewska, 2000). Therefore, one might want to consider compounds that affect methylation when looking for additional chemotherapeutic drugs. However, in general, the inclusion of methylation analysis to basic, initial toxicity screens might assist in compound prioritization and could facilitate a more rational approach towards dose selection. For instance, if two potential therapeutic agents exhibit similar cytotoxicity profiles but one affects methylation and the other does not, one would be more likely to proceed with the one that does not. Analysis of methylation may aid in threshold assessment by aiding in the determination of both the high dose to be employed

for long-term toxicity studies, and appropriate doses to be employed for evaluation of the shape of the dose-response curve for safety evaluation purposes. For example, if in a standard 28-day study, the top dose causes marked histopathology while the next lower dose results in mild histopathology and if both doses lead to altered methylation, then the methylation data can be used to support the "dropping" of the high dose and placing emphasis on the next lower dose as the appropriate high dose.

Significance of altered DNA methylation in toxicity

Failure to maintain normal DNA methylation patterns is known to facilitate aberrant genetic expression leading to carcinogenesis (Counts and Goodman, 1995; Laird, 1997). Alterations in methylation are believed to be early and frequent events, occurring at multiple points of the carcinogenic process, and are sometimes detectable before the appearance of a tumor (Lehmann et al., 2002). The methylation pattern of tumors is generally characterized by global hypomethylation coexisting with regions of hypermethylation, and specific CpG islands have been found to be differentially methylated in cancerous vs. non cancerous tissue (Lin et al., 2001; Ueki et al., 2001). In some cases, distinct patterns of methylation characteristic of different stages of adenocarcinoma have been found (Eads et al., 2001). Thus, characterization of methylation alterations associated with specific types and stages of carcinogenesis provides the basis for a better understanding of biological mechanisms underlying carcinogenesis as well as an improved ability to identify early methylation changes indicative of cancer potential.

Specific types of methylation alterations have been linked to certain neurological, developmental, and immunological disorders, and methylation has been shown to be chemically-altered. However, little is known about how methylation changes elicited by experimental compounds might contribute to toxic effects other than carcinogenesis. Also, considering that over half of mammalian genes contain CpG islands in the promoter region, and that the transcription of these genes might be linked to the methylation status of these regions (Antequera and Bird, 1993), methylation alterations have the potential to cause a wide range of changes in genetic expression, leading to cancer and non-cancer related toxicities. Also, even if methylation is not directly involved in a specific toxic effect, methylation alterations might occur as a result of another mechanism and still be indicative of, though not necessarily the cause of a specific toxic effect. Thus, further characterization of methylation alterations could lead to a greater understanding of the mechanisms underlying carcinogenesis and other toxic effects. In addition, with the significant increase in the generation of new drug candidates, as well as a large number of environmental compounds requiring toxicity screening there is a need for early, accurate *in vitro* tests for toxicity. Methylation analysis might allow for a more rational basis for dose selection and compound prioritization that can assist in the screening of compounds in the environment as well as those to be used for medicines and other consumer products. In order to take an initial step to determine the value of DNA methylation as a gauge of toxic potential, I have examined the utility of a DNA methylation assessment performed on cells treated with concentrations of model compounds shown to be cytolethal or non-cytolethal based on cytotoxicity data. I describe how, when used in conjunction with cytotoxicity and

genotoxicity data, methylation data might provide the basis for the more rational ranking of these compounds based on overall assessments of toxic potential (Chapter 4).

Hypothesis and Objectives

The hypothesis that I am testing is that the ability to maintain normal patterns of methylation is related inversely to susceptibility to carcinogenesis and perhaps other toxic outcomes. As previously mentioned, work by Counts et al. (1996) had demonstrated that PB-induced decreases in global methylation as a result of PB treatment was more marked in the relatively tumor-sensitive B6C3F1 (C57BL/6 X C3H/He) stock compared to the relatively tumor-resistant C57BL/6 strain. Using this same model with the addition of the C3H/He tumor-sensitive strain, I extended this analysis to determine if there are strain differences in PB-induced changes in methylation at GC-rich regions which could potentially represent methylation-mediated inactivation of tumor suppressor genes.

In order to characterize methylation changes within the multistage model of carcinogenesis, my next project involved the characterization of global, GC-specific, and gene specific methylation changes in the 2-stage initiation-promotion SENCAR mouse skin model in which DMBA initiated animals were promoted with cigarette smoke condensate. I was interested in determining whether DNA methylation alterations could be observed in a dose-and/or time-response and whether alterations in methylation were reversible. Therefore, I examined changes in global, GC-rich, and gene specific methylation patterns in animals promoted with various amounts of CSC for different amounts of time. To determine if there is a relationship between methylation and expression change, I analyzed gene array data and found that *HoxA5* was down-regulated.

HoxA5 is not a well-known tumor suppressor, but recent research has shown that HOXA5 binds to and up-regulates the known tumor suppressor *p53* (Raman et al., 2000). Therefore, decreased expression of *HoxA5* leading to a decrease in *p53* activity could contribute to carcinogenesis. Methylation of the promoter region of *HoxA5* has been shown to be tightly related to expression (Raman et al., 2000; Hershko et al., 2003). I analyzed the methylation status of the same *HoxA5* promoter region to determine if methylation correlates to its CSC-induced down-regulation. I also assessed the methylation status of the well known tumor suppressors *p16* and *MGMT* in tumor tissue.

To examine the value of DNA methylation analysis as a gauge of toxic potential to be used in combination with more traditional assessments of cytotoxicity and genotoxicity, I assessed the methylation patterns of rat hepatoma (H4IIE) cells that were treated with various concentrations of the model compounds camptothecin, 5-fluorouracil, rotenone, and staurosporine. Cytotoxicity assessments, including measurements of ATP and cell number were performed, and cytolethal and non-cytolethal concentrations were determined for each compound in the same manner. Global and GC-rich methylation analysis was performed on cells treated with cytolethal and non-cytolethal concentrations of each compound, and given the cytotoxicity data , genotoxicity data available on toxicity databases, and DNA methylation data, we determined the relative toxic potentials of each model compound to determine if DNA methylation analysis provided useful information. These studies are described in detail in the four chapters that make up the body of this dissertation. Chapters 1 and 2 have been published in *Toxicological Sciences*, and Chapters 3 and 4 will be submitted for publication in the very near future.

Specific Aims

1. To examine the effects of phenobarbital (PB) on DNA methylation in GC-rich regions of hepatic DNA from mice that exhibit different levels of susceptibility to liver tumorigenesis.

    a) To determine if differences in cancer susceptibility are linked to differences in the ability to maintain normal patterns of methylation in response to PB.

    b) To compare methylation status at GC-rich regions with previous measurements of global methylation change.

2. To assess methylation changes during stages of carcinogenesis using an initiation/promotion SENCAR mouse model.

    a) To determine threshold doses of CSC necessary to detect changes in methylation at particular timepoints.

    b) To determine whether changes in methylation at GC-rich regions preceded global decreases or vice versa.

    c) To assess the potential for reversibility of altered methylation in precancerous tissue.

    d) To ascertain whether particular methylation changes correlate to tumor formation.

3. To assess gene-specific methylation patterns and determine how DNA methylation might be correlated to gene expression in an initiation/promotion SENCAR mouse

skin model

a) To identify a tumor suppressor gene that was down-regulated due to treatment with CSC.

b) To determine if methylation status of the promoter region of the down-regulated tumor suppressor identified in a correlated with expression.

c) To determine the methylation status in the promoter region of known tumor suppressor genes in skin tumor tissue.

4. To determine the value of DNA methylation analysis in basic, initial toxicity assessments.

a) To assess global and GC-specific DNA methylation patterns at doses found to be cytolethal and non-cytolethal based on more traditional in vitro toxicity assays (measurements of cell number, ATP, GST, and MTT)

b) To combine cytolethality, genotoxicity and DNA methylation data in order to determine the toxic potential of the model compounds.

c) To determine how DNA methylation analysis contributes to the assessment of the relative estimated toxic potentials of the model compounds.

Experimental models

Mouse liver DNA from the relatively tumor-sensitive C3H/H3 and B6C3F1 mice, as well as from the relatively tumor resistant C57BL/6 strain was used to assess methylation status following a 2 wk treatment with 0.05% w/w PB, a dose known to promote tumors in rodents.

In order to assess methylation changes in the promotion stage of carcinogenesis, I used the SENCAR mouse skin model. Female SENCAR mice were initiated with 75 μg dimethy(*a*)benzanthacene (DMBA) and different doses of cigarette smoke condensate (CSC) promoter were applied for various amounts of time. DMBA is a known initiating agent that forms DNA adducts. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most potent carcinogen in cigarettes, as well as various formulations of CSC have been shown to behave as promoting agents in carcinogenesis (Yano et al., 2001; Gaworski et al., 1999). Animals were sacrificed immediately following completion of the promotion schedule with the exception of recovery group animals that were promoted with 27 mg CSC for 9 wks, and sacrificed following a 9 wk recovery period. These animals were used to ascertain whether changes in DNA methylation are reversible.

H4IIE rat hepatoma cells were used for *in vitro* cytotoxicity assays as well as methylation analysis. These cells are easily maintained and are frequently used in studies of hepatic gene function and *in vitro* toxicity tests (Cockerell et al., 2002; Klemm et al., 1996; Pitot, H.C., 1964). Cells were grown in 96-well plates for cytotoxicity analysis and in 6-well plates in preparation for methylation analysis.

REFERENCES FOR INTRODUCTION, SUMMARY, AND DISCUSSION SECTIONS ARE LISTED ON PAGES 212-226.

CHAPTER 1

Watson, Rebecca E., and Goodman, Jay I. (2002). Effects of phenobarbital on DNA methylation in GC-rich regions of hepatic DNA from mice that exhibit different levels of susceptibility to liver tumorigenesis. *Toxicological Sciences* **68**, 51-58.

ABSTRACT

DNA methylation is an important epigenetic mechanism involved in transcriptional control and altered patterns of methylation might lead to the aberrant gene expression contributing to carcinogenesis. Three groups of mice were used in the current study: the relatively liver-tumor-sensitive C3H/He strain and B6C3F1 stock (C57BL/6 X C3H/He), as well as the relatively resistant C57BL/6 strain. For a two-week period, animals from each group were given drinking water containing a tumor-promoting dose of phenobarbital (PB), a nongenotoxic rodent carcinogen. Methylation-sensitive restriction digests using *HpaII* or *MspI* were followed by PCR amplification using an arbitrary primer or primer pair, binding preferentially to guanine and cytosine (GC) -rich regions of DNA, including CpG islands. This procedure allows for assessment of methylation at the internal and/or external cytosine of the 5'-CCGG-3' sites recognized by *MspI* and *HpaII*. Results with the single primer indicated marked differences in PB-induced hypermethylation at external and internal cytosines of 5'-CCGG-3' sites: C3H/He>>B6C3F1>C57BL/6. Results with the arbitrary primer pair indicated PB-induced hypermethylation at the external cytosine of 5'-CCGG-3' site: B6C3F1> C3H/He, and a low level of hypomethylation at internal and external cytosine sites in C57BL/6. Thus, there was a clear indication of more methylation changes in GC-rich regions of DNA, primarily hypermethylation, in the tumor-sensitive groups of mice in response to PB treatment. Therefore, this study supports our hypothesis that the capacity to maintain normal methylation patterns is related inversely to tumor susceptibility.

INTRODUCTION

DNA methylation plays a key role in the regulation of transcription. In the mammalian genome, approximately 3-5% of cytosine residues are present as 5-methylcytosine, which is often, but not exclusively, within CpG dinucleotides of both promoter and non-promoter regions (Momparler and Bonenzi, 2000; Bird, 1992). CpG-rich stretches of DNA 200 bp or longer with a GC content of 50% or greater are termed CpG islands (Gardiner-Garden and Frommer, 1987). These are commonly found at 5' flanking, promoter regions of genes (Robertson and Jones, 2000).

In general, the density of methylation is related inversely to gene expression (Laird and Jaenisch, 1994). This relationship is particularly commonplace in CpG islands at promoter regions, where DNA methylation may block transcription factors from accessing their cognate *cis* elements and/or indirectly suppress transcription through methylated DNA binding proteins which recruit histone deacetylases, leading to chromatin condensation and subsequent gene silencing (Nan *et al.*, 1998; Jones *et al.*, 1998). In addition, a large amount of CpG methylation at non-CpG island regions is found within foreign DNA elements. Methylation of these CpG dinucleotides is related inversely to the expression of parasitic transposons, and is believed to protect genomic integrity (Robertson and Wolffe, 2000). However, in certain non-promoter regions of imprinted genes there is a direct correlation between increased methylation and gene expression. For instance, methylation of a specific non-promoter region is required for expression of the tumor-suppressor *Igf2r* gene on the maternal allele (Birger et al., 1999). Similarly, methylation of a differentially methylated non-promoter region is necessary for paternal expression of *Igf2* (Bell and Felsenfeld, 2000). Therefore, in examining

changes in DNA methylation and how this might relate to gene expression it is important to consider alterations in the methylation status of guanine and cytosine (GC)-rich sequences in both promoter and non-promoter regions of genes.

Methylation patterns in tumor tissues characteristically exhibit a decrease in global methylation accompanied by some increased methylation in selected regions of DNA (Robertson and Jones, 2000). More specifically, hypermethylation in promoter regions of tumor suppressors, associated with silencing of these genes, is a common finding in cancer (Lin et al., 2001). Hypermethylation and transcriptional silencing of the tumor suppressor *p16* promoter region is seen in B6C3F1 lung adenocarcinomas and other types of cancers (Patel et al., 2000; Esteller et al., 2001a). Similarly, loss of *pRb* expression in pituitary adenocarcinomas has been shown to be associated with methylation of *RB1* promoter regions (Simpson et al., 2000). Additionally, hypomethylation also plays an important role in carcinogenesis. Hypomethylation may facilitate aberrant gene expression of *raf* and other oncogenes normally silenced by methylation (Ray et al., 1994). Furthermore, hypomethylation could lead to a decrease in genomic integrity by reducing the methylation-mediated silencing of foreign genomic elements (Laird, 1997).

Consistent with the observation of global hypomethylation in tumor samples and precancerous lesions as compared to normal tissue (Gama-Sosa et al., 1983), studies in our laboratory have shown that hepatic DNA samples from mice which received a tumor promoting dose of the rodent carcinogen phenobarbital (PB) have lower levels of global methylation compared to controls. This difference is substantially more prominent in the

relatively tumor-sensitive C3H/He and B6C3F1 (C57BL/6 X C3H/He) mice than in the relatively tumor-resistant C57BL/6 strain (Counts et al., 1996).

In the current study we have extended our analysis of global PB-induced methylation change in these three groups of mice varying in tumor susceptibility to examine the status of methylation in selected regions of DNA, i.e. GC-rich sequences. Assessment of methylation status was performed using an arbitrarily-primed PCR approach. The primers used were designed to bind to GC-rich sequences that are particularly prevalent at CpG islands (Gonzalgo et al., 1997). PB induced an increase in methylation in GC-rich regions which was more pronounced in the relatively tumor sensitive C3H/He and B6C3F1mice than in the relatively tumor resistant C57BL/6 strain. While our previous investigations indicated that global hypomethylation occurs as a result of PB treatment and the current study detects hypermethylation in GC-rich sequences, results are quite compatible and, indeed, complementary. The important point is that a variety of alterations in methylation might facilitate carcinogenesis and the methylation patterns of PB-treated C3H/He and B6C3F1 animals deviate more from their control counterparts than what is seen in the C57BL/6 strain.

MATERIALS AND METHODS

Animals

Male C57BL/6, C3H/He and B6C3F1 (C57BL/6 X C3H/He) mice were obtained from Charles River Laboratories (Wilmington, MA). All animals were 43-63 days old, and weighed 22-24 g. Animals were housed in a temperature-controlled environment and given food and water *ad libitum*. Treatment animals were given a tumor-promoting dose of PB (0.05% w/w) in the drinking water for a two-week period. Animals were sacrificed by CO_2 asphyxiation, and their livers were snap-frozen at –80°C.

DNA Isolation and Restriction Digests

DNA was extracted by a phenol/chloroform procedure (Strauss, 1990). For each DNA sample, two restriction digests were performed: one with *RsaI* and *MspI* and one with *RsaI* and *HpaII*. All enzymes used were from Boehringer-Mannheim. *RsaI* is methylation-insensitive, while *MspI* and *HpaII* are methylation-sensitive. Both *MspI* and *HpaII* cut between cytosine residues at 5'-CCGG-3' sites. *MspI* will not cut if the external cytosine is methylated, and *HpaII* will not cut if the internal cytosine is methylated. (Mann and Smith, 1977). Restriction digests were performed with 1 μg of DNA and 5.0 units of *RsaI* in Boehringer-Mannheim buffer L. After a 1 h incubation with shaking in a water bath at 37°C, two, 2.5 unit aliquots of *MspI* or *HpaII* were added, 2 h apart. The total incubation time was 18 h. The enzymes were inactivated by a 10 min incubation at 65°C and the digests were stored at 4°C until use.

Arbitrarily primed (AP)-^{33}P PCR

PCR was performed on restriction digests using a single arbitrary primer, 5'-AACCCTCACCCTAACCCCGG-3', or a combination of arbitrary primers: 5'-AACCCTCACCCTAACCGCGC-3' and 5'-AACCCTCACCCTAACCCGCG-3' (Gonzalgo et al., 1997). While both the single primer and the primer pair were designed to bind CpG rich regions of DNA, they do not bind identical regions of DNA, thus PCR products produced are distinct. Each PCR sample was prepared in a sterile laminar flow hood on ice with appropriate negative no DNA template controls. Reactions were composed of 10 µl of the restriction digest (containing 1 µg digested DNA), 0.4 mM each primer, 2.5 units of Taq polymerase (Gibco BRL), 1.5 mM $MgCl_2$, 60 mM Tris, 15 mM ammonium sulfate, 3.3 µCi ^{33}P (New England Nuclear), and glass-distilled water to volume. Samples were heated for 5 min at 94°C before addition of dNTPs to minimize the possibility of primer-dimer formation. Cycling conditions included a single denature cycle for 2 min at 94°C, followed by 5 cycles of the following conditions: 30 s. at 94°C, 1 min at 40°C, 1.5 min at 72°C; then 40 cycles of 94°C for 30 s. 55°C for 15 s. and 72° for 1 min., a time delay cycle for 5 min at 72°C, and a soak cycle at 4°C. The 40 cycle run was used in order to maximize the opportunity to amplify regions of interest. PCR products (5 µl from each reaction) were separated on a Stratagene Castaway Precast Sequencing Gel (6% polyacrylamide, 1X TBE, 7M urea) at 50W. The gel was soaked for 10 minutes in a fixing solution containing 5% acetic acid and 5% methanol, then rinsed for 10 minutes in glass-distilled water. The gels were dried and exposed to a Kodak phosphoimage screen. A short exposure of 2-5 days, followed by a longer exposure of 5-9 days was performed for each gel. Images from short and long exposures were analyzed

separately. This procedure led to the separation of PCR products ranging from approximately 75-1500 bp. as verified by separating a labeled DNA marker on the same type of gel under identical conditions.

Analysis of Phosphoimages

Phosphoimages were analyzed with a Molecular Dynamics phosphoimager and Quantity One software (Bio-Rad). Banding patterns of ^{33}P-PCR product phosphoimages were examined to determine the methylation status at external and internal cytosines at 5'-CCGG-3' sequences. Segments of DNA between or at sites of primer annealing are amplifyable by PCR unless a site within the region is cut with *HpaII* or *MspI*. Thus, bands seen in both *MspI* and *HpaII* digest lanes are indicative of the absence of unmethylated 5'-CCGG-3' sites. Bands present in *HpaII* digest lanes but not in *MspI* digest lanes represent methylation of the internal cytosine of a 5'CCGG 3' site. Conversely, bands seen more prominently in *MspI* digested lanes are indicative of methylation of the external cytosine. A hypothetical example is presented in Figure 1. We considered a control vs. treatment group difference of 1-3, 4-6, and >6 bands seen in the *MspI* or *HpaII* lanes of one group which are either not present or seen less distinctly in the other as a small, moderate and large amount of methylation change, respectively. Data from Quantity One were exported to Excel where the percent intensity/ total intensity of the lanes were calculated and graphed using SPSS Sigma Plot 2000.

RESULTS

Phosphoimages of ^{33}P-PCR products from PB-treated and untreated samples from C57BL/6, C3H/He, and B6C3F1 using the single arbitrary primer are represented in Figure 2a, b, and c, respectively. These results are tabulated in Table 1. In the tumor-resistant C57BL/6 strain (Figure 2a), the banding pattern of untreated and PB-treated samples is similar. However, in each phosphoimage shown there are 2 bands seen in the PB-treated *RsaI/MspI*-digested samples, and in one phosphoimage there is 1 band seen in the PB-treated *RsaI/HpaII*-digested samples that are seen less distinctly in the untreated samples. This is indicative of a small amount of PB-induced hypermethylation at the external and internal cytosine of 5'-CCGG-3' sites. In the tumor-prone C3H/He strain (Figure 2b), the banding pattern in the PB-treated samples is more markedly different as compared to the untreated samples. There were 5-9 bands seen in the *RsaI/MspI* and *RsaI/HpaII* lanes of PB-treated samples not seen in the untreated samples. These data are indicative of hypermethylation at numerous external and internal cytosines in 5'-CCGG-3' sites in the PB-treated C3H/He mice. The banding pattern of the B6C3F1 phosphoimage (Figure 2c) was more similar to C3H/He than C57BL/6; a moderate amount of hypermethylation was seen at the external cytosine site of PB-treated samples. Phosphoimages of ^{33}P-PCR products from PB-treated and untreated samples from C57BL/6, C3H/He, and B6C3F1 using the arbitrary primer pair are represented in Figure 3a, b, and c, respectively. These results are tabulated in Table 2. In each group of PB-treated v. control mice, fewer differences were observed using the arbitrary pair of primers as compared to the data derived from the single arbitrary primer. This is likely to be due to the difference in PCR products produced by using the single primer vs. the

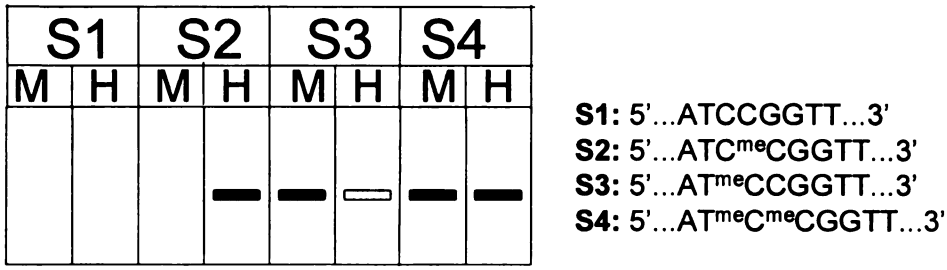


Figure 1. Illustration of an analysis of arbitrarily primed ^{33}P-PCR hypothetical results. The correlation between hypothetical bands seen in *RsaI/MspI* (M) and *RsaI/HpaII* (H) digest lanes and the methylation status of a 5'-CCGG-3' sequence located between primer annealing sites is presented. Samples 1-4 (S1-S4) each represent one of the four patterns of cytosine methylation possible at a 5'-CCGG-3' sequence. In S1, neither cytosine is methylated. Thus, both *MspI* and *HpaII* will cleave the CCGG sequence and no bands will be seen in either the *MspI* or *HpaII* digest lanes. In S2, the internal cytosine is methylated so that *HpaII* cannot cleave, but *MspI* can (*MspI* cleaves regardless of the methylation status of the internal cytosine). In S3, the external cytosine is methylated so that *MspI* cannot cleave and *HpaII* cleaves at a much reduced rate, resulting in an intense band in the *MspI* digest lane, and a faint band in the *HpaII* digest lane. In S4, both cytosines are methylated and neither enzyme is able to cleave; therefore, bands are seen in both the *MspI* and *HpaII* digest lanes.

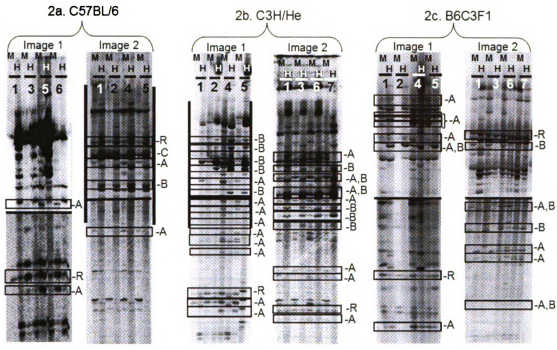


Figure 2. Methylation status of GC-rich regions of hepatic DNA in C57BL/6 (a), C3H/He (b), and B6C3F1 (c) mice was assessed by ^{33}P-PCR using a single arbitrary primer. *RsaI/MspI* and *RsaI/HpaII* digests are presented in lanes indicated by and **M** and **H**, respectively. Numbers underneath the bars on the top of the gel indicate individual animals. For a, b, and c: animals 1-3 were untreated, and animals 4-6 or 7 were PB-treated. Thus, data shown is representative of 3 untreated and 3-4 treated animals. Analysis was repeated for 2 controls and 1-2 treated animals from each group to test for reproducibility. Two digests were performed per DNA sample. '
A' indicates a row of bands where the M lanes of treated animals are more prominent than in the controls, 'B' indicates a row of bands where the H lanes of treated animals are more prominent than in the controls and 'C' indicates a row of bands where the M lanes of treated animals are less prominent than in the controls. 'R' indicates a reference row of bands that are reasonably constant and highlighted to illustrate loading differences.

Sections of images above and below the thick black line approximately at the middle of each image are of the same gel; however, the exposure times were different in order to better visualize the separated PCR products. The lower portion represents a longer, 7-9 d exposure while the upper portion represents a shorter, 2-5 d exposure. The vertical heavy black lines alongside phosphoimages in 2a and 2b indicate regions subjected to image analysis and represented graphically in Figure 4.

Table 1. Summary of Phosphoimage Data from PB-Treated and Untreated PCR Samples Using the Single Arbitrary Primer

Strain/stock	Number of 'A' rows		Number of 'B' rows		Number of 'C' rows	
	Image 1	Image 2	Image 1	Image 2	Image 1	Image 2
C57Bl/6	2	2	0	1	0	1
C3H/He	9	7	5	6	0	0
B6C3F1	7	4	1	4	0	0

Note. A rows are rows of bands where the *RsaI/MspI* lanes of treated animals are more prominent than in the controls. B rows are rows of bands where the *RsaI/HpaII* lanes of treated animals are more prominent than in the controls. C rows are rows of bands where the *RsaI/MspI* lanes of treated animals are less prominent than in the controls.

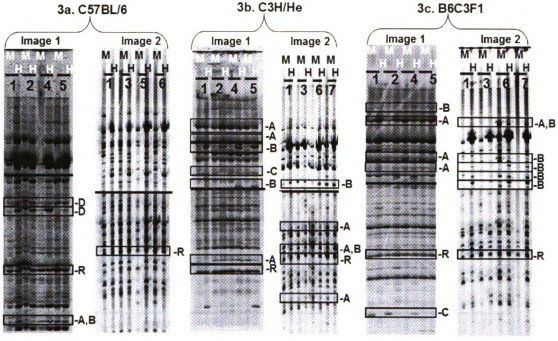


Figure 3. Methylation status of GC-rich regions of hepatic DNA in C57BL/6 (a), C3H/He (b), and B6C3F1 (c) mice was assessed by ^{33}P-PCR using an arbitrary primer pair. *RsaI/MspI* and *RsaI/HpaII* digests are presented in lanes indicated by and **M** and **H**, respectively. Numbers underneath the bars on the top of the gel indicate individual animals. For a, b, and c: animals 1-3 were untreated, and animals 4-6 or 7 were PB-treated. Thus, data shown in representative of 3 untreated and 3-4 treated animals. Analysis was repeated for 2 controls and 1-2 treated animals from each group to test for reproducibility. Two digests were performed per DNA sample. 'A' indicates a row of bands where the M lanes of treated animals are more prominent than in the controls, 'B' indicates a row of bands where the H lanes of treated animals are more prominent than in the controls, 'C' indicates a row of bands where the M lanes of treated animals are less prominent than in the controls, and 'D' indicates a row of bands where the H lanes of treated animals are less prominent than in the controls. 'R' indicates a reference row of bands that are reasonably constant and highlighted to illustrate loading differences.

Sections of images above and below the thick black line approximately at the middle of each image are of the same gel; however, the exposure times were different in order to better visualize the separated PCR products. The lower portion represents a longer, 7-9 d exposure while the upper portion represents a shorter, 2-5 d exposure.

Table 2. Summary of Phosphoimage Data from PB-Treated and Untreated PCR Samples Using the Arbitrary Primer Pair

Strain/stock	Number of 'A' rows		Number of 'B' rows		Number of 'C' rows		Number of 'D' rows	
	Image 1	Image 2	Image 1	Image 2	Image 1	Image 2	Image 1	Image 2
C57Bl/6	1	0	1	0	0	0	2	0
C3H/He	3	3	2	2	1	0	0	0
B6C3F1	2	1	1	5	1	0	0	0

Note. 'A' rows are rows of bands where the *RsaI/MspI* lanes of treated animals are more prominent than in the controls. 'B' rows are rows of bands where the *RsaI/HpaII* lanes of treated animals are more prominent than in the controls. 'C' rows are rows of bands where the *RsaI/MspI* lanes of treated animals are less prominent than in the controls. 'D' rows are rows of bands where the *RsaI/HpaII* lanes of treated animals are less prominent than in the controls.

also a very small amount of hypermethylation at the internal and external cytosine sites seen on one of the C57BL/6 phosphoimages. A small and moderate amount of hypermethylation was seen in the internal cytosines of C3H/He (Figure 3b) and B6C3F1 (Figure 3c), respectively. A small amount of hypermethylation at the external cytosine was seen in both B6C3F1 and C3H/He. Thus, using both the single arbitrary primer and the arbitrary primer pair, a greater amount of hypermethylation was seen in the PB-treated animals of the tumor-sensitive C3H/He and B6C3F1 mice as compared to what was seen in the C57BL/6 strain.

In order to correct for differences in the overall lane intensities, the percent intensity of radioactive signal adjusted for the overall lane intensity was ascertained (Figure 4 and Figure 5). In agreement with the data presented in Figure 2, these data indicate that there is a greater amount of PB-induced hypermethylation in the tumor-sensitive C3H/He strain as compared to the tumor-resistant C57BL/6 strain.

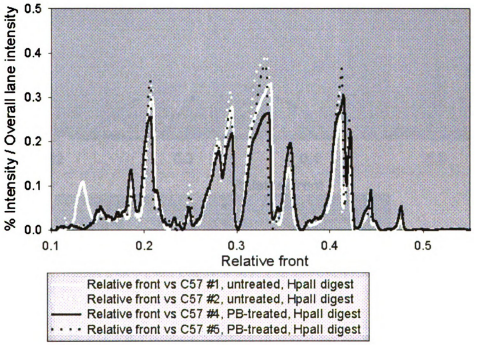


Figure 4. Image analysis of regions of the C57BL/6 phosphoimage presented in Figure 2a. This graph illustrates the intensity of the ^{33}P signal for points down the length (relative front) of each *HpaII* lane in the images after subtracting the lane backgrounds and thus correcting for differences in overall lane intensities. The pixel intensity is plotted against the relative front of each lane. Increased methylation is reflected as a greater pixel intensity.

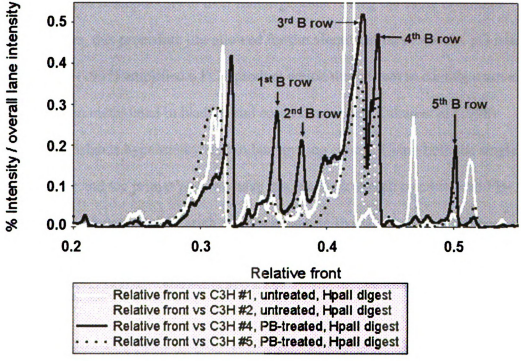


Figure 5. Image analysis of regions of the C3H/He phosphoimage presented in Figure 2b. This graph illustrates the intensity of the 33P signal for points down the length (relative front) of each HpaII lane in the images after subtracting the lane backgrounds and thus correcting for differences in overall lane intensities. Peaks corresponding to the first 5 'B' rows from the top of the image shown in Figure 2b are indicated. The pixel intensity is plotted against the relative front of each lane. Increased methylation is reflected as a greater pixel intensity. This is seen more prominently in the tumor sensitive C3H/He mice than in the C57BL/6 mice (See Figure 4).

DISCUSSION

An arbitrarily-primed PCR approach has enabled us to examine PB-induced methylation changes at GC-rich regions of DNA, including CpG islands, in three groups of mice varying in susceptibility to liver tumorigenesis. Using the same or similar arbitrary primers, this procedure has allowed for the identification of novel CpG islands. Gonzalgo et al. (1997) amplified a PCR product which was shown to contain a novel CpG island often methylated in bladder and colon tumors and Kohno et al (1998) identified CpG islands hypermethylated in human lung cancer. Using both the single arbitrary primer and the primer pair, we have discerned an overall trend toward PB-induced hypermethylation of GC-rich regions of mouse liver DNA which is more pronounced in the relatively tumor-susceptible C3H/He and B6C3F1 mice compared to the relatively resistant C57BL/6.

Highly methylated sequences in promoter regions inhibit transcription through methylated DNA-binding proteins which interfere with the binding of transcription factors to their cognate cis elements. Two such proteins, MeCP1 and MeCP2, have been found to bind preferentially to methylated DNA and repress transcription *in vivo* and *in vitro* (Bird & Wolffe, 1999). Furthermore, MeCP2 has been found to co-immunoprecipitate with Sin3A, a protein that interacts with histone deacetlyase (Bird & Wolffe, 1999). Therefore, methylated regions may be more apt to become deacetylated, and the associated regions become more tightly wrapped around the histones, making transcription less probable. Hypermethylation at GC-rich sequences in promoter regions has been shown to be linked to the silencing of several known tumor suppressor genes,

including *p16* (Esteller et al., 1999), *p14* (Esteller et al., 2001b), and *O^6-methylguanine-DNA methyltransferase* (Patel et al., 2000), in a variety of cancers.

Previous studies performed in our laboratory examined global, average methylation status in B6C3F1 and C57BL/6 mice and demonstrated a greater amount of global hypomethylation in hepatic DNA of PB-treated animals as compared with the C57BL/6 strain (Counts et al., 1996). However, a limitation of this earlier study is the fact that the methodology employed did not permit us to discern treatment-induced increased methylation in particular regions of DNA. The arbitrarily-primed PCR procedure used in the current investigation is advantageous because it extends the analysis of methylation change by allowing for the detection of specific methylation alterations in GC-rich regions of the genome. This analysis aids in understanding the multitude of effects of tumor promoters on methylation.

A unique feature of the current study is that through the use of the methylation-sensitive enzymes *MspI* and *HpaII* we were able to detect hypermethylation occurring at both the internal and external cytosines of the 5'-CCGG-3' site. While most methylation reported to occur in mammals is located within the symmetrical dinucleotide CpG, CpNpG and has been shown to occur in mammalian cells (Stirzaker et al., 1997; Clark et al., 1997; Clark et al., 1995; Ray et al., 1994). Using the single arbitrary primer, a greater or equivalent amount of PB-induced ^{me}CpCpG compared to ^{me}CpG was found in B6C3F1 and C3H/He. Using the arbitrary primer pair, there was no distinct difference detected in the amount of methylation at CpG and CpCpG sites. Such a difference may be more difficult to detect with the primer pair because there was less overall variation between band patterns of treated vs. control samples.

It is possible that mechanisms, in particular the methyltransferases, responsible for maintenance of methylation at CpG and non-CpG sites vary. In mammalian embryonic stem cells, the level of de novo methyltransferase Dnmt3a has been correlated with the presence of non-CpG methylation (Ramsahoye et al., 2000). When treated with certain tumor promoters, specific methylases may be more or less affected than others leading to differences in the level of methylation change at CpG and non-CpG sites.

A tumor-promoting dose of PB has been shown to lead to an increase in liver cell proliferation 1-2 weeks after administration as well as a decrease in the levels of S-adenosyl methionine (SAM), the co-factor for methylation reactions (Shivapurkar and Poirier, 1982). These effects vary between C57BL/6 and B6C3F1. While the increase in liver cell proliferation is more marked in C57BL/6, the decrease in global methylation is more prominent in B6C3F1. Hepatic DNA from B6C3F1 mice also exhibits a higher level of global hypomethylation following a choline/methionine deficient diet (Counts et al., 1996).

Genetic differences between strains of mice are likely to contribute to these variations in the ability to maintain patterns of DNA methylation. The lifetime rate of spontaneous tumor formation is less than 5% in C57BL/6 mice, but up to 80% in C3H/He mice (Buchmann et al., 1991;Grasso and Ginsler, 1975). C3H/He mice are 20-50X more susceptible than C57BL/6 mice to induction of cancer by the carcinogens N-diethylnitrosamine (Drinkwater and Ginsler, 1986) and N-ethyl-N-nitrosourea (Hanigan et al., 1988). It has been proposed that multiple susceptibility loci within an *Hcs* (hepatocarcinogen sensitivity site) account for approximately 85% of this difference in sensitivity (Drinkwater et al., 1986). Furthermore, mutational activation of *Ha-ras* is

more often seen in C3H/He and B6C3F1 tumors than in C57BL/6 (Buchmann et al., 1991) and strain differences in the methylation status of this gene may play a role in the expression of this oncogene (Counts and Goodman, 1994).

Several factors are involved in maintaining methylation, including the availability of methyl groups, methylation co-factors including SAM, and the utilization of methyl groups by methyltransferases and demethylases. Dnmt 1 is believed to act primarily as a maintenance methylase (although it also may act as a de novo methylase) (Bestor et al., 1988) while Dnmt 3a/3b is believed to act as a de novo methylase (Okano et al., 1999). A genetic variation within the multiple susceptibility loci which affects any part of any one of these factors could have a large effect on the capacity to maintain normal patterns of methylation. For instance, a strain difference in the activity of a particular methyltransferase could potentially lead to the observed changes in global and regional methylation, both of which are more markedly altered in the tumor sensitive C3H/He and B6C3F1 mice than in the tumor sensitive C57BL/6. It is possible that Dnmt1, Dnmt3, and perhaps other methyltransferases compete for and/or have some specificity for methylation at particular regions of DNA. If a predominantly globally-acting methyltransferase in C3H/He and B6C3F1 was more sensitive to dietary and PB-challenge than C57BL/6, compensatory expression of a methyltransferase acting preferentially at CpG islands may explain why we see PB induced global methylation and CpG island hypermethylation predominantly in these mice.

The results reported here in combination with previous studies on global levels of methylation (Counts et al, 1996) indicate that specific hypermethylation in GC-rich regions of DNA in response to a tumor-promoting dose of PB occurs concurrently with a

decrease in global levels of methylation. Both hyper- and hypomethylation contribute significantly to carcinogenesis and, importantly, the simultaneous occurrence of these events is not mutually exclusive (Counts and Goodman, 1995). Indeed, altered methylation might be viewed as a secondary mechanism underlying carcinogenesis (Goodman and Watson, 2002). Furthermore, the capacity of PB to affect DNA methylation is greater in the tumor sensitive C3H/He and B6C3F1 mice as compared to the relatively resistant C57BL/6 strain. Taken together, these sets of data support the hypothesis that susceptibility to carcinogenesis might be related inversely to the capacity to maintain normal methylation patterns.

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CHAPTER 2

Watson, Rebecca E, Curtin, Geoff M., Doolittle, David J., and Goodman, Jay I. (2003). Progressive alterations in global and GC-rich DNA methylation during tumorigenesis. *Toxicological Sciences* **75**, 289-299.

ABSTRACT

DNA methylation plays a key role in the regulation of gene expression, and failure to maintain normal patterns of methylation often contributes to carcinogenesis. We have characterized progressive methylation changes during the promotion stage of carcinogenesis using a SENCAR mouse skin initiation/promotion tumorigenesis model. Mice were initiated with a dermal application of 75 μg dimethylbenz[a]anthracene (DMBA) and promoted with 9, 18, 27, and 36 mg cigarette smoke condensate (CSC) thrice weekly for time periods up to 29 wks, when a large increase in tumor number was produced by the highest three doses. Global and GC-specific methylation were assessed using *SssI* methylase and arbitrarily primed PCR, respectively. Changes in GC-specific methylation were dose- and time-dependent. CSC doses required to detect these changes were 27 mg at 6 wks and 18 mg at 9 wks. This effect appears to be reversible; changes in GC-specific methylation were less marked after 9 wks promotion with 27 mg CSC followed by 6 wks recovery in comparison to 9 and 15 wks promotion with 27 mg CSC and no recovery period. Both tumor and non-tumor tissue promoted with 27 mg CSC for 29 wks exhibited changes in GC-specific methylation that were more pronounced in tumors. Tumor tissue was globally hypomethylated whereas non-tumor tissue did not exhibit changes in global methylation. In conclusion, as expected for a mechanism underlying tumor promotion, CSC alters methylation in a threshold-exhibiting, reversible, progressive fashion during promotion. Progressive alterations in global and GC-rich methylation appear to be mechanistically important during tumor promotion.

INTRODUCTION

Carcinogenesis is a multistage, multistep process with three experimentally defined stages: initiation, promotion, and progression (Pitot and Dragan, 1994). Initiation involves a heritable alteration to the genome of a normal cell that provides a selective growth advantage over surrounding cells in response to promoting agents. Initiation is thought to be irreversible and due to mutation; however, epigenetic factors may also play a role (Goodman and Watson, 2002). During promotion, initiated cells clonally expand and increasingly aberrant subclones develop. Promoting agents might facilitate this expansion by increasing the proliferation rate and/or decreasing the rate of apoptosis in these cells (Schulte-Hermann et al., 1990). The promotion stage is reversible, and the dose-response relationship for promoters exhibits a threshold (Pitot and Dragan, 1994; Goodman, 2001). In progression, continued subclone expansion no longer requires a promoting agent, and more extensive alterations to the genome such as chromosomal damage and aneuploidy are observed. (Pitot and Dragan, 1994).

Changes required for the basic cancer phenotype include evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential and sustained angiogenesis (Hanahan and Weinberg, 2000). While these events might occur via mutation, epigenetic events can play a fundamental role in carcinogenesis (Watson and Goodman, 2002a). Epigenetic regulation of gene expression occurs through heritable transcriptional modulation superimposed on the primary DNA sequence. Thus, epigenetic mechanisms such as DNA methylation, i.e. the 5-methylcytosine content of DNA, have the capacity to change transcriptional levels without changing the sequence (Holliday, 1994). Genes commonly

found to have altered transcriptional levels in cancer, such as the often underexpressed tumor suppressor *p53* and the often overexpressed oncogene *ras*, can be altered by mutation or epigenetic mechanisms (Hosaka et al., 2002). Importantly, a mutated oncogene needs to be expressed in order to contribute to carcinogenesis (Hahn et al., 1999), and the expression level might be governed by epigenetic mechanisms.

Methylation facilitates a remodeling of chromatin to an inactive state. Increased methylation in GC-rich promoter regions of genes is generally associated with decreased transcription and vice-versa (Ballestar and Esteller, 2002). Much of the promoter-specific methylation occurs at CpG islands, 200 bp or longer stretches of DNA with a 50% or greater GC content and a higher than expected CpG content (Gardiner-Garder and Frommer, 1987). In several types of cancers, increased methylation in the promoter regions of tumor suppressors such as *p16, E-cadherin,* and *O^6-methylguanine DNA methyltransferase* (*MGMT*) is associated with, and believed to be the cause for, decreased expression of these genes (Esteller et al., 2001). Both hyper- and hypomethylation may contribute to carcinogenesis via silencing of tumor suppressor genes, upregulation of oncogenes, and/or decreased genome stability (Goodman and Watson, 2002; Counts and Goodman, 1995). Tumors characteristically exhibit increases in methylation at GC-rich regions with a decreased overall, or global methylation (Gama-Sosa et al., 1983) that can facilitate oncogene expression. Changes in methylation precede tumor formation, indicating that these alterations might contribute to tumorigenesis (Robertson and Jones, 2000). There has been limited research on mouse skin methylation, but a few reports indicate methylation differences between normal and tumor skin tissue (Winter et al., 1990; Ramsden et al., 1985).

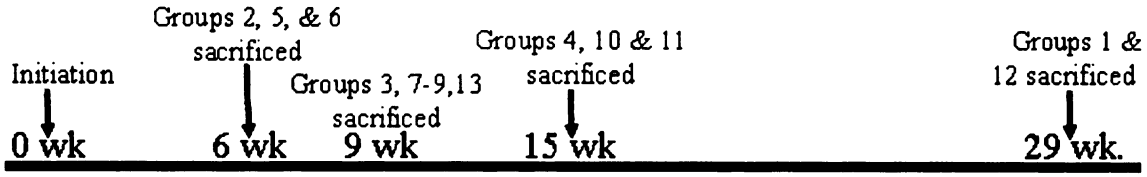
We have examined both global and GC-specific methylation using a SENCAR mouse skin initiation/promotion model of tumorigenesis. The SENCAR mouse stock was generated in the 1960's and 1970's from selective breeding of mice sensitive to epidermal papilloma formation in response to 7,12-dimethylbenz[a]anthracene (DMBA) initiation and the croton oil (containing TPA) promoting agent (Stern and Conti, 1996). These mice are extremely sensitive to carcinogenesis, and generally respond more rapidly and uniformly to the induction of skin tumors than other available strains or stocks. Importantly, the initiation and promotion stages are clearly demarcated, thus facilitating the study of biochemical and molecular mechanisms involved in a particular stage of carcinogenesis (Slaga et al., 1996).

SENCAR mice were initiated with a dermal application of DMBA, followed by administration of various doses of cigarette smoke condensate (CSC), a presumptive tumor promoter, for different lengths of time. We are testing the hypothesis that specific types of methylation alterations play a role during the promotion stage of carcinogenesis. Four specific aims were addressed: 1) to assess methylation status during tumorigenesis in this classic two-stage model system, 2) to ascertain whether particular methylation changes correlate to tumor formation in a sequential fashion, 3) to determine whether changes in methylation exhibit a dose-response relationship with regard to promoter treatment, and 4) to assess the potential for reversibility of altered methylation in precancerous tissue.

MATERIALS AND METHODS

Animals

Weanling female SENCAR mice were obtained from the National Cancer Institute, at the Frederick Cancer Research and Development Center (Frederick, Maryland). Mice (5-7 wks of age at receipt) were allowed 2 weeks to acclimate to the testing environment, and then randomly assigned to treatment groups according to body weight (Figure 1). To ensure groups of similar mean body weight, all groups were compared by ANOVA and least significant difference criteria, and were demonstrated not to be significantly different at a 5 percent, two-tailed assumption. Animals were housed and cared for in accordance with the Institute of Laboratory Animal Resources (ILAR), Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Experimental animals were initiated with either 75 μg DMBA or acetone (vehicle control). Initiation was followed by thrice-weekly CSC promotion at concentrations of 0, 9, 18, or 27 mg CSC per application (in acetone) for 6 or 9 wks or a concentration of 27 mg for 15 or 29 wks. Another group of 5-7 wk old SENCAR mice were initiated with 75 μg DMBA or acetone and promoted with 36 mg CSC for 29 wks in the same laboratory during this same time period for a concurrent study. Though we did not use tissues from this last group, we do present tumor incidence data from these animals (Figure 7). Following completion of the exposure regimen, animals were euthanized with 70% CO_2, and skin collected from the chemical application site was snap-frozen at –80°C until use. DNA was isolated by a phenol/chloroform procedure as described in Strauss, 1990.



Group #	Initiator (+/-)	Promoter dose (mg)	Duration of treatment (wks)	Sacrifice time (wks)
1	-	-	N/A	29
2	+	-*	6	6
3	+	-*	9	9
4	+	-*	15	15
5	+	18	6	6
6	+	27	6	6
7	+	9	9	9
8	+	18	9	9
9	+	27	9	9
10	+	27	9	15
11	+	27	15	15
12	+	27	29	29
13	-*	27	9	9

Figure 1. Experimental design indicating control and CSC-promoted groups of SENCAR mice used in this study. Initiation was performed with a single dermal application of 75 μg DMBA. Promotion with CSC (cigarette smoke condensate) was performed with thrice weekly dermal applications of the various doses indicated. The duration of treatment and sacrifice time are presented. There were at least 7 animals in each group.
+ indicates initiation, -* indicates acetone administration.

Preparation of Cigarette Smoke Condensate (CSC)

Cigarettes supplied by R. J. Reynolds were conditioned to the laboratory environment (64.4-78.8°F and 30-70% relative humidity) and smoked using modified AMESA smoke generators operated under Federal Trade Commission standard conditions. Mainstream smoke collected from the vacuum port of the smoke machines was delivered to a condensate collection system that consisted of glass-filled impingers maintained at temperatures approximating -10°C, -50°C and -70°C. Condensate was extracted from the glass beads using high purity acetone, then subjected to rotary evaporation to reduce acetone and water content; this procedure was designed to yield a condensate sample with a total water content approximating 8%. To reduce variation in condensate composition, several daily condensate collections were combined to create a "pooled condensate" sample. CSC dosing solutions were prepared as needed by serial dilution of the pooled condensate using an "8% water in high purity acetone solution" to create dosing solutions of 45, 90, and 135 mg "tar"/mL. Both the pooled condensate and the prepared CSC dosing solutions were stored in amber glass bottles at -20 ± 4°C.

Global DNA methylation analysis: *SssI* methylase assay

SssI methylase utilizes S-adenosyl methionine as a methyl group donor to methylate the 5' position of cytosine at unmethylated CpG sites in DNA. Thus, the level of global DNA methylation can be determined by the amount of tritiated methyl groups from [^{3}H-CH_3] S-adenosyl-L-methionine incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the original degree of

methylation. DNA (1 μg) was incubated with 2 μCi [^{3}H-CH_3] S-adenosyl-L-methionine (New England Nuclear, Boston, MA) and 3 units of *SssI* methylase (New England Biolabs, Beverly, MA) for 1 h at 30°C. Results are presented as counts per minute per microgram (cpm/μg) DNA. Five replicates were performed per sample. Graphical presentation was performed using Excel®. Statistical analysis was performed with Excel using 2-tailed t-tests to compare the average cpm/μg DNA measurements between treatment groups and controls. A p value of < 0.05 was considered statistically significant.

Methylation analysis of GC-rich regions

Restriction digests

For each DNA sample, 3 restriction digests were performed as follows: *RsaI* alone, *RsaI* and *MspI*, and *RsaI* and *HpaII*. *RsaI* is a methylation-insensitive enzyme used to cut the DNA into smaller fragments. Both *MspI* and *HpaII* are methylation-sensitive enzymes that cut between cytosine residues at 5'-CCGG-3' sites. *MspI* will not cut if the external cytosine is methylated, while *HpaII* will not cut if the internal cytosine is methylated; both will cut if the site is unmethylated (Mann and Smith, 1977). All enzymes used were from Roche (Indianapolis, IN). Restriction digests were performed with 1 μg of DNA and 5.0 units of *RsaI* in Roche buffer L. After a 1 h incubation (with shaking) in a water bath at 37°C, two 2.5 unit aliquots of *MspI* or *HpaII* were added, 2 h apart. The total incubation time was 18 h. The enzymes were inactivated by a 10 min incubation at 65°C, and the digests were stored at 4°C until use.

Arbitrarily primed (AP)-[^{33}P] PCR

PCR was performed on restriction digests using a single primer (5'-AACCCTCACCCTAACCCCGG-3') that arbitrarily binds within GC-rich regions of DNA (Gonzalgo *et al.*, 1997). Reactions were composed of 5 µl of the restriction digest (containing 1 µg digested DNA), 0.4 µM each primer, 1.25 units of Taq polymerase (Gibco BRL, Rockville, MD), 1.5 mM $MgCl_2$, 60 mM Tris, 15 mM ammonium sulfate, 1.65 µCi α-[^{33}P]-dATP (New England Nuclear, Boston, MA), and glass-distilled water to volume. Samples were heated for 5 min at 94°C before addition of dNTPs in order to minimize the possibility of primer-dimer formation. Cycling conditions included a single denature cycle for 2 min at 94°C, followed by 5 cycles under the following conditions: 30 s at 94°C, 1 min at 40°C, 1.5 min at 72°C; then 30 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min, a time delay cycle for 5 min at 72°C, and a soak cycle at 4°C. PCR products (5 µl of each) were separated on a 6 % polyacrylamide sequencing gel at 45 watts for 2 ¼ -2 ½ h. The gel was soaked for 10 min in a fixing solution with 5% acetic acid and 5% methanol, rinsed for 10 min in glass-distilled water, dried, and placed into a cassette with a storage phosphoimage screen to visualize labeled PCR products. Compared to larger DNA fragments on the upper halves of gels, smaller fragments on the lower halves of gels sometimes required a longer exposure to clearly discern bands. Thus, a short exposure of 3 d followed by a longer exposure of 8 d was often performed on a gel. Phosphoimages were analyzed using Quantity One® Bio-Rad software.

Quantification of band intensity

Regions of the phosphoimages in which bands appear more or less prominently compared to controls were boxed and numbered. Bands within these regions were outlined and measured for pixel number and intensity using NIH image. The total pixel intensity units for each band were obtained by multiplying the pixels in the band by the mean intensity units within the outlined region. Reference rows of bands (R) with reasonable lane-to-lane consistency were chosen to represent lane-to-lane background and/or loading differences. In order to compensate for any differences in lane background levels, the ratio of band intensity for each numbered region to the band intensity within the corresponding lane's reference (R) region was determined. Ratio fold change differences between CSC-promoted and control animals were calculated by dividing the ratios of the CSC-promoted animals by the ratios of the control animals in corresponding regions.

RESULTS

Global methylation was assessed for non-tumor tissue from treatment groups 1, 4, 9, and 11, and tumor and non-tumor tissue from treatment group 12 (Figures 1 and 2). Only the tumor samples from treatment group 12 exhibited a statistically significant difference in the level of global methylation compared to untreated controls (group 1). The global methylation levels of non-tumor tissue from animals given the identical 27 mg CSC for 29 wks treatment did not exhibit a statistically significant difference from controls. Similarly, tissues from animals promoted with 27 mg for 9 or 15 wks (groups 9 and 11, respectively) and sacrificed immediately afterwards did not exhibit a level of global methylation which was significantly different from either untreated (at 29 wks) or initiated-only animals (groups 1 and 4, respectively).

Analysis of GC-rich methylation of tumor and non-tumor tissue (29 wks promotion) from treatment group 12 is shown in Figure 3. Increased methylation at the external C of 5'-CCGG-3' sites is expected to result in more prominent bands in *RsaI/MspI*-treated samples, while increased methylation at the internal C is expected to result in more prominent bands in *RsaI/HpaII*-treated samples. In tumor tissues there were 7 regions in which bands in the RsaI/MspI lanes were seen more prominently compared to controls, indicative of methylation at the external cytosine of the 5'-CCGG-3' site. In 4 of these 7 regions, bands in the *RsaI/MspI* lanes of the non-tumor tissue from CSC-promoted animals were also more prominent than what was seen in the untreated controls (Figure 3a). A quantitative representation of band intensity for each of the 7 regions is provided in Figure 3b. Within a particular region, an increase in the

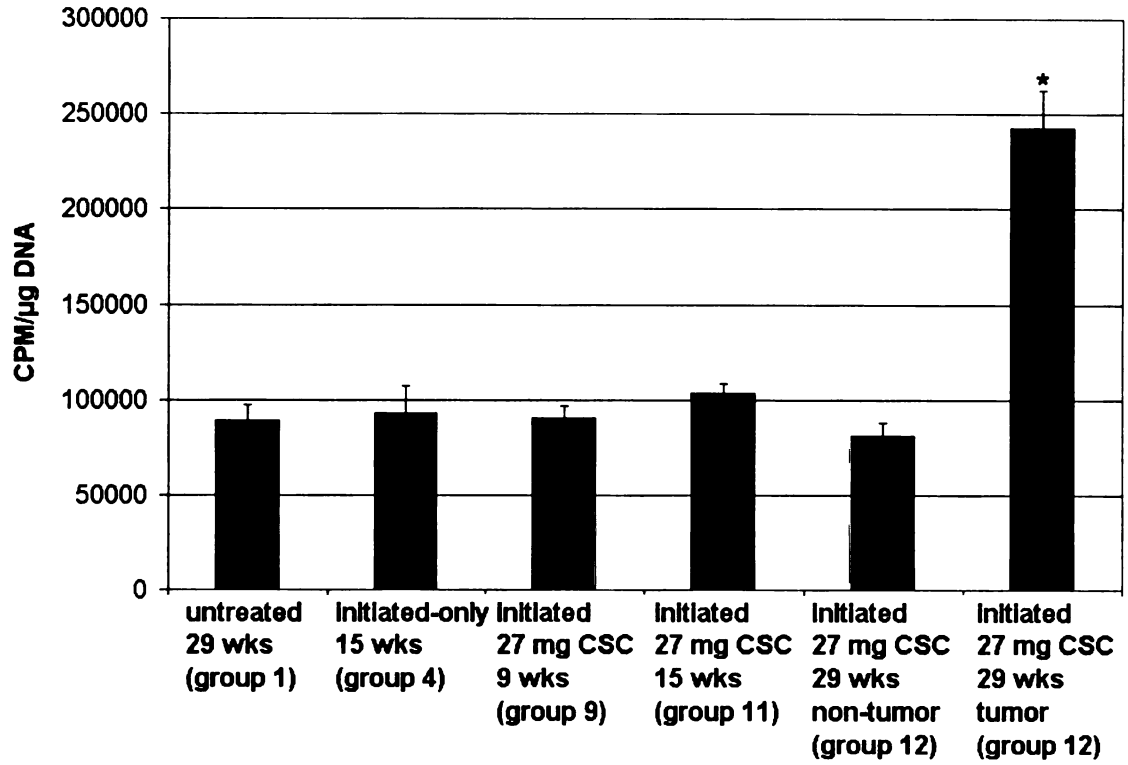
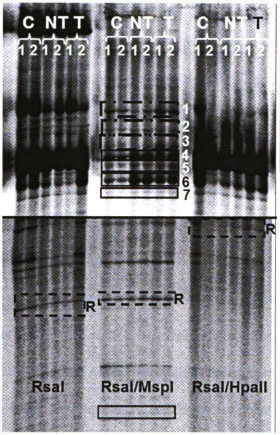


Figure 2. Global methylation status in tumor and non-tumor mouse skin DNA. Global methylation in tumor and non-tumor mouse skin DNA from initiated animals receiving 27 mg CSC promoter for 29 wks, as well in non-tumor mouse skin DNA from initiated animals receiving 27 mg CSC promoter for 9 or 15 wks and sacrificed immediately afterwards is presented. Initiated-only and no treatment controls sacrificed at 15 and 29 wks, respectively, are shown. Mean and standard error values are indicated, with each darkened bar representing the mean of the 3-7 animals shown for each group. *indicates a statistically significant difference ($p < 0.05$) between the tumor group and both untreated samples.

3a.



3b.

Region	C	NT	Fold Change	T	Fold Change
1	1.75	0.99	0.57	5.47	3.13
2	1.13	1.34	1.19	1.53	1.35
3	1.46	1.50	1.03	2.02	1.38
4	1.95	4.79	2.46	4.02	2.06
5	1.24	1.74	1.40	1.77	1.43
6	1.77	5.80	3.28	3.12	1.76
7	0.43	0.78	1.81	0.69	1.60

Figure 3. GC-rich methylation status in tumor and non-tumor mouse skin DNA from initiated animals receiving 27 mg CSC promoter for 29 wks. Control animals were neither initiated nor promoted during the corresponding 29 week period. 'C' indicates control animals, 'NT' indicates non-tumor skin, and 'T' indicates tumor skin. Numbers underneath the brackets at the top of the gel indicate individual animals. For each sample shown in *a*, *RsaI, RsaI/MspI*, and *RsaI/HpaII* restriction digests were performed. Numbered solid boxes indicate rows of bands seen more prominently in initiated, promoted tumor and non-tumor tissues compared to untreated controls. Numbered dotted boxes indicate rows of bands seen most prominently in the tumor tissues compared to non-tumor and control samples. Dashed boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. The area of the gel above the black line is from a 3-d exposure, while the area of the gel below the black line is from an 8-d exposure of the same gel. Results shown for the 2 animals per group that are presented are representative of 6-7 animals per group. *b:* The quantification of the pixel intensity of bands that appear more prominently in the *RsaI/MspI* lanes of treated animals compared to controls is shown. For each group, the average ratio of the band pixel intensities in the numbered region to the band pixel intensities in the reference lanes of corresponding lanes is indicated. The NT and T fold change values were obtained by dividing the ratios treated non-tumor and tumor groups by the control ratio in the same region.

average ratio of CSC-promoted samples compared to corresponding controls (untreated at 29 wks, or acetone-promoted at earlier time points) represents a more prominent band in the CSC-promoted samples, indicative of CSC-induced hypermethylation. Conversely, a decrease in the average ratio of the CSC-promoted samples compared to controls represents a less prominent band in the CSC-promoted samples, indicative of CSC-induced hypomethylation. In this case, the higher ratios shown for the CSC-promoted tumor and non-tumor groups compared to untreated controls indicates hypermethylation at the external cytosine of the 5'-CCGG-3' site. Additional regions of GC-rich DNA were methylated at the external cytosine in tumor compared to non-tumor tissue.

The fact that we observe a very different degree of inhibition of restriction by both *MspI* and *HpaII*, which share a common 5'-CCGG-3' recognition site, rules out the likelihood that adduct formation, rather than methylation changes, at the recognition site underlie the changes in band intensity observed. Specifically, we have observed many examples of sites where *MspI* digestion was inhibited and relatively few examples of sites where *HpaII* digestions was inhibited. In some cases, *MspI* digestion was inhibited within the same region where *HpaII* was not and vice-versa.

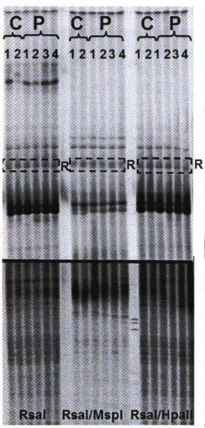
In order to determine whether the effect of promoter treatment requires initiation to induce increases in methylation, the GC-rich methylation patterns of non-tumor tissue collected at 9 weeks from animals initiated with DMBA and promoted with 27 mg CSC (group 9) were compared with non-tumor tissue from animals initiated with acetone and similarly promoted with CSC (group 13). Regardless of whether the skin was initiated or not, increases in methylation were detected at the external cytosine site, indicating that prior application of an initiator was not necessary for CSC to effect methylation at the 9

wk time point (data not shown). Furthermore, there were no differences seen in the GC-rich methylation patterns between DMBA-initiated, acetone-promoted (9 or 15 wks) and untreated (29 wks) animals (data not shown).

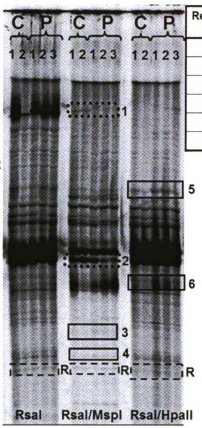
Next, we examined GC-rich methylation in non-tumor tissue from animals treated with various doses following 6 (Figure 4) and 9 (Figure 5) wks promotion to determine the concentration of CSC necessary to induce detectable changes at each time point. The lowest dose found to cause changes in GC-rich methylation at 6 wks was 27 mg (Figure 4b). At this dose, there were 2 regions in both *RsaI/MspI* and *RsaI/HpaII* lanes at which bands were more prominent in CSC-promoted animals compared to acetone-promoted controls. The corresponding increases in pixel intensity ratios of the promoted animals (Figure 4c) are indicative of increased methylation at both the internal and external cytosine sites. There were also two regions at which bands were seen less prominently in the *RsaI/MspI* lanes of the CSC-promoted animals compared to controls. The corresponding decreases in pixel intensity ratios (Figure 4c) are indicative of a decrease in methylation at the external cytosine site. No changes in GC-rich methylation were detected with 18 mg CSC promotion at 6 wks (Figure 4a). Thus, the threshold dose needed to elicit detectable changes in GC-rich methylation at 6 wks is between 18 and 27 mg CSC.

DMBA-initiated non-tumor skin promoted with 18 mg CSC for 9 wks induces increases in band intensity in 3 regions in the *RsaI/MspI* lanes compared to acetone-promoted controls (Figure 5b). The corresponding increases in pixel intensity ratios of the CSC-promoted animals (Figure 5c) are indicative of increases GC-rich methylation at the external cytosine. Treatment of initiated tissues with 9 mg CSC does not elicit

4a.



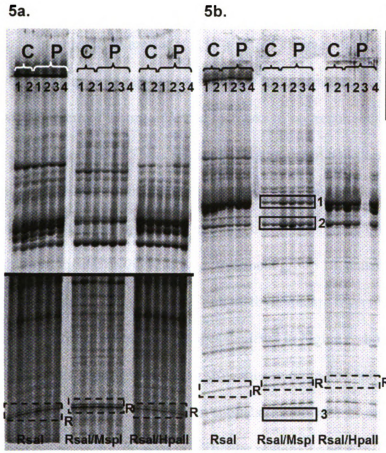
4b.



4c.

Region	C	P	Fold Change
1	1.69	1.26	0.75
2	4.92	3.12	0.63
3	0.95	1.17	1.23
4	0.90	1.35	1.50
5	1.16	1.70	1.47
6	5.66	7.82	1.38

Figure 4. GC-rich methylation in initiated non-tumor mouse skin DNA promoted with 18 (*a*) or 27 (*b*) mg CSC for 6 wks. 'C' indicates control animals initiated with DMBA and promoted with acetone; 'P' indicates DMBA-initiated, CSC-promoted animals. Numbers underneath the brackets at the top of the gel indicate individual animals. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Numbered solid boxes indicate rows of bands seen more prominently in CSC-promoted animals compared to controls. Numbered dotted boxes indicate rows of bands seen less prominently in CSC-promoted animals compared to controls. Dashed boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. In 4a, the area of the gel above the black line is from a 3-d exposure, while the area of the gel below the black line is from an 8-d exposure of the same gel. In 4b, the entire image shown is from a gel exposed for 3 d. Results shown for the 2 animals in the control group and the 3-4 animals in the promoted groups are representative of 6 animals in the control and 18 mg CSC-promoted groups, and 3 animals in the 27 mg CSC-promoted group. *c*: The quantification of the pixel intensity of bands which appear more or less prominently in the *RsaI/MspI* and *RsaI/HpaII* lanes of CSC-promoted animals compared to controls in *b* is shown. For each group, the average ratio of the band pixel intensities in numbered regions to the band pixel intensities in the reference lanes of corresponding lanes is indicated.



Region	C	P	Fold Change
1	1.71	2.49	1.46
2	2.02	2.72	1.35
3	1.29	1.66	1.29

Figure 5. GC-rich methylation status of initiated non-tumor mouse skin DNA promoted with 9(*a*) or 18(*b*) mg CSC for 9 wks. 'C' indicates control animals initiated with DMBA and promoted with acetone; 'P' indicates DMBA-initiated, CSC-promoted animals. Numbers underneath the brackets at the top of the gel indicate individual animals. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Numbered solid boxes indicate rows of bands seen more prominently in CSC-promoted animals compared to controls. Numbered dashed boxes indicate reference (R) rows of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. The area of the gel above the black line is from a 3-d exposure, while the area of the gel below the black line is from an 8-d exposure of the same gel. Results shown for the 2 animals in the control groups and the 4 animals in the promoted groups are representative of 6 animals per group. *c*: The quantification of the pixel intensity of bands that appear more or less prominently in the *RsaI/MspI* lanes of treated animals compared to controls in *b* is shown. For each group, the average ratio of the band pixel intensities in numbered regions to the band pixel intensities in the reference lanes of corresponding lanes is indicated.

observable changes in methylation (Figure 5a), indicating that the threshold dose needed for detectable increases in GC-rich methylation at 9 wks is between 9 and 18 mg CSC.

In order to determine if CSC-induced changes in GC-rich methylation were reversible, we compared the effects of a 9 wk treatment of 27 mg (group 9, Figure 6a), a 9 wk treatment of 27 mg followed by 6 wks of no treatment (group 10, Figure 6b), and a 27 mg treatment for 15 wks (group 11, Figure 6c) in non-tumor tissue. In all of these groups, an increased amount of methylation at the external cytosine was observed. More prominent increases in external cytosine methylation were observed for treatment group 9 and 11 compared to those seen for treatment group 10, consistent with the finding that the CSC-induced changes in GC-rich methylation are reversible. Table I presents quantification of the phosphoimages depicted in Figure 6 showing that the most prominent increases in pixel intensity ratios in promoted animals compared to corresponding controls are seen in animals treated with 27 mg for 9 and 15 wks (Figure 6a and 6c, respectively, note in particular regions 1 and 4 in Table I) in comparison to animals which were promoted with 27 mg CSC for 9 wks and sacrificed after a 6 wk recovery period (Figure 6b, note region 3 in Table 1). These data support the conclusion that the increases in methylation induced by CSC are reversible.

Finally, when we examined tumor incidence after 29 wks of CSC promotion (Figure 7), we found that there was a very low incidence of tumors in uninitiated animals treated with 36 mg of CSC, demonstrating that initiation is required for a marked increase in tumor formation in response to CSC promotion under the experimental conditions examined. Also, the most significant increase in tumor number was seen between 9 and

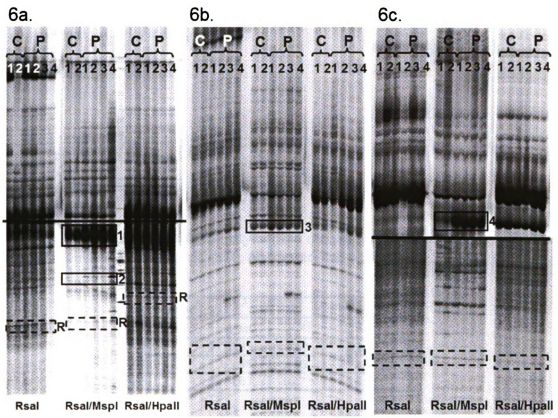


Figure 6. Reversibility of GC-rich methylation changes. The methylation status of GC-rich regions of initiated non-tumor mouse skin DNA promoted with 27 mg of CSC for 9 wks (*a*), 27 mg of CSC for 9 wks followed by 6 wks of recovery (*b*), and 27 mg of CSC for 15 wks (*c*) is presented. 'C' indicates control animals initiated with DMBA and promoted with acetone (sacrificed at 9 weeks in *a* and 15 wks for *b* and *c*); 'P' indicates DMBA-initiated, CSC-promoted animals. Numbers underneath the brackets at the top of the gel indicate individual animals. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Numbered solid boxes indicate rows of bands seen more prominently in CSC-promoted animals compared to controls. Dashed boxes indicate reference (R) rows of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. In 6a and 6c, the area of the gel above the black line is from a 3-d exposure, while the area of the gel below the black line is from an 8-d exposure of the same gel. In 6b, the entire image shown is from a gel exposed for 3 d. shown for the 2 animals in the control group and the 4 animals in each promoted group are representative of 6 animals per group.

Table 1. Average ratios of pixel intensity units in each region to corresponding reference bands for each group in Figure 6a (left), 6b (center) and 6c (right).

Region[a]	C[b]	P[c]	Fold Change[e]
1	6.94[d]	14.48	2.09
2	0.55	1.12	2.04
3	3.98	6.02	1.51
4	4.07	10.72	2.63

[a]Regions 1 and 2 are shown in Figure 6a, region 3 is shown in Figure 6b, and region 4 is shown in 6c.
[b]C: control: DMBA initiated only
[c]P: promoted with 27 mg CSC for 9 wks (regions1and 2), 27 mg CSC for 9 with a 6 wk recovery period (region 3), and 27 mg CSC for 15 wks (region 4)
[d]Pixel intensity ratio of the bands in the designated regions as compared to the bands in the corresponding reference regions (Figure 6a-c).
[e]Fold change values were calculated by dividing the pixel intensity ratio in the CSC-promoted samples (P) by that of the control samples (C) within the same region.

18 mg CSC (Figure 7), an observation that parallels the threshold dose required to discern increases in GC-rich methylation at 9 wks (Figure 5).

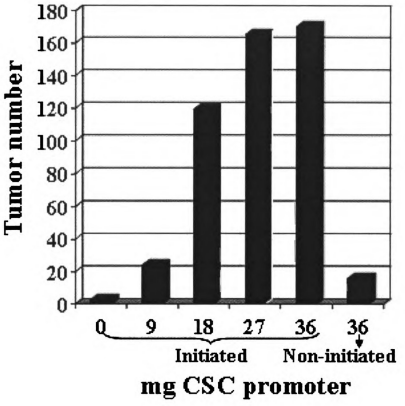


Figure 7. Effects of various doses of promoter on tumor incidence. Tumor incidence among approximately 39-40 initiated animals treated with 0, 9, 18, 27 and 36 mg of promoter three times a week for 29 wks (solid bars) and non-initiated animals treated with 36 mg promoter three times a week is shown (hatched bar). Some animals were sacrificed early due to tumor load. These animals and their tumors are included in the figure.

DISCUSSION

We have characterized changes in global and GC-specific methylation that occur as a result of promotion with various doses of CSC for different time periods in a two-stage initiation/promotion SENCAR mouse skin tumorigenesis model. Our goal was to determine the overall effect of the promoter on genome-wide patterns of methylation in order to discern particular aspects of methylation that are dysregulated during tumorigenesis. A frequent finding in tumor tissue is that global levels of methylation are decreased, while there appear to be selective increases and/or decreases in the GC-rich promoter regions of genes (Baylin et al., 1998). This is consistent with our observation that DMBA-initiated, CSC-promoted tumor tissue is globally hypomethylated with increases in GC-rich methylation. Additionally, our study reveals a progressive increase in GC-rich methylation, in a time- and dose-dependent, threshold-exhibiting manner that precedes the appearance of tumors. Global hypomethylation appears to be a relatively late event that is observed in tumor tissue and not in surrounding non-tumor tissue. Therefore, distinct mechanisms might underlie alterations in global and GC-rich patterns of methylation.

Maintaining normal patterns of methylation is dependent on multiple, interdependent factors including maintenance and *de novo* methylation, demethylation not linked to DNA replication, the availability of methyl group sources (S-adenosyl methionine is the proximate methyl donor), cellular proliferation, and cellular differentiation (Goodman and Watson, 2002). Alteration of one or more of these factors may lead to hyper- and/or hypomethylation, both of which have been shown to contribute to carcinogenesis. Maintenance methylation following DNA replication is accomplished

by Dnmt1, which acts preferentially at hemimethylated sites in DNA, while *de novo* methylation is primarily accomplished by Dnmt 3a and b (Okano et al., 1998). Changes in the cellular proliferation rate challenge a cell's methylation machinery to adjust the maintenance methylation rate accordingly, and cellular differentiation is controlled by changes in DNA methylation. In addition, methylation can be directed to specific regions of DNA. For example, the leukemia-promoting promyeloid leukemia retinoic acid receptor fusion protein has been shown to induce gene hypermethylation and silencing by recruiting DNA methyltransferases to target promoters (DiCroce et al., 2002). A unique feature of our study is that through the use of *HpaII* and *MspI*, methylation of both the internal and external cytosines of the 5'-CCGG-3' site was assessed. We report that the majority of persistent methylation changes found within the GC-rich regions occur at the external C of 5'-CCGG-3' sites, indicating that CSC promotion might have a targeted effect on this particular type of methylation. While the bulk of methylation research focuses on methylation within the symmetrical CpG dinucleotides, CpNpG methylation has been detected in mammalian cells (Clark et al1997; Stirzaker et al., 1997). The specific basis for CpNpG methylation in mammalian systems is not known. However, in *Arabidopsis*, CpNpG-specific methylation occurs through an interaction of the DNA methyltransferase with histone 3, which first must be methylated by a specific methyltransferase (Jackson et al., 2002).

Altered methylation of the GC-rich promoter regions of genes is a common event in carcinogenesis, and is detectable prior to the appearance of a clinically evident tumor (Lehmann et al., 2002). For instance, methylation of the promoter regions of *p16* and *MGMT* tumor suppressor genes has been detected in the sputum DNA of all patients with

squamous cell carcinoma of the lung up to 3 yrs before clinical diagnosis (Palmisano et al., 2000). Furthermore, methylation of tumor suppressor genes *p16, MINT1 (methylated in tumor 1), MINT2, MINT31*, *MGMT*, or *hMLH1* are frequent observations in colorectal cancer (Chan et al., 2002). Increases in promoter methylation of at least one of these genes was the only molecular abnormality identified in 16% of aberrant crypt foci, which are postulated to be the earliest precursor lesions in colorectal carcinogenesis (Chan et al., 2002). The arbitrarily primed PCR procedure used in our study has been shown to amplify GC-rich, CpG-containing promoter regions of a variety of genes (Gonzalgo et al., 1997; Kohno et al, 1998).

Both hyper- and hypomethylation of promoter regions might contribute to carcinogenesis by facilitating the transcriptional silencing of suppressor genes and enhanced expression of oncogenes, respectively (Laird, 1997). Furthermore, hypomethylation of non-promoter regions may lead to a decreased stability of the genome due to an increase in the expression of transposons that are typically silenced by methylation (Robertson and Jones, 2000). Therefore, alterations in DNA methylation may play a variety of roles in carcinogenesis (Counts and Goodman, 1995).

The SENCAR mouse skin model allows for demarcation of the initiation and promotion stages of carcinogenesis (Slaga et al., 1996). Additionally, the rate of tumor formation in animals treated with initiator only has been shown to be virtually the same as that in untreated animals (Ewing et al., 1988). Consistent with this observation, our studies demonstrate a clear dose response relationship for tumor formation following promotion with CSC, while treating uninitiated animals with a high dose of promoter resulted in minimal tumor incidence, indicating that CSC does not appear to possess a

significant initiating potential. (Figure 7). Therefore, CSC appears to be acting primarily as a tumor promoter, and the SENCAR model is ideal for permitting examination of both qualitative and quantitative effects on methylation during the promotion stage. We have demonstrated that the promoter effects on GC-rich methylation exhibit a threshold. Moreover, threshold doses required for detectable GC-rich methylation decreased with increased time of promotion, indicating that the effects of the promoter were both time- and dose-dependent, and that the altered methylation observed fits well with the classic criteria for a mechanism involved in tumor promotion (Pitot and Dragan, 1991; 1994). The promoting effects of CSC on methylation are similar to those elicited by the classic rodent liver tumor promoter phenobarbital (PB), which also causes global hypomethylation (Counts et al., 1996) and hypermethylation of GC-rich regions at both the external and internal cytosine sites at 5'-CCGG-3' sequences (Watson and Goodman, 2002b). In addition, the effects of both PB and CSC are reversible, a hallmark characteristic of a tumor promoter (Pitot and Dragan, 1991;1994).

Furthermore, we have found that the threshold dose required to induce detectable changes in GC-rich methylation (18 mg, Figure 5) at 9 wks is the same threshold dose required to elicit a dramatic increase in tumor incidence at 29 wks (Figure 7). This suggests that methylation changes at early times might be predictive of future tumorigenesis. Indications that methylation changes might serve as biomarkers of carcinogenesis have become increasingly more prevalent. For instance, it has been reported that aberrant methylation of *p16* is an early event in lung cancer and a potential biomarker for early diagnosis (Belinsky et al., 1998). Here, the GC-rich alterations detected prior to global decreases in methylation might be indicative of methylation-

mediated silencing of particular tumor suppressor genes, followed by facilitation of expression of oncogenes and transposable elements. This model supports a causal role for altered methylation in skin tumorigenesis. CSC acts as a classic promoter, inducing methylation changes in a progressive, threshold-exhibiting, progressive and reversible manner, as expected for a mechanism underlying tumor promotion. It is important to stress the fact that methylation change(s) per se, particularly at early times following chemical treatment, do not indicate that tumor formation is inevitable, since these changes are potentially reversible.

Carcinogenesis involves a progressive clonal selection/expansion of cells that are increasingly abnormal, both genetically and phenotypically. The specific sequence by which key heritable alterations to the genome occur may be an important determinant of carcinogenesis. However, it appears likely that the individual crucial alterations to critical genes stem from a stochastic process, and one can expect this to be enhanced under conditions where control of DNA methylation is decreased. Indeed, whether a particular modification predominates, e.g., hypo- vs. hypermethylation and/or alterations in global and/or GC-rich regions, at a certain stage of tumor development can depend upon the species, target organ and chemicals involved (Counts and Goodman, 1995). The current characterization of stepwise, progressive, promoter-induced alterations in methylation in the SENCAR two-stage mouse skin tumorigenesis model provides further support for the multiple roles that aberrant methylation may play in this process. Multiple changes in methylation are observed during CSC tumor promotion; increased methylation of GC-rich regions precedes global decreased methylation. Hence, progressive

alterations in global and GC-rich methylation appear to be mechanistically important in tumor promotion.

ACKNOWLEDGEMENT

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CHAPTER 3

INCREASED DNA METHYLATION IN THE *HOXA5* PROMOTER REGION CORRELATES WITH DECREASED EXPRESSION OF THE GENE DURING TUMOR PROMOTION

This chapter represents a manuscript that was submitted to *Carcinogenesis* in January, 2003. Authors include: Watson, Rebecca E., Curtin, Geoff M., Hellman, Gary M., Doolittle, David J., and Goodman, Jay I..

ABSTRACT

DNA methylation is an important regulator of gene transcription, and promoter-region methylation typically decreases expression. A two-stage SENCAR mouse skin carcinogenicity model was used to examine gene-specific changes in methylation during tumor promotion. Following initiation with 75 μg 7,12-dimethylbenz[a]anthracene, methylation analysis was performed on skin promoted with 9, 18, 27 or 36 mg cigarette smoke condensate (CSC) for 9 wks, or 27 mg CSC for 9 wks and sacrificed 6 wks afterwards (recovery group). Additionally, tumors that arose following promotion with 27 mg CSC for 29 wks were assessed. Gene array analysis identified genes that were differentially expressed during treatment. Expression of the *HoxA5* gene, which has characteristics of a tumor suppressor, was markedly decreased following 9 weeks of treatment with 27 mg and expression increased to control levels in the recovery group. Methylation status of the *HoxA5* promoter was measured using the enzymatic regional methylation assay (ERMA). DNA was bisulfite-modified and PCR amplified with primers containing *dam* sites (GATC), then incubated with [^{14}C-methyl] S-adenosyl-L-methionine (SAM) and *dam* methyltransferase to standardize DNA quantity. DNA was incubated with [^{3}H-methyl] SAM and *SssI* methylase to quantify methylation status. Higher ^{3}H/^{14}C ratios indicate increased methylation. The ^{3}H/^{14}C ERMA ratios of animals promoted with 27 or 36 mg CSC (48.2 $\pm$ 6.9 and 24.2 $\pm$ 6.1, respectively) were higher than the control or recovery group ratios (12.3 $\pm$ 0.1 and 12.6$\pm$0.3, respectively); sequence analysis supported these findings. Furthermore, increased methylation of either *p16* or *O^6 methylguanine methyltranferase (MGMT)* was detected in 4/8 (50%) of the tumor samples retrieved from mice promoted with 27 mg CSC for 29 weeks. These data

suggest that increased DNA methylation contributes to the down-regulation of *HoxA5*, and this, combined with hypermethylation of *p16* or *MGMT*, might facilitate the progressive clonal expansion of increasingly aberrant cells during tumor promotion.

INTRODUCTION

DNA methylation is an example of an epigenetic mechanism, whereby a heritable change in gene expression occurs without a change in DNA sequence (Wolffe and Matzke, 1999). Approximately 4% of cytosine bases in mammalian DNA are methylated at the 5' position (Ehrlich et al., 1982). The presence of 5-methylcytosine in the promoter region of a gene generally decreases expression because methyl-CpG binding proteins prevent transcription factors from binding to the promoter region, either by interfering with the recognition of *cis* elements or by introducing steric hindrance (Curradi et al., 2002). Moreover, these proteins recruit histone deacetylases that facilitate the remodeling of chromatin to an inactive state (Nakao, 2001). Often, methylation is found within CpG islands, which are defined as 200 bp or longer stretches of DNA with a 50% or greater GC content and a higher than expected CpG content (Gardiner-Garden and Frommer, 1987). CpG islands are present in the promoter region of approximately 60% of all mammalian genes (Antequera and Bird, 1993), and it has been shown that DNA methylation in these regions can facilitate carcinogenesis by silencing tumor suppressor genes (Jones and Laird, 1999). DNA methylation likewise silences the expression of transposable elements, which would otherwise lead to transcriptional interference and contribute to genomic instability (Yoder et al., 1997; Carnell and Goodman, 2003). Finally, hypomethylation can increase oncogene expression, with multiple alterations of methylation potentially contributing to carcinogenesis (Counts and Goodman, 1995).

Carcinogenesis is a multistage, multistep process with three experimentally-defined stages: initiation, promotion and progression (Pitot and Dragan, 1994). Initiation involves a heritable alteration that confers a selective growth advantage to a cell during

the promotion stage. Promoting agents facilitate replication of the initiated cell by increasing the proliferation rate and/or by decreasing the rate of apoptosis in these abnormal cells (Schulte-Hermann et al., 1990). Similarly, during the promotion stage, proliferating cells acquire further genetic alterations that effectively confer an additional growth advantage over the surrounding cells. This process repeats so that increasingly abnormal subclones develop, eventually leading to cells that can proliferate autonomously and progress to frank carcinomas (Pitot and Dragan, 1994). The alterations leading to autonomous growth can be the result of mutations or epigenetic changes (Goodman and Watson, 2002). For instance, both mutation and hypermethylation can lead to an inactive tumor suppressor gene; in the functional sense, these events may be equivalent. An important feature of the promotion stage is that it is reversible, and the dose-response relationship for a promoter exhibits a threshold (Pitot, 1982). The progression stage is characterized by widespread genomic instability and chromosomal aberrations (Pitot, 1991).

The two-stage SENCAR (sensitive to mouse carcinogenesis) mouse skin model allows for the temporal separation of initiation and promotion stages, thus facilitating the study of molecular mechanisms involved at particular stages of carcinogenesis (Slaga et al., 1996). Using this model, previous work in our laboratory characterized global and GC-rich changes in methylation patterns during the promotion of 7, 12-dimethylbenz[a]anthracene (DMBA)-initiated mouse skin with various doses of cigarette smoke condensate (CSC). Global DNA methylation was decreased only in tumor tissue, while GC-rich methylation was increased in a progressive (i.e., time- and dose-dependent) and reversible manner in the promotion stage (Watson et al., 2003). The

observed increases in GC-rich methylation might lead to the silencing of tumor suppressor genes.

In this study, the SENCAR model was used to perform a more in-depth analysis of the relationship between gene-specific increases in GC-rich methylation and changes in gene expression. Gene array analysis was performed using an 1176-gene cDNA array (Clontech Atlas™ Mouse 1.2 I array) to identify genes that were differentially regulated during the process of tumor promotion, particularly those that were down-regulated in a reversible fashion. *HoxA5*, which possesses characteristics of a tumor suppressor (Raman et al., 2000), was identified as a promising candidate for this purpose. Increased methylation of the promoter region of *HoxA5* is a frequent observation in a number of tumor tissues (Cillo et al., 2001), particularly in lung adenocarcinomas (Shiraishi et al., 2002) and breast cancer tissue (Maroulakou and Spyropoulos, 2003). Recent evidence has shown that HOXA5 binds to a consensus sequence on the tumor suppressor *p53* gene and up-regulates its expression (Raman et al., 2000). Importantly, methylation of the *HoxA5* is inversely related to *HoxA5* expression in breast cancer cells (Raman et al., 2000), as well as with fluctuations in *HoxA5* expression during development (Hershko et al., 2003). Expression of *HoxA5* was decreased in animals promoted with 27 or 36 mg CSC for 9 wks and sacrificed immediately afterwards, but not in animals promoted with 27 mg CSC for 9 wks and given a 6 wk recovery period. Therefore, methylation analysis of the promoter region of the gene was performed to determine if the observed down-regulation might be a consequence of increased methylation.

In addition, promoter-region methylation status of the well-known tumor suppressor genes *O^6 Methylguanine methyltransferase (MGMT)* and *p16* was assessed in

tumor samples from mice promoted with 27 mg CSC for 29 wks. Methylation has been shown to down-regulate both *MGMT* and *p16* in a number of cancer types (Esteller, 2002). *MGMT* specifically repairs guanine adducts, and down-regulation of this gene would be expected to contribute to an increase in unrepaired DNA adducts, leading to mutation (Bhakat and Mitra, 2003). Since *p16* is a key regulator of the cell cycle, a decrease in expression would lead to an increase in cell proliferation. Hypermethylation of *p16* is thought to be an early event in several cancer types, as well as an early diagnostic marker for lung cancer (Belinsky et al., 1998).

Results from gene-specific methylation analyses conducted during the current effort indicated that: 1) increased methylation is related inversely to *HoxA5* mRNA expression 2) increased methylation of the promoter region of *HoxA5* is reversible, indicating a process involved in the promotion stage, and 3) 50% of tumor samples exhibited an increase in methylation in the promoter region of either *p16* or *MGMT*, compared to no increases for DMBA-initiated controls. Thus, we propose that methylation of *HoxA5* can contribute to tumorigenesis by decreasing *HoxA5* expression, and in turn, reducing *p53* expression. Continued clonal expansion of abnormal cells could likewise be facilitated by an increase in methylation at the promoter regions of *p16* and *MGMT*.

●MATERIALS AND METHODS

Animals

Weanling female SENCAR mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, Maryland). Mice (5-7 wks of age at receipt) were allowed 2 wks to acclimate to the testing environment, and then randomly assigned to treatment groups according to body weight. To ensure groups of similar mean body weight, all groups were compared by ANOVA and least significant difference criteria, and were demonstrated not to be significantly different at a 5%, two-tailed assumption. Animals were housed and cared for in accordance with the Institute of Laboratory Animal Resources (ILAR), Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Three mice per group were initiated with 75 μg DMBA, followed by thrice-weekly promotion with 9, 18, 27 or 36 mg CSC or acetone vehicle control for 9 wks; additional experimental groups included DMBA-initiated animals promoted with 27 mg CSC for 9 wks and allowed 6 wks recovery, and DMBA-initiated animals promoted with 27 mg CSC for 29 wks. Animals were euthanized with CO_2 within 30 hrs of the last application of promoter, and skin collected from the chemical application site snap-frozen at –80°C until use. DNA was isolated by a phenol/chloroform procedure as described in Strauss (1990).

Gene Array Analysis

RNA isolation

RNA was isolated from tissue samples (100-300 mg) using TRI Reagent® (Molecular Research Center, Inc.; Cincinnati, OH), washed with 70% ethanol dissolved

in nuclease-free water and quantified by spectrophotometry. Formaldehyde agarose gel electrophoresis was performed to assess the integrity of RNA samples.

cDNA synthesis and hybridization

cDNA synthesis and array analysis were conducted using Clontech Atlas™ Mouse 1.2 nylon arrays, which target 1176 genes (BD Biosciences Clontech; Palo Alto, CA), following the manufacturer's protocol with modifications. Briefly, 10 μg of total RNA were incubated with 1.5 μl of 2.0 μM Bio-T_{30}-Bio (IDT; Coralville, IA) at 70°C for 2 min. Streptavidin magnetic beads (Dynal® Biotech; Oslo, Norway) were added to bind the poly A^+ RNA fraction. Following a 30-min incubation, the beads were separated using a Dynal® magnetic particle concentrator, and washed twice with 20 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM EDTA. The bead-RNA complexes were then washed twice in 1X PowerScript™ Reaction Buffer (BD Biosciences Clontech). CDS primers (BD Biosciences Clontech) were added and annealed at 65°C for 2 min. Labeled cDNA was synthesized according to the PowerScript™ (BD Biosciences Clontech) protocol at 50°C for 30 min with the inclusion of α[^{32}P]-dATP (PerkinElmer™ Life Sciences; Boston MA). Reverse transcriptase was inactivated by incubation at 70°C for 3 min. Labeled cDNA was released from the magnetic beads by incubation with 2.5 U RNase H (Invitrogen™ Life Tech.) at 37°C. The cDNA was then purified using Nucleospin™ columns (BD Biosciences Clontech).

Successively numbered arrays from the same printing lot were utilized to minimize variability Arrays were prehybridized at 42°C with 5 ml ResGen™ MicroHyb™ (Invitrogen™) containing 5 μl Cot-1 DNA (Invitrogen™) and 5 μl poly dA (Invitrogen™)

in a Robbins Scientific® Model 400 Hybridization Incubator (Sunnyvale, CA) for 2 h. The ^{32}P-labeled cDNA samples were heated to 95°C for 2 min, removed from the incubator, and placed on ice to cool. cDNA was then added to the prehybridization solution, and the solution incubated overnight at 42°C. Membranes were washed twice for 20 minutes at 50°C with 2X SSC (Invitrogen™), 1% SDS (Sigma-Aldrich), and once for 20 minutes at 55°C with 0.5X SSC containing 1.0% SDS.

Image capture and analysis

The cDNA arrays were exposed overnight to Molecular Dynamics storage phosphor screens (Amersham Biosciences; Sunnyvale, CA). Phosphor screens were scanned using a Molecular Dynamics Storm 860 (Amersham) phosphorimager at 50 micron resolution. Images were quantified using ImageQuant™ software version 5.2 (Amersham), and further analyzed in Excel (Microsoft Corp.; Redmond, WA) as described previously (Hellmann et al., 2001).

Statistical and clustering analysis of microarray data

Expression data were analyzed after transformation to a log to the base 2 scale. This simplifies interpretation, since a difference of 1 unit equates to a doubling of expression. Prior to comparisons, blots were normalized by subtracting the mean of the log values for each blot. If two blots were identical except for a constant multiplicative ratio, their adjusted values would be identical after this treatment. This adjustment was deemed appropriate for animals receiving different treatments, since 1) only a small proportion of the 1200 genes measured from the blot were expected to be affected by the

experimental treatment, and 2) use of the log scale minimizes the impact of these differences. No background subtractions were performed prior to analysis, since this introduces significant variability to genes expressed at low levels.

Following normalization, the log values for each gene were compared among treatment groups using analysis of variance with $p<0.05$ required for significance. All expression differences flagged by calculation were verified by visual inspection of original blots. Fold change differences were converted to % expression changes by the following formulas: A % increase = (Fold increase-1) X 100, with a 1.5 fold increase = (1.5-1) X 100 = a 50% increase; A % decrease = (1-1/absolute value of fold decrease) X 100, with a 1.5 fold decrease = (1-1/1.5) X 100 = a 33% decrease. Hierarchical cluster analysis was performed for down-regulated genes using GeneSpring™ (Silicon Genetics, Inc. Redwood City, CA).

Bisulfite Modification of DNA

Bisulfite modification of DNA deaminates unmethylated cytosine bases to uracil bases, while methylcytosine bases remain unchanged (Frommer et al., 1992). During PCR amplification, methylcytosine and uracil bases in the bisulfite-modified DNA template are replaced with cytosine and thymine bases, respectively, in the PCR products. Thus, PCR products from DNA that was more highly methylated prior to bisulfite conversion would contain a greater cytosine content compared to less methylated DNA. DNA, 2 μg per reaction, was bisulfite modified using the Chemicon® CpGenome™ kit

(Temecula, CA) for analysis of *p16* and *MGMT*, while the ZYMO EZ DNA Methylation Kit ™ (Orange, CA) was used for *HoxA5* analysis.

Enzymatic regional methylation assay (ERMA)

HoxA5 methylation analysis using the ERMA

PCR was performed in preparation for the ERMA (Galm et al., 2002) using primers with 11 bp tags (underlined below) containing *dam* methylase 5' GATC 3' recognition sites. Primers were specific for bisulfite modified DNA and did not bind to CpG sites. The forward primer [5'-AAG ATC TGA TCA TAA TTG GTA TAT TTA ATG GAA TTG-3'] and reverse primer [5'-AAG ATC TGA TCA AAT TAT AAA AAT AAC TAA AAC ATA TAC TC-3'] were used to amplify the region -345 to +32 bp relative to the transcriptional start site. PCR reactions contained 0.75 μg bisulfite modified DNA, 10 pmol each primer, 1X Failsafe™ Buffer H (Epicentre®; Madison, WI) 1 μl dimethylsulfoxide (DMSO; Sigma®, St. Louis, MO), 2 U Taq polymerase (Invitrogen™), and glass-distilled water to 20μl. PCR conditions were: 98°C for 3 min, followed by 40 cycles of 94°C forl min, 46°C for 35 s, and 72°C for 1 min. A final extension step was performed at 72°C for 5 min. Amplification of the target 399 bp target fragment was verified by electrophoresis on a 3% agarose gel .

PCR products were purified using the ZYMO Clean and Concentrator™ kit and quantified fluorometrically (Yamamoto et al., 1989). The ERMA was performed with 450 ng PCR product, 4 replicates per sample. Incubation with *dam* methylase (New England Biolabs, Inc.®; Beverly, MA) and [^{14}C] S-adenosyl methionine (SAM; Amersham) labeled the *dam* methylase recognition sites in the primer tags with ^{14}C,

allowing for quantification of PCR product in each reaction. A second incubation with *SssI* methylase (New England Biolabs, Inc.®) and a mixture of 15 μM non-radiolabeled SAM (New England Biolabs, Inc.®) and 1.0 μCi [^{3}H]-SAM (Amersham) labeled C's 5' to G's with ^{3}H such that more highly methylated samples incorporate more radioactivity. Reactions were spotted onto DE81 ion exchange filters (Whatman®; Maidstone, England) washed 3 X with a 0.5 M phosphate buffer, 1 X with 70% ethanol, and 1 X with 100% ethanol prior to scintillation counting (Packard; Meriden, CT). ^{3}H/^{14}C dpm ratios were calculated.

MGMT methylation analysis using the ERMA

To obtain enough *MGMT* PCR product for the ERMA, nested PCR was performed. Both primer sets were specific for bisulfite modified DNA and did not bind to CpG sites. The outer reaction forward primer [5'-TTG ATG TTT AGG ATG GGT AAA GAA T-3'] and outer reverse primer [5'-ATA CCC CAA AAC TCA CCA ACT TAC-3'] were used to amplify a region between -315 and +85 bp relative to the transcriptional start site. PCR reactions contained 0.75 μg bisulfite-modified DNA, 10 pm each primer, 1X Failsafe™ Buffer G (Epicentre®), 1U Taq polymerase (Invitrogen™) and glass-distilled water to 20 μl. PCR conditions were: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 53°C for 35 s, and 72°C for 1 min. A final extension step was performed at 72°C for 5 min. Nested *MGMT* PCR was performed using primers tagged with *dam* methylase recognition sites (underlined below) in preparation for the ERMA. The forward primer: [5'-<u>AAG ATC TGA TC</u>G AAG AAG AGG TTT GTT TTA GGA ATA-3'] and reverse primer [5'-<u>AAG ATC TGA TC</u>C AAC

TTA CAA ACT ACA AAC AAC AAC-3'] were used to amplify the region -274 to +69 bp relative to the transcriptional start site. Reactions contained 3µl outer *MGMT* PCR product, 10 pmol each primer, 1X Failsafe buffer G (Epicentre®), 1U Taq polymerase (Invitrogen™) and glass-distilled water to 25 µl. PCR conditions were: 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 61°C for 35 s, and 72°C for 1 min. A final extension step was performed at 72°C for 5 min. Amplification of the 365 bp target fragment was verified by electrophoresis on a 3% agarose gel.

The remainder of the ERMA was performed as described with *HoxA5* above except the amounts of non-radiolabeled SAM and ^{3}H-SAM were 20µM and 1.25 µCi, respectively.

_p16_ methylation analysis using methylation specific PCR (MSP)

Semi-nested PCR was performed to verify that the *p16* region was correctly bisulfite-modified. Inner and outer primer sets were specific for DNA that was bisulfite converted and did not bind to CpG sites. The outer forward primer [5'-GTT GTG TAT AGA ATT TTA GTA TTG-3'] and reverse outer primer [5'-CCA CCC TAA CCA ATC TAT CTA CAA C-3'] were used to amplify the region -753 to +33 bp relative to the translational start site (Patel et al., 2000). Reactions contained 1µg of bisulfite-modified DNA, 10 pmol each primer, 1 U Taq polymerase (Promega®; Madison, WI), 1 U Taq polymerase (Invitrogen™), 1 µl DMSO (Sigma®), 1.5 mM $MgCl_2$ (Promega®), 1X PCR buffer B (Promega®), 0.25 µM dNTP (Invitrogen™) and glass-distilled water to 20 µl. Cycling conditions were: 2 min at 94°C, followed by 24 cycles of 1 min at 94°C, 35 sec at 52°C, and 1 min at 72°C. The inner PCR reaction was performed using 2 µl of the

outer PCR product, the forward inner primer [5'-TTT TTA GAG GAA GGA AGG AGG GAT TT-3'] and the reverse outer primer described above. The inner reaction amplified the region -107 to +33 bp relative to the translational start site. Inner PCR components and conditions were otherwise the same as indicated above for the outer reaction except PCR was performed with 40 cycles with a 56°C annealing temperature. Amplification of the 140 bp target fragment, indicative of successful bisulfite conversion as well as successful outer and inner PCR reactions, was verified by electrophoresis on a 2% agarose gel.

For each sample, MSP (Herman et al., 1996) was performed on the same outer PCR reaction used in a successful inner PCR reaction. The M primer used in MSP [5'-TTA GCG TGG GTA GTA GGC GG-3'] is specific for DNA that was methylated at 2 CpG sites (underlined) prior to bisulfite conversion. Similarly, the forward U primer [5'-GTT AGT GTG GGT AGT AGG TGG-3'] is specific for DNA that was unmethylated at the same 2 sites prior to bisulfite conversion. The M or U primer was used with the outer reverse primer (described above) to amplify the region -356 (M primer) or -357 (U primer) to +33 bp relative to the translational start site. Reactions contained 2µl outer *p16* PCR product, 10 pm each primer, 1X Failsafe™ Buffer J (Epicentre®), and 1 U Taq polymerase (Invitrogen™). PCR conditions were: 94°C for 2 min, followed by 1 min at 94°C, 35 s at 59°C, and 1 min at 72°C for 35 cycles, and a final extension step at 72°C for 5 min. To determine if the target fragment was amplified, samples were analyzed by electrophoresis on a 2% agarose gel.

Sequencing

Automated sequencing of purified tagged *HoxA5* PCR products was performed at the Genomics Technology Support Facility at Michigan State University with an ABI PRISM®3100 Genetic Analyzer using the *HoxA5* outer forward primer (described above), as the sequencing primer. Sequencing reactions were composed of 20 ng PCR product and 30 pmol of *HoxA5* outer forward primer (described above) and glass distilled water for a reaction volume of 12 µl. Consensus sequences and sequence comparisons were preformed using Wisconsin GCG™ SeqWeb® software.

RESULTS

In light of our earlier observation indicating a general increase in methylation at CpG islands occurring between 6 and 9 wks following the start of promotion of DMBA initiated SENCAR mouse skin with CSC (Watson et al., 2003), we focused on down-regulated genes in this study. The number of individual genes that were down-regulated by at least 1.5-fold compared to initiated-only controls is presented in Figure 1. There is a distinct increase in the number of genes down-regulated in mice promoted with 27 and 36 mg CSC vs. those promoted with 9 and 18 mg CSC. The number of down-regulated genes is decreased in the recovery group compared to animals promoted with the same dose and sacrificed immediately afterwards. *HoxA5* was identified as a down-regulated gene that would be expected to play a role in tumorgenesis if its expression was reduced. While *HoxA5* was expression was not affected in animals promoted with 9 and 18 mg CSC, it was significantly down-regulated in animals promoted with 27 and 36 mg CSC by group averages of 61% and 66%, respectively (Figure 2). By contrast, *HoxA5* was not down-regulated in animals promoted with 27 mg CSC for 9 wks, but allowed a 6-wk recovery period. The methylation status of *HoxA5* was assessed in order to determine if the observed reduction in gene expression was linked to promoter-region hypermethylation.

HoxA5 methylation status was assessed with the ERMA and sequencing. A map of the *HoxA5* gene indicating the location of the ERMA primers and the region sequenced is presented in Figure 3. The ability of *HoxA5* ERMA to discriminate between methylated and nonmethylated templates was first assessed using untreated control DNA and *in vitro SssI* methylated DNA (Figure 4a). The $^{3}H/^{14}C$ ratio in the methylated sample

was significantly higher than that of the control (27.2 ± 1.8 vs. 7.5 ± 0.4), thus the procedure could distinguish between highly methylated and less methylated samples. Examination of DNA samples from the experimental groups revealed a significantly higher $^{3}H/^{14}C$ ratio in the group promoted with 27 and 36 mg CSC and sacrificed immediately afterwards (48.2 ± 6.9 and 24.2 ± 6.1, respectively), compared to either the recovery or initiated-only control groups (12.3 ± 0.1 and 12.6 ± 0.3, respectively) (Figure 4b). These data indicated a correlation between increased HoxA5 methylation status and a decreased expression level. DNA sequence analysis of the bisulfite-modified templates indicated that the sequences of groups promoted with 27 and 36 mg contain 11 and 10 cytosine bases, respectively, while the recovery and initiated-only control groups contained no cytosines (Figure 5). This is in complete agreement with the ERMA results presented in Figure 4, and provides further evidence that a decreased expression of *HoxA5* is correlated with an increased methylation status in the promoter region. A summary of *HoxA5* expression and methylation data is tabulated in Table 1.

Since promoter-region methylation frequently decreases expression of MGMT and/or p16, the methylation status of these genes in tumor DNA from mice treated with 27 mg CSC for 29 wks and non-tumor DNA from initiated-only controls was compared. The methylation status of MGMT and *p16* promoter sequences was examined in tumor DNA from mice treated with 27 mg CSC for 29 wks. First, the methylation status of *MGMT* was examined using the ERMA. A map of the *MGMT* promoter indicating the location of the ERMA primers is presented in Figure 6a. Verification of the *MGMT* ERMA was performed as described for the *HoxA5* ERMA (above). The $^{3}H/^{14}C$ ratio in the methylated sample was significantly higher than that of the initiated-only control

(44.1 ± 0.3 vs. 24.1± 1.1), indicating that the *MGMT* ERMA could detect differences in methylation status (Figure 6b). The average $^{3}H/^{14}C$ ratio of the initiated-only control samples was 26.1 ± 0.7 with a 95% confidence interval of 22.1-30.2 (Figure 6c). The $^{3}H/^{14}C$ ratios of 2/8 tumor samples were 41.8 ± 0.7 and 43.1 ± 0.7, both values above the 95% confidence interval of the control group, and closer to the ratios of the *SssI* methylated controls (44.1 ± 0.3 and 43.8 ±1.5), indicating an increased amount of methylation (Figure 6d).

The *p16* ERMA was performed on the same samples used for *MGMT* methylation analysis, above. Data in the literature indicated a discrete subset of CpGs where methylation plays a role in controlling transcription (Patel et al., 2000), and this was amenable to MSP. A map of *p16* indicating the location of the MSP primers is presented in Figure 7a. Using the primer set specific for DNA that was unmethylated at 2 CpG sites (U primer set), the target 390 bp fragment was amplified in all tumor and control samples, indicating that all the samples contained DNA that was unmethylated at these 2 sites (Figure 7b). With the primer set specific for DNA that was methylated at the same sites (M primer set), the target 389 bp fragment was amplified, indicative of methylated CpG sites, in 2/8 (25%) of the tumor samples and none of the controls (Figure 7c). These samples were not the same 2 samples found to have an increased amount of methylation in the promoter region of *MGMT*. Therefore, 4/8 tumors (50%) exhibited an increase in methylation at the promoter region of either *p16* or *MGMT*.

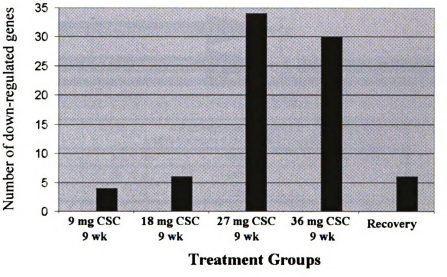
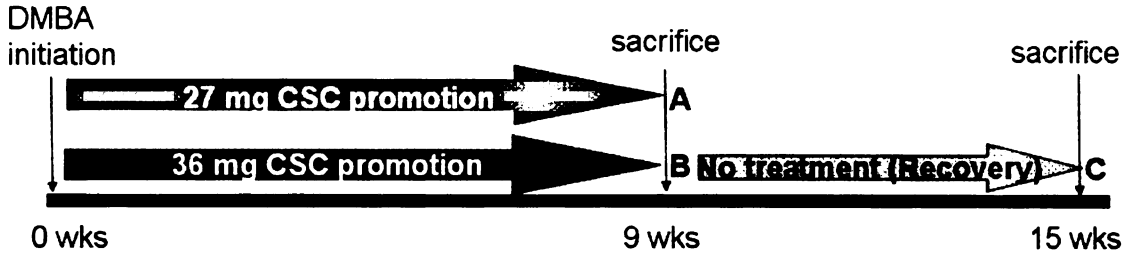


Figure 1. Number of down-regulated genes in CSC promoted animals compared to controls. The number of individual genes that were down-regulated by at least 1.5 fold, as detected by gene array analysis, is presented for animals promoted with 9, 18, 27 or 36 mg CSC for 9 wks and sacrificed immediately afterwards, as well as for animals promoted with 27 mg CSC and sacrificed 6 wks afterwards (recovery group) (n = 3 per group).



Animal	Group	*HoxA5* expression compared to DMBA/acetone 9 wk control % change	Group mean % change
1	A	-65%	-61%
2	A	-65%	
3	A	-52%	
1	B	-74%	-66%
2	B	-59%	
3	B	-65%	
1	C	16%	+2%
2	C	-25%	
3	C	15%	

Figure 2. Initiation / Promotion and sampling schedule. Promotion schedules and sacrifice times for groups of animals used in this study are presented. Values for *HoxA5* expression levels are presented as % increases (↑) and decreases (↓) compared to initiated-only controls. Gene expression analysis was performed for 3 randomly selected animals per group.

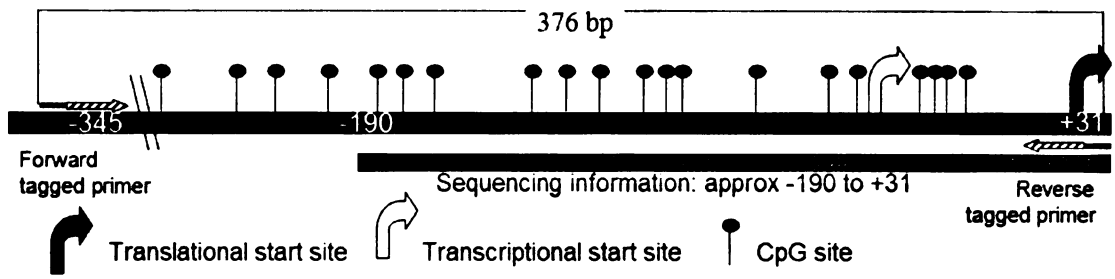
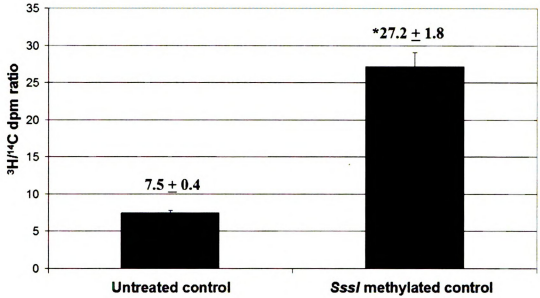


Figure 3. Map of the murine *HoxA5* promoter region. The location of the *HoxA5* transcriptional and translational start sites, the binding sites of tagged ERMA primers and the region of the gene that was sequenced following PCR of bisulfite-modified DNA are presented. Distances in bp shown are relative to the transcriptional start site. The most 5' region of the sequence used for *HoxA5* analysis is described in Zakany *et al.* (1988) and the remainder is listed under the accession number NM010453 in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/).

Figure 4. Enzymatic Regional Methylation Analysis (ERMA) of *HoxA5* Promoter. In *a*, verification of the *HoxA5* ERMA procedure is presented. ERMA was performed on an untreated control sample, and DNA from the same sample that which was *SssI* methylated *in vitro* prior to bisulfite modification, i.e., to determine if the procedure was optimized so that differences in methylation status could be detected. Here, the $^{3}H/^{14}C$ ratio was significantly increased when the sample was *SssI* methylated *in vitro* prior to bisulfite modification, verifying that the procedure can detect methylation differences within the *HoxA5* promoter region. Once the procedure was verified, ERMA was performed on samples from mice treated with 27 or and 36 mg CSC for 9 wks and sacrificed immediately afterwards, recovery group mice treated with 27 mg CSC for 9 wks and given a 6 wk recovery period prior to sacrifice, and initiated-only controls (b).

* Statistically significant difference from control and reversibility group, 2-tailed t-test, $p < 0.01$.

4a.


35
30
25
20
15
10
5
0
$^3H/^{14}C$ dpm ratio
*27.2 ± 1.8
7.5 ± 0.4
Untreated control
Sssl methylated control

4b.

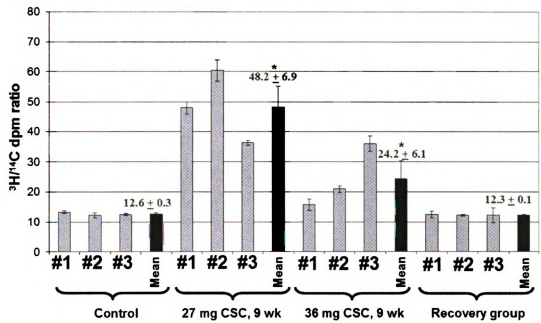

80
70
60
50
40
30
20
10
0
$^3H/^{14}C$ dpm ratio
12.6 ± 0.3
*
48.2 ± 6.9
*
24.2 ± 6.1
12.3 ± 0.1
#1 #2 #3 Mean #1 #2 #3 Mean #1 #2 #3 Mean #1 #2 #3 Mean
Control
27 mg CSC, 9 wk
36 mg CSC, 9 wk
Recovery group

Figure 5. Examination of *HoxA5* Promoter Methylation Status by DNA Sequence analysis of Bisulfite-Modified Templates. PCR samples used in the ERMA experiment presented in Figure 4 were sequenced in the region -190 to +32 relative to the *HoxA5* transcriptional start site (a). The presence of cytosine residues in the PCR products are indicative of sites in the original template DNA that were methylated. Consensus sequences were determined for each group of 3 mice and group consensus sequences were compared to each other, as well as to the GenBank *HoxA5* sequence (NM 010453) with all of the C's converted to T's (GenBank C-T), as would be anticipated following bisulfite modification (b). Sites at which there are differences in methylation status seen between consensus sequences are boxed. A "." in the consensus sequence indicates that different bases were present in each of the individual sequences. The percent of methylated C's within CpG's of the 16 possible methylated CpGs present within the sequenced fragment, as well as the percent methylated non-CpGs of the possible non-CpG cytosines within the fragment is presented.

* Statistically significant difference from control and reversibility group, 2-tailed t-test, $p < 0.01$.

5a.

```
                                  -190
27 mg CSC     GTGGTAATGG GTGGTGGGGA TGTGTTTAGG GCGCGCGCGT TATTTTTTTT GT..........TTTTATTT
36 mg CSC     GTGGTAATGG GTGGTGGGGA TGTGTTTAGG GCGCGCGCGT TATTTTTTTT GT..........TTTTATTT
Control       GTGGTAATGG GTGGTGGGGA TGTGTTTAGG GTGTGTGTG T TATTTTTTT GT..........TTTTATTT
Recovery      GTGGTAATGG GTGGTGGGGA TGTGTTTAGG GTGTGTGT G T TATTTTTTT GT..........TTTTATTT
GenBank C-T   GTGGTAATGG GTGGTGGGGA TGTGTTTAGG GTGTGTGTG T TATTTTTTT GT..........TTTTATTT

27 mg CSC     AATTTTTTTA TTAGTGTACG AGTTTATTTT TAGAGGTTAT TAGGTAGGAT TTACGGATTGG ATAATAAAAG
36 mg CSC     AATTTTTTTA TTAGTGTATG AGTTTATTTT TAGAGGTTAT TAGGTAGGAT TTACGGATTGG ATAATAAAAG
Control       AATTTTTTTA TTAGTGTATG AGTTTATTTT TAGAGGTTAT TAGGTAGGAT TTATGGATTGG ATAATAAAAG
Recovery      AATTTTTTTA TTAGTGTATG AGTTTATTTT TAGAGGTTAT TAGGTAGGAT TTATGGATTGG ATAATAAAAG
GenBank C-T   AATTTTTTTA TTAGTGTATG AGTTTATTTT TAGAGGTTAT TAGGTAGGAT TTATGGATTGG ATAATAAAAG

27 mg CSC     TACGTGATTCG GAAGTCGTAT TTTATATTTG GGTGTTTACG TAGGAGGGAA TCGAGTATAT
36 mg CSC     TACGTGATTCG GAAGTCGTAT TTTATATTTG GGTGTTTACG TAGGAGGGAA TCGAGTATAT
Control       TATGTGATTTT GAAGTTGTAT TTTATATTTG GGTGTTTATG TAGGAGGGAA TTGAGTATAT
Recovery      TATGTGATTTT GAAGTTGTAT TTTATATTTG GGTGTTTA G TAGGAGGGAA . TGAGTATAT
GenBank C-T   TATGTGATTTT GAAGTTGTAT TTTATATTTG GGTGTTTATG TAGGAGGGAA TTGAGTATAT

                                                                  +32
27 mg CSC     GTTTTAGTTA TTTTTATAAT TT
36 mg CSC     GTTTTAGTTA TTTTTATAAT TT
Control       GTTTTAGTTA TTTTTATAAT TT
Recovery      GTTTTAGTTA TTTTTATAAT TT
GenBank C-T   GTTTTAGTTA TTTTTATAAT TT
```

5b.

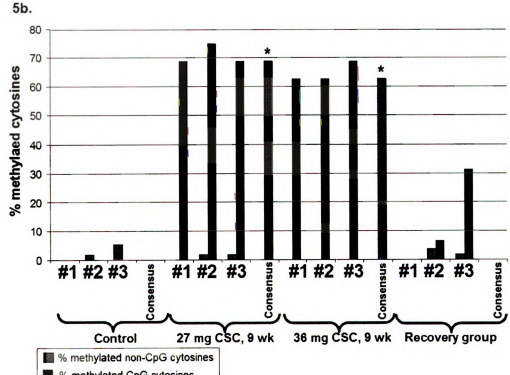


Table 1. Summary of *HoxA5* expression and methylation data

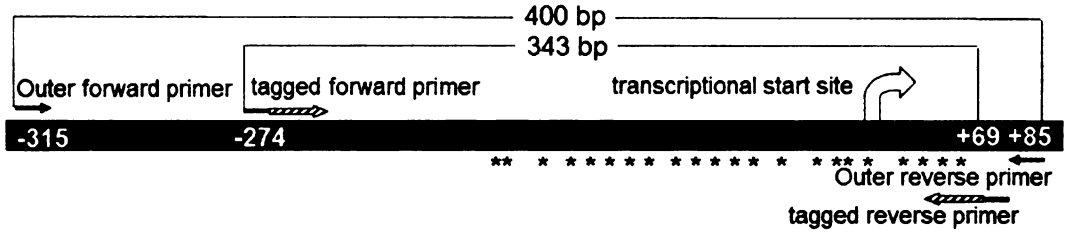
Animal	Group	expression change (%)	ave group expression change (%)	ERMA $^{3}H/^{14}C$ ratio	ave group ERMA $^{3}H/^{14}C$ ratio	% ^{me}CpGs in individual sequences [a, b]	% ^{me}CpGs in consensus sequence [c]
#1	control	N/A	N/A	13.3 ± 0.42	12.6 ± 0.3	0	0
#2	control	N/A		12.2 ± 0.73		0	
#3	control	N/A		12.4 ± 0.39		0	
#1	27 mg, 9wk	65 ↓	61↓	47.9 ± 1.99	48.2 ± 6.9	68.8	68.8
#2	27 mg, 9wk	65 ↓		60.3 ± 3.60		75.0	
#3	27 mg, 9wk	52 ↓		36.3 ± 0.78		68.8	
#1	36 mg, 9wk	74 ↓	66↓	15.8 ± 1.94	24.2 ± 6.1	62.5	62.5
#2	36 mg, 9wk	59 ↓		20.9 + 1.02		62.5	
#3	36 mg, 9wk	65 ↓		36.0 ± 2.69		68.8	
#1	recovery	16↑	2↑	12.5 ± 1.06	12.3 ± 0.1	0	0
#2	recovery	25↓		12.2 ± 0.32		6.3	
#3	recovery	15↑		12.2 ± 2.5		31.1	

[a] % ^{me}CpGs refers to the % methylated CpGs within the sequenced region as a percentage of the 16 potentially methylated CpG sites.
[b] methylated non-CpG sites were uncommon and are therefore not indicated in this table.
[C] consensus is based upon consistency and not a simple numerical average.

Figure 6. DNA methylation in the promoter region of *MGMT*. A diagram of the *MGMT* promoter, indicating location of PCR primers and CpG sites is presented in *a*. Outer PCR is performed with *MGMT* outer forward and reverse primers. Nested PCR using the tagged primers was performed in preparation for ERMA. The sequence used for *MGMT* analysis is listed under the accession number S82865 in the NCBI GenBank nucleotide database. In *b*, verification of the *MGMT* ERMA procedure is presented. ERMA was performed on an untreated control sample, and DNA from the same sample that was *SssI* methylated *in vitro* prior to bisulfite modification to determine if the procedure was optimized for detecting differences in methylation status. Here, the $^{3}H/^{14}C$ ratio was significantly increased when the sample was *SssI* methylated *in vitro* prior to bisulfite modification, verifying that the procedure can detect methylation differences within the *HoxA5* promoter region. *MGMT* ERMA results for initiated-only control samples are presented in *c*. MGMT ERMA results for tumor samples promoted with 27 mg CSC for 29 wks are presented in *d*.

* Statistically significant difference from (non-*SssI* methylated) control(s), 2-tailed t-test, $p < 0.01$.
** indicates a $^{3}H/^{14}C$ ratio above the 95% confidence interval of the initiated-only control group, and closer to what is seen in the *SssI* methylated controls, suggesting that these samples are from a population distinct from the control group.

6a.


400 bp
343 bp
Outer forward primer
tagged forward primer
transcriptional start site
-315
-274
+69 +85
Outer reverse primer
tagged reverse primer

6b.

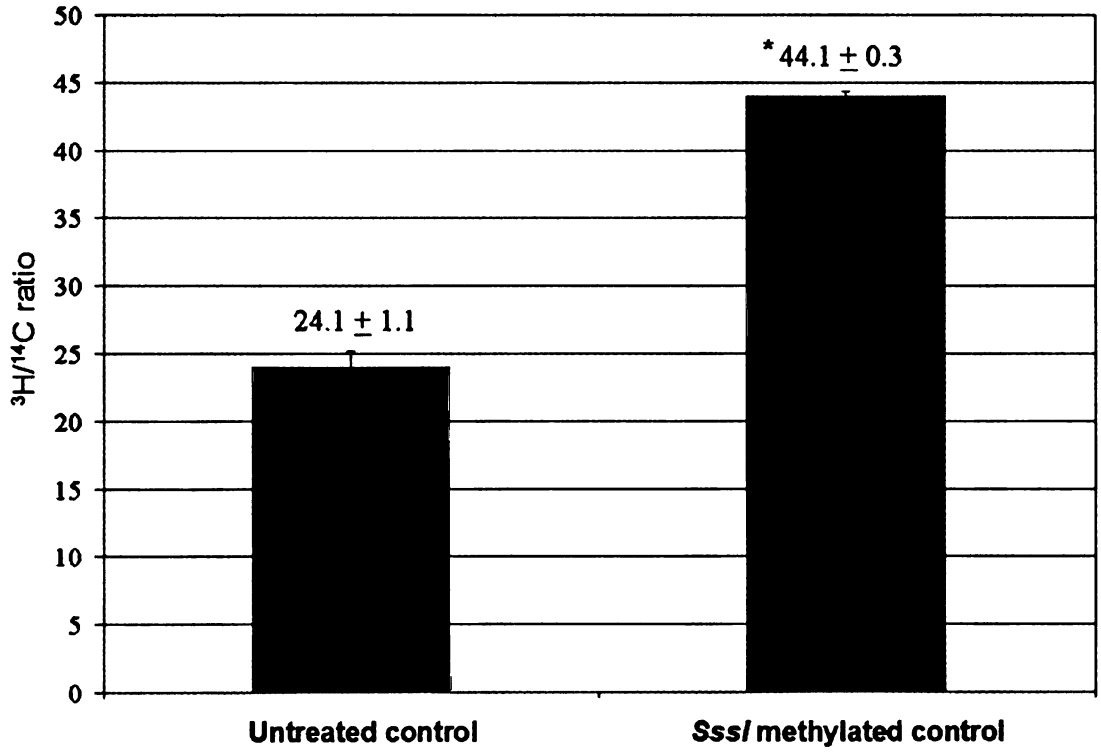
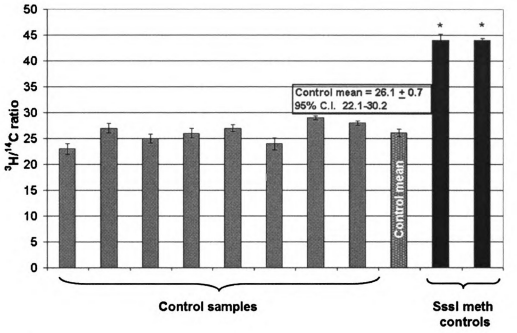

50
45
40
35
30
25
20
15
10
5
0
$^{3}H/^{14}C$ ratio
*44.1 ± 0.3
24.1 ± 1.1
Untreated control
*Sss*I methylated control

Figure 6. DNA methylation in the promoter region of *MGMT*. A diagram of the *MGMT* promoter, indicating location of PCR primers and CpG sites is presented in *a*. Outer PCR is performed with *MGMT* outer forward and reverse primers. Nested PCR using the tagged primers was performed in preparation for ERMA. The sequence used for *MGMT* analysis is listed under the accession number S82865 in the NCBI GenBank nucleotide database. In *b*, verification of the *MGMT* ERMA procedure is presented. ERMA was performed on an untreated control sample, and DNA from the same sample that was *SssI* methylated *in vitro* prior to bisulfite modification to determine if the procedure was optimized for detecting differences in methylation status. Here, the $^{3}H/^{14}C$ ratio was significantly increased when the sample was *SssI* methylated *in vitro* prior to bisulfite modification, verifying that the procedure can detect methylation differences within the *HoxA5* promoter region. *MGMT* ERMA results for initiated-only control samples are presented in *c*. MGMT ERMA results for tumor samples promoted with 27 mg CSC for 29 wks are presented in *d*.

* Statistically significant difference from (non-*SssI* methylated) control(s), 2-tailed t-test, $p < 0.01$.
** indicates a $^{3}H/^{14}C$ ratio above the 95% confidence interval of the initiated-only control group, and closer to what is seen in the *SssI* methylated controls, suggesting that these samples are from a population distinct from the control group.

6c.


50
45
40
35
30
25
20
15
10
5
0
$^3H/^{14}C$ ratio
Control mean = 26.1 ± 0.7
95% C.I. 22.1-30.2
Control mean
*
*
Control samples
SssI meth controls

6d.

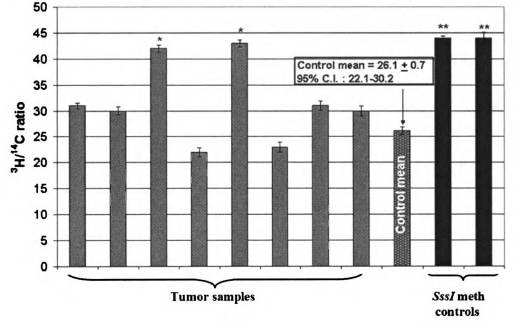
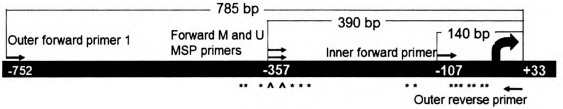

50
45
40
35
30
25
20
15
10
5
0
$^3H/^{14}C$ ratio
Control mean = 26.1 ± 0.7
95% C.I. : 22.1-30.2
Control mean
*
*
**
**
Tumor samples
Sssl meth controls

Figure 7. DNA methylation in the promoter region of *MGMT*. A diagram of *MGMT* indicating location of PCR primers and CpG sites is presented in *a* (previous page), Outer PCR is performed with *MGMT* outer forward and reverse primers. Nested PCR using the tagged primers was performed in preparation for ERMA. In *b* (previous page), verification of the *MGMT* ERMA procedure is presented. ERMA was performed on an untreated control sample, and DNA from the same sample which was *SssI* methylated *in vitro* prior to bisulfite modification to determine if the procedure was optimized so that differences in methylation status could be detected. Here, the $^{3}H/^{14}C$ ratio was significantly increased when the sample was *SssI* methylated *in vitro* prior to bisulfite modification, verifying that the procedure can detect methylation differences within the *HoxA5* promoter region. *MGMT* ERMA results for initiated-only control samples are presented in *c*. MGMT ERMA results for tumor samples treated with 27 mg CSC for 29 wks are presented in *d*.

* Statistically significant difference from (non-*SssI* methylated) control(s), 2-tailed t-test, $p < 0.01$.
** indicates a $^{3}H/^{14}C$ ratio above the 95% confidence interval of the initiated-only control group, and closer to what is seen in the *SssI* methylated controls, suggesting that these samples are from a population distinct from the control group.

7a.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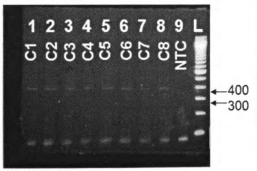
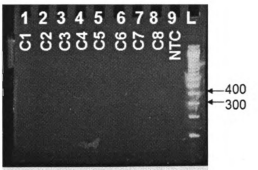
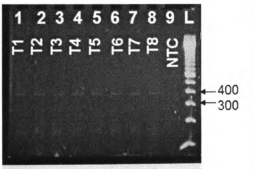
390 bp
140 bp
Outer forward primer 1
Forward M and U
MSP primers
Inner forward primer
-752
-357
-107
+33
Outer reverse primer

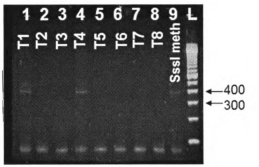
7b.


1 2 3 4 5 6 7 8 9 L
C1 C2 C3 C4 C5 C6 C7 C8 NTC
400
300

7c.


1 2 3 4 5 6 7 8 9 L
C1 C2 C3 C4 C5 C6 C7 C8 NTC
400
300


1 2 3 4 5 6 7 8 9 L
T1 T2 T3 T4 T5 T6 T7 T8 NTC
400
300


1 2 3 4 5 6 7 8 9 L
T1 T2 T3 T4 T5 T6 T7 T8 Sssi meth
400
300

DISCUSSION

While the study of carcinogenesis often focuses on mutation, epigenetic events such as DNA methylation, also play an important role in the process. Both hyper- and hypomethylation can contribute to tumorigenesis. Hypomethylation can increase the expression of oncogenes and transposable elements, posing a threat to genomic stability (Counts and Goodman, 1995; Carnell and Goodman, 2003). Conversely, hypermethylation can silence tumor suppressor genes (Laird, 1997). Specific patterns of methylation change are likely to vary according to the chemical, target tissue, and species involved (Counts and Goodman, 1995). For instance, relatively tumor-sensitive B6C3F1 mice treated with the rodent tumor promoter phenobarbital exhibit global hypomethylation in liver tissue as early as 1 wk after starting treatment (Counts et al., 1996). In a previous study using the SENCAR initiation-promotion model, progressive increases in methylation at GC-rich regions were observed during the promotion stage, and this persisted in papillomas, while hypomethylation was only observed only in papillomas (Watson et al., 2003).

Promoter-region hypermethylation represents a frequent occurrence in preneoplastic tissue (Feng et al., 2001; Nuovo et al., 1999), with down-regulation of tumor suppressor genes often correlated with promoter-region hypermethylation in neoplastic tissues (Laird, 1997; Esteller, 2002). The importance of DNA methylation in the transcriptional repression of particular genes has been demonstrated through re-activation of these genes following application of the demethylating agent 5-azacytidine (Patel et al., 2000; McGregor, 2002). Within individual tumor suppressor genes, methylation increases in a progressive fashion; additionally, the silencing of an increasing

number of tumor suppressor genes correlates with more severe clinicopathology (Lee et al., 2003). Thus, characterization of gene-specific methylation change can provide insight into which genes are altered at what point during tumorigenesis and how this might contribute to transformation, and whether or not this is reversible during the promotion stage.

In this study, the two-stage SENCAR mouse skin carcinogenesis model was used to characterize methylation changes during the process of tumor promotion. The ERMA was used to quantify promoter region methylation density in the *HoxA5* promoter. The specific region of the promoter examined is that which has been reported to contain critical sites of methylation involved in the regulation of expression (Raman et al., 2000; Hershko et al., 2003). ERMA data indicated that *HoxA5* was more heavily methylated in groups promoted with 27 or 36 mg CSC and sacrificed immediately afterwards compared to either the recovery (promoted with 27 mg CSC for 9 wks and sacrificed 6 wks later) or control (initiated-only) groups (Figure 4b), indicating that methylation correlates with gene expression, both of which were reversible.

The ERMA results were verified by sequencing (Figure 5). However, the differences between the two treatment groups in methylation status indicated by sequencing were not as large as those indicated by ERMA. We must keep in mind that we are dealing with a heterogeneous population of cells. The ERMA reflects average methylation of the region of interest, while sequencing data of the PCR product used for the ERMA is presented as methylation of particular cytosine residues in an "all-or-none" fashion, e.g. if cytosines at a particular site in 40% of the cells were methylated, while 60% were unmethylated, the sequencing data would indicate an unmethylated cytosine.

Therefore, the sequencing data can verify the ERMA without there necessarily being a 1:1 comparison. During the experiment, it was observed that there was somewhat more irritation in the skin of the animals treated with 36 mg CSC compared to the 27 mg group which could account for the differences observed. Furthermore, gene expression data indicated a higher degree of toxicity/apoptosis at the 36mg dose, and death of initially responding cells would reduce the population exhibiting the methylation changes. This may explain why a higher level of methylation was seen with the 27 mg dose of promoter compared to the 36 mg dose.

In addition to *HoxA5*, the methylation status of the promoter regions of *MGMT* and *p16* were examined . The ERMA assay was employed to measure the methylation status in the promoter region of *MGMT* in tumor and initiated-only control tissues. The MSP assay (Herman et al., 1996) was used to assess the methylation status of the *p16* promoter region in the same tumor and initiated-only samples, because the sequence was particularly amenable to the design of primers specific for CpG sites within a region where methylation was shown to play an important role in gene silencing (Patel et al., 2000). It was found that 50% of the tumors, and none of the initiated-only tissue analyzed exhibited an increase in methylation at the promoter region of either *p16* or *MGMT*.

The gene-specific methylation changes observed extend and complement the dose- and time- dependent, reversible increases in GC-rich methylation previously reported for this system (Watson et al., 2003). We hypothesized that GC-rich methylation increases might reflect, at least in part, methylated tumor suppressor promoters; the observed hypermethylation of *p16, MGMT* and *HoxA5* reported here supports this contention. Notably, this is the first study, to our knowledge, describing

that both methylation and down-regulation of *HoxA5* occurs in a reversible manner during tumorigenesis. The reversibility of *HoxA5* methylation also mirrors the reversible increases reported for genome-wide GC-rich methylation patterns (Watson et al., 2003), which in turn tracks with the number of individual down-regulated genes in a dose-responsive, reversible pattern.

Reversible changes in methylation can occur by three mechanisms: 1) "active demethylation" whereby demethylase activity results in changing a 5-methylcytosine residue to a cytosine (Frémont et al., 1997; Ramchandani et al., 1999); 2) failure to maintain patterns of methylation following replication; and 3) cell turnover. Active demethylation is clearly seen in development (Ehrlich, 2003) and in T-cell activation (Bruniquel and Schwartz, 2003), suggesting that this mechanism is possible in a variety of cases. It is unlikely that cell turnover alone would explain the reversibility observed in *HoxA5* methylation because previous experiments with this same model using a less sensitive arbitrarily primed PCR technique detected a small degree of GC-rich methylation remaining in the recovery group (Watson et al., 2003); this indicated that it was possible for particular methylation patterns to be at least partially retained following the 6 wk recovery period. Regardless of the mechanism(s) involved, both methylation of the *HoxA5* promoter, and the average methylation status of GC-rich DNA, were clearly reversible, a finding indicative of a process involved in the promotion stage (Pitot and Dragan, 1994; Goodman, 2001).

A potential role for the methylation changes we observed in carcinogenesis, within the context of the multistage nature of the process, is illustrated using a hypothetical schematic presented in Figure 8. We start with a situation where DMBA

initiation could stem from a mutation of *H-ras*. This has been shown to be involved in the initiation stage of skin carcinogenesis (Yuspa, 1986; DiGiovanni, 1992) Treatment with a promoter, e.g. CSC, can stimulate proliferation of a population of cells with DMBA-induced damage. Genetically damaged cells, *H-ras* mutated in this example, would thus have a growth advantage, and clonally expand. Since HOXA5 binds to a target element in the *p53* promoter thereby up-regulating its transcription (Raman et al., 2000), *HoxA5* hypermethylation, leading to down-regulation of the gene, would decrease *p53* activity and thus, the capacity for apoptosis within a proliferating population of *H-ras* mutated cells. Therefore, cells that bear DMBA-induced damage and exhibit *H-ras* mutation, plus *HoxA5* down-regulation could continue to clonally expand under the influence of the promoting agent with additional genetic damage accumulating. Subsequent methylation-mediated down-regulation of *MGMT* and/or *p16* could facilitate the further clonal expansion of increasingly abnormal cells. This may be accomplished by decreasing DNA repair and increasing proliferation rate, respectively. This could facilitate more genetic alterations, eventually leading to papilloma formation. The decreased level of global methylation observed in the papillomas (Watson et al., 2003), could further encourage clonal expansion of increasingly abnormal cells by up-regulating oncogenes and increasing expression of transposable elements (Counts and Goodman, 1996). Global decreases in methylation are frequently observed within the same genome exhibiting increases in GC-rich methylation tissue (Ehrlich, 2002). Eventually, a subset of the papillomas may acquire additional genetic alterations leading to frank carcinoma.

Hypermethylation of *HoxA5*, *MGMT* and *p16* likely represent a subset of tumor suppressors methylated and down-regulated throughout the process of promotion,

possibly facilitated by an increased activity of Dnmt1. This study is the first report on the reversibility of *HoxA5* methylation patterns regulating expression, and provides a possible explanation for how *HoxA5*, *p16*, and *MGMT* hypermethylation contribute to tumorigenesis within the context of the multistage model of carcinogenesis.

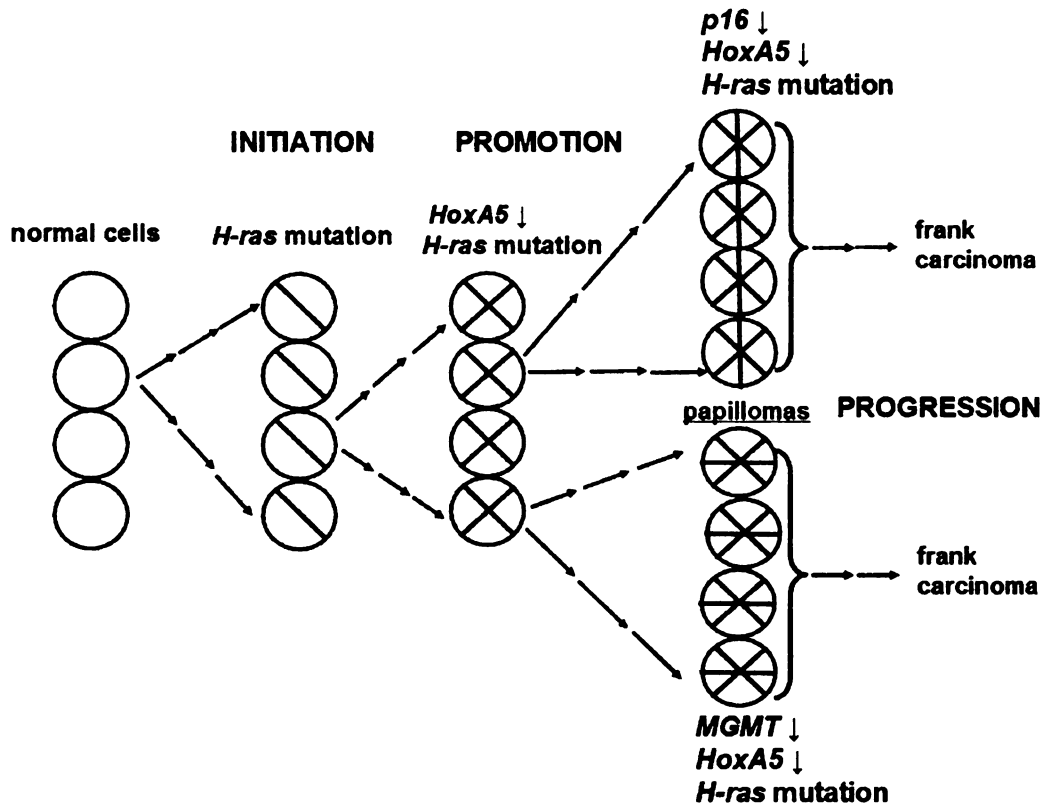


Figure 8. **DNA methylation might play a role as a key driver for the stepwise clonal expansion of increasingly abnormal cells in the promotion stage of carcinogenesis.** Each line through a cell denotes a critical, heritable change to the genome, caused by a mutation or epigenetic change; arrows indicate cellular replication. In this hypothetical example, initiation stems from mutation of *H-ras*. Promoter treatment would selectively stimulate proliferation of cells bearing the *H-ras* mutation. Methylation of *HoxA5,* leading to decreased expression, could constitute a "critical event" in the promotion stage of carcinogenesis. Reduced levels of HOXA5 could decrease *p53* expression, reducing the rate of apoptosis in cells with genetic damage and an increased rate of cellular proliferation. A decreased expression of *p16* mediated by hypermethylation would further increase proliferation giving the cells an additional growth advantage. Alternatively, methylation of *MGMT* resulting in its decreased expression would lead to a reduced ability to repair DNA, contributing to mutation. Thus, increased methylation of either gene would further contribute to the formation of additional abnormal cell populations, leading to papillomas, and eventually frank carcinomas.

decrease *p53* activity and capacity for apoptosis. Therefore, cells that bear DMBA-induced damage and exhibit *p53* haploinsufficiency plus *HoxA5* down-regulation could continue to clonally expand under the influence of the promoter. Subsequent methylation-mediated down-regulation of *MGMT* and/or *p16* could facilitate the further clonal expansion of increasingly abnormal cells. This may be accomplished by decreasing DNA repair and increasing proliferation rate, respectively, eventually leading to papilloma formation. The decreased level of global methylation observed in the papillomas (Watson et al., 2003), could further encourage clonal expansion of increasingly abnormal cells by up-regulating oncogenes and increasing expression of transposable elements (Counts and Goodman, 1996). Global decreases in methylation are frequently observed within the same genome exhibiting increases in GC-rich methylation tissue (Ehrlich, 2002). Eventually, a subset of the papillomas may acquire additional genetic alterations leading to frank carcinoma.

Hypermethylation of *HoxA5*, *MGMT* and *p16* most likely to represent a subset of tumor suppressors methylated and down-regulated throughout promotion, possibly facilitated by an increased activity of Dnmt1. This study is the first report of the reversibility of *HoxA5* methylation patterns regulating expression and describes how *HoxA5*, *p16*, and *MGMT* hypermethylation could contribute to tumorigenesis within the context of the multistage model of carcinogenesis.

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CHAPTER 4

THE VALUE OF DNA METHYLATION ANALYSIS IN BASIC, INITIAL TOXICITY ASSESSMENTS

This chapter represents a manuscript that was submitted to *Toxicological Sciences* in December, 2003. Authors include:Watson, Rebecca E., McKim, James M., Cockerell, Gary L., and Goodman, Jay I..

ABSTRACT

DNA methylation is an epigenetic mechanism that regulates patterns of gene expression. Here, our goal was to take a first step towards determining if assessment of DNA methylation might be a useful tool when used in conjunction with initial, basic *in vitro* tests, to provide a more informative preliminary appraisal of the toxic potential of chemicals in order to prioritize them for further evaluation. Our aim was to provide a better picture of a compound's toxic potential and an indication of its possible mechanism of action at an earlier time, thus contributing to a rational approach toward an overall reduction in testing by helping to make improved decisions early in the process. Given that alteration in DNA methylation in and of itself is not necessarily indicative of toxicity, global and GC-rich patterns of DNA methylation were evaluated in conjunction with more traditional cytolethality measurements, e.g., cytolethality and genotoxicity assessments, on rat hepatoma (H4IIE) cells. The relative "toxic" potential of model compounds camptothecin, 5-fluorouracil, rotenone, and staurosporine was estimated by employing DNA methylation assessments combined with our cytolethality data plus genotoxicity information gleaned from the literature. The overall contribution of the methylation assessment was threefold: 1) it strengthened a ranking based upon genotoxicity, 2) it provided an indication that a compound might be more potentially problematic than what cytolethality and genotoxicity assessments alone would indicate, and 3) for nongenotoxic compounds, those that are more potent with regard to ability to alter methylation, particularly at non-cytolethal concentrations, may be more potentially toxic.

INTRODUCTION

There is an increasing need for more informative preliminary tests to predict the toxic potential of chemicals to prioritize them for further evaluation. This is pertinent for the screening of environmental compounds as well as for the development of medicines and consumer products. For practical purposes, when faced with large numbers of small amounts of compounds, initial evaluations will be based upon results of *in vitro* studies. Clearly, the initial assessments should be predictive of *in vivo* toxic effects and amenable to dose (concentration)-response analysis. In light of the recent change in the paradigm of drug discovery leading to the development of very limited quantities of numerous potential lead compounds using combinatorial chemistry, the need for enhanced *in vitro* approaches for basic, initial assessment of toxicity is particularly acute in the pharmaceutical industry (Furka, 2002). In this context, it is important to note that a high percentage of potential new medicines currently fail due to toxicity, often during preclinical or clinical trials, resulting in a significant waste of time and resources (Cockerell et al., 2002).

Typically, initial assessments of toxicity include measurements of cytolethality and genotoxicity (including mutagenicity). Knowledge concerning the mutagenic potential of a compound is an important component of a basic, initial safety assessment (Ames, 1979; Rueff et al., 1996). However, different mutagenicity assays performed on the same compound can produce markedly disparate results (Choi et al., 1996). Structure-activity relationships often provide a basis for selection of potential drug candidates in the pharmaceutical industry, and this approach has also been used to try to identify compounds acting at sites known to elicit a toxic response (Woo et al., 1995).

Toxicogenomics holds out the potential to develop into a useful screening tool for identification of the toxic potential of chemicals (Tennant, 2002). However, a substantial effort is necessary in order to evaluate this approach, including data analyses, more thoroughly before it can be employed on a routine basis.

We propose that DNA methylation analysis might be a useful tool when used in conjunction with initial, basic *in vitro* tests, e.g., cytolethality and genotoxicity assessments. This can provide increased knowledge of a chemical's toxic potential and contribute to an enhanced ability to prioritize compounds for further evaluation. Methylation of cytosine residues of DNA is an epigenetic mechanism that regulates gene expression as well as tissue-specific, developmental, immunological and neurological processes (Robertson and Jones, 2000). Both hypo- and hypermethylation may lead to deleterious effects. In general, increases in methylation at promoter regions leads to transcriptional silencing by directly hindering the binding of transcription factors or by recruiting proteins that bind methylated cytosines, e.g., chromatin deacetylase (Attwood et al., 2002). Conversely, hypomethylation may lead to the increased expression of certain genes and/or the loss of genomic stability via expression of transposable elements that are normally silenced by methylation (Counts and Goodman, 1995; Carnell and Goodman, 2003). Alterations to normal patterns of methylation have been shown to play a role in cancer (Counts and Goodman, 1995) as well as in developmental, neurological, and immunological disorders (as reviewed in Watson and Goodman, 2002a). Thus, altered methylation can lead to aberrant transcription of genes and, therefore, might form a basis for a variety of toxic outcomes. However, there is a possible positive side in that compounds that are found to affect methylation might be useful in cancer therapy; a

currently employed anticancer drug, azacytosine, acts by decreasing DNA methylation (Goffin and Eisenhauer, 2002).

Here, we performed DNA methylation analysis in conjunction with more traditional cytolethality assessments on rat hepatoma (H4IIE) cells treated with the known demethylating agent azacytidine, as well as the model compounds camptothecin, 5-fluorouracil, rotenone, and staurosporine. Our goal was to take a first step towards determining if assessments of DNA methylation might assist in improving basic, initial, toxicological screens.

In our view, the appropriate initial approach should be a general one, involving an evaluation of global methylation status and an assessment of methylation in GC-rich regions of the genome, rather than attempting gene-specific evaluations. Our aim is to determine if assessment of DNA methylation can provide a useful added dimension to basic, initial toxicity assessment of a compound's toxic potential and an earlier indication of its possible mechanism of action. This could aid in selecting and prioritizing those compounds that should be considered for further evaluation and contribute to an overall reduction in testing by helping to make improved decisions early in the process.

MATERIALS AND METHODS

Cell culture and DNA purification

H4IIE rat hepatoma cells (between passages 7-9) were grown in 96- and 6-well plates for *in vitro* toxicity analysis and for methylation analysis, respectively. We have conducted experiments to ascertain that results from these *in vitro* toxicity assessments do not vary between 96 and 6 well plates (data not shown). Cells to be used for methylation analysis were dosed with concentrations of compounds deemed to be cytolethal and non-cytolethal based on a battery of *in vitro* cytolethality assessments. After a 72 hour incubation, cells were washed twice with PBS, trypsinized, centrifuged, and frozen at -80°C until use. DNA was extracted using Trizol® reagent (Sigma-Aldrich®, St. Louis, MO) and stored at 4°C until use.

Proof of principle compound: 5-aza-2'deoxycytidine

Our initial studies focused on our proof-of-principle compound 5-aza-2'deoxycytidine (dAzaC, purchased from Sigma Aldrich®), a cytosine analog known to cause demethylation by incorporating into DNA and irreversibly binding DNA methyltransferase, thus inhibiting methylation of newly replicated DNA (Lu and Randerath, 1984).

Model Compounds

Following the initial studies with dAzaC, four model compounds with varying modes of action and different toxic effects were selected. None of these compounds were known to have any effect on DNA methylation. Camptothecin is an S-phase specific

anticancer agent that inhibits the activity of DNA topoisomerase I, leading to replication fork arrest as well as single- and double-strand DNA breaks (Morris & Geller, 1996). 5-fluorouracil (5-FU) is a pyrimidine analog that is metabolized to 5-fluorodeoxyrudine monophosphate, a compound that competes with deoxyuridine monophosplate for thymidylate synthetase. Normally, thymidylate synthetase catalyzes the conversion of deoxyuridine monophosphate to thymidine monophosphate, a precursor of thymidine triphosphate, a necessary component of DNA (Parker & Cheng, 1990). Thus, the overall effect of 5-FU is to inhibit replication. Rotenone inhibits complex I of the mitochondrial oxidative phosphorylation chain, stopping the supply of electrons to quinol cytochrome c oxidoreductase. This decreases ATP production and the release of cytochrome c from the mitochondria as well as the increased permeability of the mitochondrial membrane leads to caspase-mediated apoptosis (Pei et al., 2003). Staurosporine is a nonspecific inhibitor of protein kinases which promotes apoptosis through both caspase-dependent and independent mechanisms (Belmokhtar et al., 2001). Staurosporine also inhibits the catalytic activity of topoisomerase II by blocking the transfer of phosphodiester bonds form DNA to the active tyrosine site (Lassota et al., 1996). All compounds described were purchased from Sigma-Aldrich®.

In vitro Toxicity Assessments

In vitro toxicity assessments for each compound included measurements of adenosine triphosphate (ATP), cell number, glutathione-S-transferase (GST), and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as part of the Tox Cluster battery of assays described by McKim et al. (2001).

ATP assay

ATP serves as the principal immediate donor of free energy and is present in all metabolically active cells (Crouch et al., 1993). Levels of ATP decline rapidly when cells are injured, and this can be easily measured using an ATP bioluminescence assay in which a luciferin ATP substrate was added which interacts with ATP and oxygen to form oxyluciferin, AMP, PP_i, CO_2, and light (Crouch et al., 1993). The ATPLite-M™ Packard ATP bioluminescence assay kit was used to measure the amount of ATP in the H4IIE cells. The amount of ATP is extrapolated from the amount of light emitted as measured by a spectrophotometer (Packard®, Palo Alto, CA). Results are expressed as percentage of control values.

Cellular Proliferation Assay

Measurements of cellular proliferation provide a general measure of toxicity. Cell number was assessed using the CyQUANT cell proliferation assay kit from Molecular Probes® (Eugene, OR), a highly sensitive, fluorescence based microplate assay for determining the number of cultured cells (Jones et al., 2001). Cells were rinsed with PBS to remove dead cells no longer adhering to the plate, lysed, and the DNA was stained using the CyQUANT fluorescent dye. Fluorescence was measured using a Packard Spectracount fluorescence reader. Using a standard curve generated from fluorescence readings of known amounts of H4IIE cells, the cell number in our samples was extrapolated.

GST assay

GST leakage is linked to a loss of membrane integrity and necrosis in hepatocytes, and thus, the amount of GST is related to cell viability (Giannini et al., 2000). To measure GST release into the serum, we used the Biotrin® (Czech republic) Rat Alpha GST Enzyme Immunoassay. After 72 h, serum from the cells was removed, diluted 1:4 with media, and 100 µl/well of the diluted serum was placed into 96-well plates coated with IgG antibody. The cells were incubated for 1 h at room temperature using a rotary mixer. Plates were then washed 6 times using the Biotrin Wash Buffer. After removing all the fluid from the plate, 100µl/well of the Biotrin Conjugate was added. This conjugate binds to the IgG-bound GST. Plates were incubated with the conjugate for 1 h at room temperature using a rotary mixer, and then washed 6 times using Biotrin Wash buffer. After removing all the fluid from the plate, 100 µl of Biotrin TMB substrate was added to each well. The plates were incubated for 15 min. at room temperature using a rotary mixer. Following incubation, 50 µl stop solution was added to each well and plates were read using a Packard Spectracount spectrophotomer. The % damaged GST-releasing cells and % non-damaged cells (not releasing GST above basal values) is determined using a standard curve generated from standards containing known percentages of control and 50µM digitonin-treated cells. Digitonin damages cells and elicits GST release. GST Results are presented as the % of control cells not releasing GST above basal values.

MTT assay

Measurements of MTT provide a general measurement of mitochondrial dehydrogenase activity and cell viability (Rodriguez and Acosta, 1997). The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue MTT formazan product by mitochondrial dehydrogenases (Mossman, 1983). Each well of H4IIE cells within 96 well plates was incubated with 100 µl of a 0.5 mg/ml MTT solution for 3 h. Following the MTT incubation, the media was removed by aspiration and 200 µl of isopropanol was added to each well to dissolve and solubilize the intracellular MTT formazan product. After a 20 minute incubation with isopropanol (with shaking) in the dark, the optical density of each well was assessed at 570 and 850 nm using a Packard Spectracount spectrophotometer. Results are expressed as a percentage of control values. MTT was purchased from Sigma-Aldrich®.

Rationale by Which Cytolethal and Non-cytolethal Concentrations of Model Compounds Were Selected

Based upon dose-response analysis, the threshold concentration was estimated to be the first concentration below which there was no statistically significant change compared to measurements in untreated control cells and above which there was a significant change in at least two of the parameters. A concentration equal to 10-25% of this value was used as the non-cytolethal concentration. The cytolethal concentration was selected as the first concentration at which the percent control values for at least two of the assays was between 25 and 40%. Thus, non-cytolethal and cytolethal concentrations were chosen in a uniform manner for each model compound. See Figure

3 for an illustration of concentration-response curves and concentration selection for each model compound.

Global DNA methylation analysis: *SssI* methylase assay

SssI methylase utilizes S-adenosyl methionine as a methyl group donor to methylate the 5' position of cytosine at unmethylated CpG sites in DNA. Thus, the level of global DNA methylation can be determined by the amount of tritiated methyl groups from [^{3}H-CH_3] S-adenosyl-L-methionine incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the original degree of methylation (Balaghi and Wagner, 1993). DNA (1 μg) was incubated with 2 μCi [^{3}H-CH_3] S-adenosyl-L-methionine (New England Nuclear, Boston, MA) and 3 units of *SssI* methylase (New England Biolabs, Beverly, MA) for 1 h at 30°C. Results are presented as counts per minute per microgram (cpm/μg) DNA. Five replicates were performed per sample. Graphical presentation was performed using Excel®. Statistical analysis was performed with Excel using two-tailed t-tests to compare the average cpm/μg DNA measurements between treatment groups and controls. A p value of < 0.05 was considered statistically significant.

Methylation analysis of GC-rich regions

Restriction digests

For each DNA sample, 3 restriction digests were performed as follows: *RsaI* alone, *RsaI* and *MspI*, and *RsaI* and *HpaII*. *RsaI* is a methylation-insensitive enzyme used to cut the DNA into smaller fragments. Both *MspI* and *HpaII* are methylation-

sensitive enzymes that cut between cytosine residues at 5'-CCGG-3' sites. *MspI* will not cut if the external cytosine is methylated, while *HpaII* will not cut if the internal cytosine is methylated; both will cut if the site is unmethylated (Mann and Smith, 1977). All enzymes used were from Roche® (Indianapolis, IN). Restriction digests were performed with 1 μg of DNA and 5.0 units of *RsaI* in Roche buffer L. After a 1 h incubation (with shaking) in a water bath at 37°C, two 2.5 unit aliquots of *MspI* or *HpaII* were added, 2 h apart. The total incubation time was 18 h. The enzymes were inactivated by a 10 min incubation at 65°C, and the digests were stored at 4°C until amplified by PCR.

Arbitrarily primed (AP)-[^{33}P] PCR

PCR was performed on restriction digests using a single primer (5'-AACCCTCACCCTAACCCCGG-3') that arbitrarily binds within GC-rich regions of DNA (Gonzalgo *et al.*, 1997). Reactions were composed of 5 μl of the restriction digest (containing 1 μg digested DNA), 0.4 μM each primer, 1.25 units of Taq polymerase (Gibco BRL, Rockville, MD), 1.5 mM $MgCl_2$, 60 mM Tris, 15 mM ammonium sulfate, 1.65 μCi α-[^{33}P]-dATP (New England Nuclear, Boston, MA), and glass-distilled water to volume. Samples were heated for 5 min at 94°C before addition of dNTPs in order to minimize the possibility of primer-dimer formation. Cycling conditions included a single denature cycle for 2 min at 94°C, followed by 5 cycles under the following conditions: 30 s at 94°C, 1 min at 40°C, 1.5 min at 72°C; then 30 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min, a time delay cycle for 5 min at 72°C, and a soak cycle at 4°C. PCR products (5 μl of each) were separated on a 6 % polyacrylamide sequencing gel at 45 watts for 2 ¼ -2 ½ h. The gel was soaked for 10 min in a fixing solution with 5% acetic

acid and 5% methanol, rinsed for 10 min in glass-distilled water, dried, and placed into a cassette with a storage phosphoimage screen to visualize labeled PCR products. Compared to larger DNA fragments on the upper halves of gels, smaller fragments on the lower halves of gels sometimes required a longer exposure to clearly discern bands. Thus, a short exposure of 3 d followed by a longer exposure of 8 d was often performed on a gel. Phosphoimages were analyzed using Bio-Rad® (Hercules, CA) Quantity One® software.

RESULTS

First, we wanted to determine how the existing *in vitro* toxicity analysis compared to methylation analysis using dAzaC, a drug known to alter DNA methylation. Studies were performed using H4IIE cells treated with 10 and 100 μM concentrations of dAzaC, both concentrations found to be non-cytolethal based on *in vitro* toxicity analysis. These concentrations of dAzaC decreased global methylation levels in a dose-dependent manner (Figure 1). Arbitrarily-primed PCR results show that in the treated samples, there are 5 GC-rich regions in which there was a greater amount of methylation at the external cytosines at 5'-CCGG-3' sites and 2 GC-rich regions in which there was a greater amount of methylation at the internal cytosines at 5'-CCGG-3' sites compared to untreated controls (Figure 2).

Cytolethal and non-cytolethal concentrations were chosen for the model compounds based on *in vitro* toxicity data, as indicated in Figure 3. Global methylation status in cells treated with both cytolethal and non-cytolethal concentrations of these compounds is presented in Figure 4. Global methylation levels of cells treated with both cytolethal and non-cytolethal concentrations camptothecin were not significantly different from the untreated controls. However, treatment with a toxic dose of 5-FU and a non-cytolethal dose of staurosporine led to statistically significant ($p < 0.05$) decreases in the global level of methylation. A cytolethal concentration of staurosporine led to a reduction in global methylation levels, but this was not statistically significant ($p = 0.08$). Also, at both cytolethal and non-cytolethal concentrations, rotenone seemed to increase global levels of DNA methylation, though this was not statistically significant ($p = 0.12$ and 0.09, respectively).

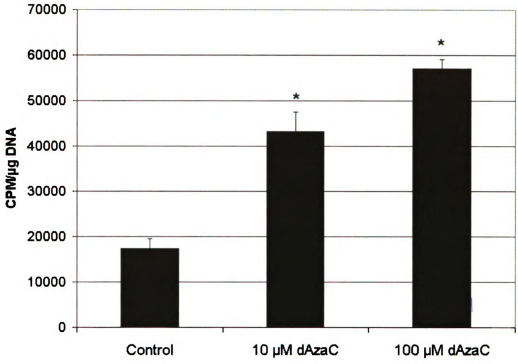


Figure 1. Global methylation levels in and H4IIE cells treated with 10 and 100 µM 5-aza-2'deoxycytidine (dAzaC) for 72 h. Control H4IIE cells were untreated and grown for 72 h. Each bar shown represents the mean CPM/µg DNA value from 4 separate wells. *Statistically significant difference ($p < 0.05$) compared to control group

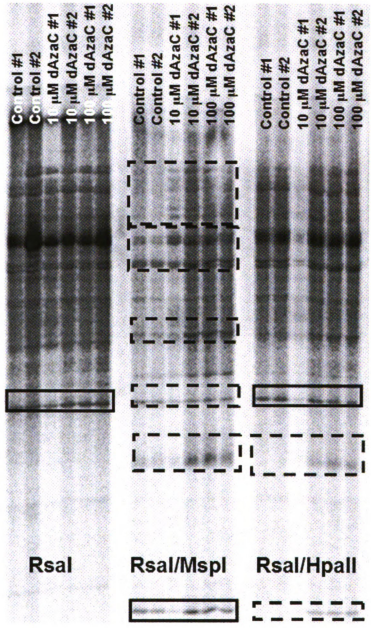
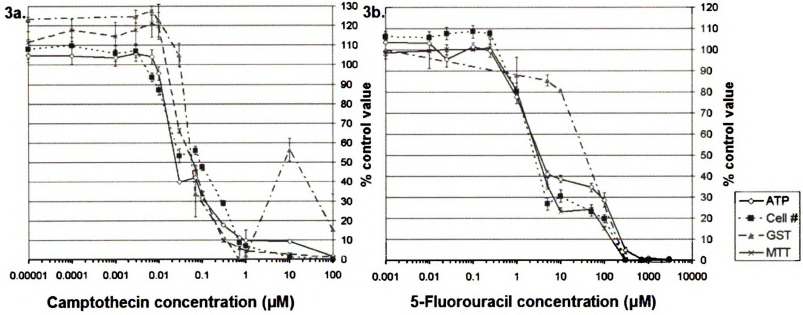


Figure 2. Methylation status of GC-rich regions in H4IIE cells treated with 10 and 100 μM 5-aza-2'deoxycytidine (dAzaC) for 72 h. Results shown indicate GC-rich methylation patterns from 2 separate wells each (#1 and #2 as labeled at the top of the gel), of untreated control cells, and cells treated with 10 μM or 100 μM of dAzaC. These results are representative of 4 separate wells of samples from each group. Dashed boxes indicate regions in which bands are seen more distinctly in the treated samples compared to controls. Solid boxes indicate reference rows of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent.

Figure 3. *In vitro* toxicity assessment of camptothecin (*a*), 5-FU (*b*), rotenone (*c*-next page), and staurosporine (*d*-next page. *In vitro* assays included measurements of ATP, cell number, GST, and MTT. Percent control values for a range of concentrations are indicated. Thick horizontal bars indicate the 25-40% control range used to select cytolethal concentrations for each compound. The thick vertical line indicates the threshold dose below which at least 3 parameters do not vary significantly from control values and above which at least 2 parameters are statistically below the % control values of the preceding concentration. The dotted vertical line indicates the non-cytolethal concentration, chosen to be 10-25% of the threshold. The dashed vertical line indicates the cytolethal concentration. Data was analyzed and cytolethal and non-cytolethal concentrations were selected as indicated in a uniform manner for each model compound. Two replicates of the full set of toxicological parameters were performed for each compound and the results were highly reproducible. A summary of threshold, cytolethal, and non-cytolethal concentrations chosen for each compound is shown in *e*.

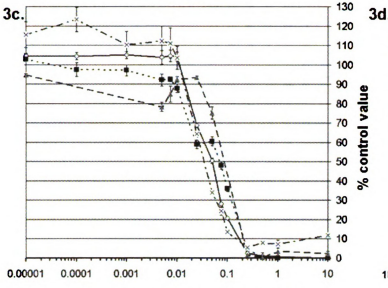


3e.

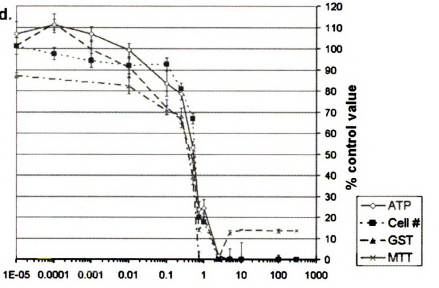
Compound	Non-cytolethal (NCL) concentration	Cytolethal (CL) concentration
Camptothecin	0.001 μM	0.1 μM
5-Fluorouracil	0.25 μM	5.0 μM
Rotenone	0.001 μM	0.07 μM
Staurosporine	0.001 μM	0.75 μM

Figure 3, continued. *In vitro* toxicity assessment of camptothecin (*a*-previous page), 5-FU (*b*-previous page), rotenone (*c*), and staurosporine (*d*). *In vitro* assays included measurements of ATP, cell number, GST, and MTT. Percent control values for a range of concentrations are indicated. Thick horizontal bars indicate the 25-40% control range used to select cytolethal concentrations for each compound. The thick vertical line indicates the threshold dose below which at least 3 parameters do not vary significantly from control values and above which at least 2 parameters are statistically below the % control values of the preceding concentration. The dotted vertical line indicates the non-cytolethal concentration, chosen to be 10-25% of the threshold. The dashed vertical line indicates the cytolethal concentration. Data was analyzed and cytolethal and non-cytolethal concentrations were selected as indicated in a uniform manner for each model compound. Two replicates of the full set of toxicological parameters were performed for each compound and the results were highly reproducible. A summary of threshold, cytolethal, and non-cytolethal concentrations chosen for each compound is shown in *e*.

3c.



3d.



3e.

Compound	Non-cytolethal (NCL) concentration	Cytolethal (CL) concentration
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Rotenone	0.001 μM	0.07 μM
Staurosporine	0.001 μM	0.75 μM

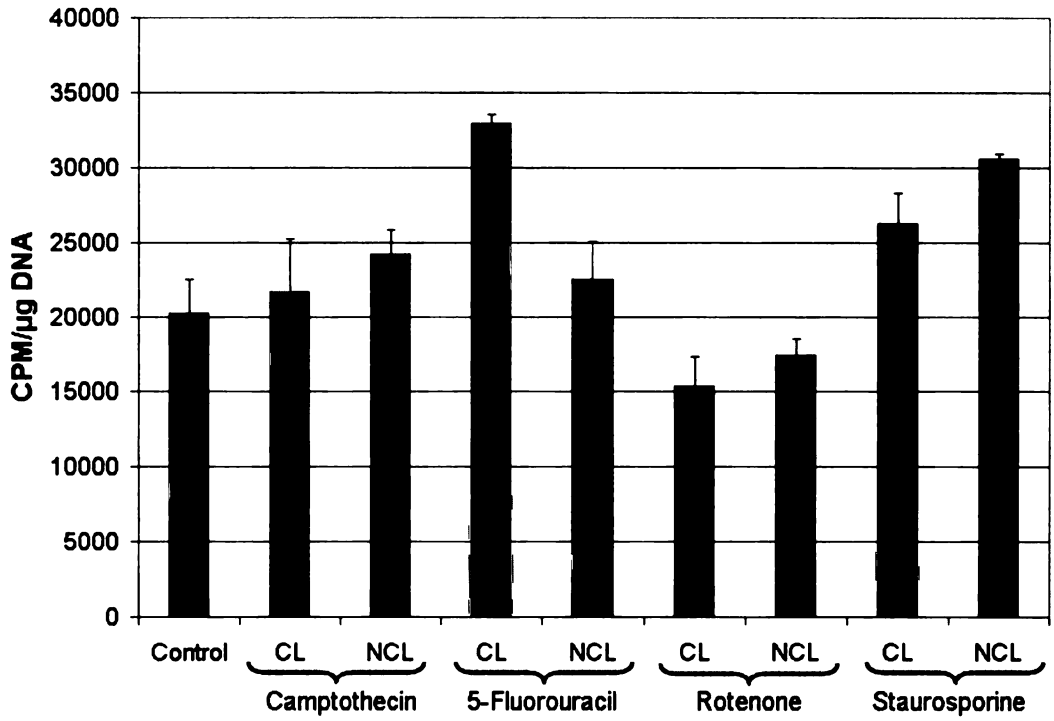
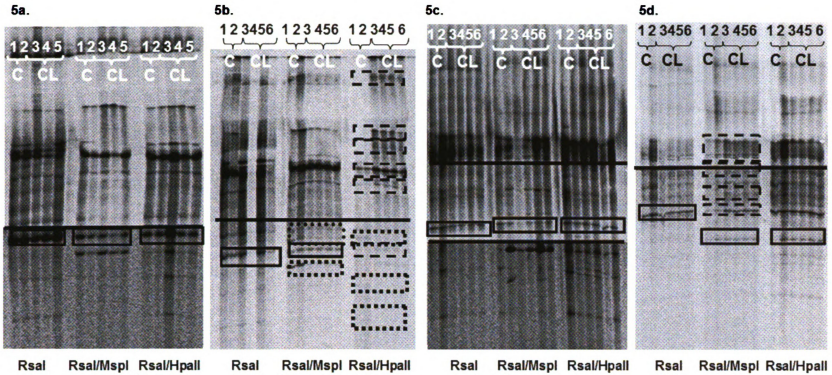


Figure 4. Global methylation levels in H4IIE cells treated for 72 h with camptothecin, 5-FU, rotenone, and staurosporine. Global methylation status in untreated control cells and cells treated with cytolethal (CL) and non-cytolethal (NCL) concentrations of camptothecin, 5-fluorouracil, rotenone, and staurosporine is presented. Mean and standard error values are indicated, with each bar representing the mean CPM/µg DNA value from DNA samples from four separate wells.
* indicates a statistically significant difference ($p < 0.05$) compared to controls.

GC-specific methylation status was assessed for cytolethal doses of all the model compounds (Figure 5). A cytolethal concentration of neither campothecin (5a), nor rotenone (5c) resulted in detectable alterations in the GC-rich methylation patterns of H4IIE cells in comparison to controls. Treatment with a cytolethal concentration of 5-fluorouracil (5b) induced hypermethylation at the internal cytosine of the 5'-CCGG-3' site in 6 GC-rich regions, hypomethylation at the internal cytosine in 3 GC-rich regions, and hypomethylation at external cytosine in 2 GC-rich regions. Treatment with a cytolethal concentration of staurosporine (5d) resulted in hypermethylation at the external cytosine of the 5'-CCGG-3' site in 5 GC-rich regions. Since treatment with cytolethal concentrations of 5-FU and staurosporine led to alterations in GC-rich methylation, the GC-rich methylation status of cells treated with non-cytolethal concentrations of these compounds was assessed (Figure 6). The non- cytolethal concentration of 5-FU did not lead to any GC-specific methylation alterations (6a), but the non- cytolethal concentration of staurosporine led to increases and decreases in methylation at the internal cytosine of the 5'-CCGG-3' site in 5 and 2 regions, respectively (6b). A summary of the results of global and GC-rich DNA methylation analysis is presented in Table 1.

Figure 5. GC-rich methylation in H4IIE cells treated with cytolethal concentrations of camptothecin (*a*), rotenone (*b*), 5-FU (*c*), and staurosporine (*d*). 'C' indicates control, untreated cells 'CL' indicates cells treated with cytolethal concentrations of each compound. Numbers underneath the brackets at the top of the gel indicate individual samples from separate wells within 6-well plates such that n = 2 for control cells and n = 4 for cells treated with a cytolethal dose of each compound. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Dashed boxes indicate rows of bands seen more prominently in treated animals compared to controls and dotted boxes indicate rows of bands seen less prominently in treated animals compared to controls. Solid boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. The top part of the images are from a 1-2 d. exposure, the middle part (in *b*) is from a 3 d. exposure, and the bottom part is from a 7 d. exposure.


5a.
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
C CL C CL C CL
RsaI RsaI/MspI RsaI/HpaII
5b.
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
C CL C CL C CL
RsaI RsaI/MspI RsaI/HpaII
5c.
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
C CL C CL C CL
RsaI RsaI/MspI RsaI/HpaII
5d.
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
C CL C CL C CL
RsaI RsaI/MspI RsaI/HpaII

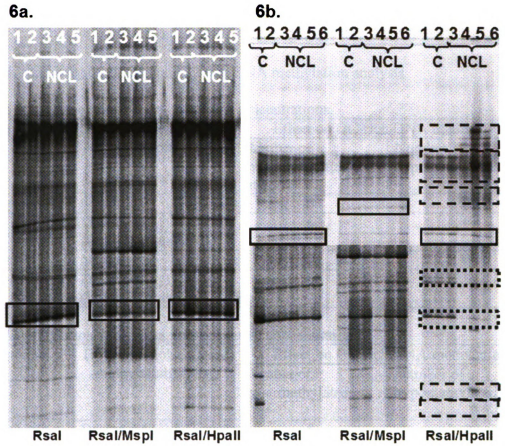


Figure 6. GC-rich methylation in H4IIE cells treated with non-cytolethal concentrations of 5-FU (*a*) and staurosporine (*b*). 'C' indicates control, untreated cells 'Tr' indicates cells treated with non-cytolethal concentrations of fluorouracil and staurosporine. Numbers underneath the brackets at the top of the gel indicate individual samples from separate wells within 6-well plates such that n = 2 for control cells and n = 4 for cells treated with a cytolethal dose of each compound. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Dashed boxes indicate rows of bands seen more prominently in CSC-promoted animals compared to controls and dotted boxes indicate rows of bands seen less prominently in CSC-promoted animals compared to controls. Solid boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. The image shown in *b* is from a 4 d exposure. The top section of the image shown in *a* is from a 2 d. exposure and the bottom section is from a 7 d. exposure.

Table 1. Summary of global and GC-rich DNA methylation analysis

a. DNA methylation analysis at cytolethal concentrations.

Compound	Effect on global DNA methylation status	Effect on GC-rich DNA methylation	
		Number of hypermethylated sites	Number of hypomethylated sites
Camptothecin	No change	0	0
5-FU	Decreased (↓) [a] (Hypomethylation)	6	0
Rotenone	No change	0	0
Staurosporine	Decreased (↓) [b] (Hypomethylation)	6	5

b. DNA methylation analysis at non-cytolethal concentrations.

Compound	Effect on global DNA methylation status	Effect on GC-rich DNA methylation	
		Number of hypermethylated sites	Number of hypomethylated sites
dAzaC	Decreased (↓) [a] (Hypomethylation)	8	0
Camptothecin	No change	Not analyzed [c]	Not analyzed [c]
5-FU	No change	0	0
Rotenone	No change	Not analyzed [c]	Not analyzed [c]
Staurosporine	Decreased (↓) [b] (Hypomethylation)	5	2

Note. These data are presented in Figures 1-6.
[a] Statistically significant, 2-tailed t-test, $p < 0.05$.
[b] There is a trend toward a decrease in global DNA methylation though not statistically significant, 2-tailed t-test, $p = 0.08$.
[c] GC-rich DNA methylation status was not analyzed for camptothecin and rotenone at non-cytolethal concentrations because no such changes were observed at cytolethal concentrations.

DISCUSSION

Based upon the results of our investigation, we believe that the inclusion of an assessment of methylation status as a component of initial, preliminary toxicity testing can help in the prioritization of compounds at early screening stages and contribute to a better understanding of possible mechanisms underlying toxicity. For instance, if two compounds exhibit similar results from cytotoxicity and genotoxicity assays, but one compound affects methylation, and the other does not, this could provide a basis for considering the latter to be less potentially toxic. More effective prioritization is expected to result in an overall decrease in time, cost and testing.

We performed methylation analysis by examining both global and GC-rich methylation in H4IIE cell DNA. These approaches assess different and, importantly, complementary aspects of genome-wide methylation. A focus on gene-specific methylation would not be appropriate during initial toxicity testing. One would not know which gene(s) to examine unless compound-specific changes could be anticipated. Global and GC-rich methylation patterns might be regulated by different methyltransferases and might be affected as a result of administration of a specific compound. For example, our data show that dAzaC, a demethylating agent which irreversibly binds and thus inactivates methyltransferases, decreased global methylation as expected, but also increased methylation at GC-rich sites. Other studies have reported that dAzaC treatment reduces global levels of methylation while increasing methylation at select regions (Grassi et al., 2003; Broday et al., 1999). The basis for the latter effect not known, but this finding indicates that dAzaC might affect methylation at GC-rich regions in a manner secondary to its known mode of action, and provides an additional

example of the importance of examining both global and GC-specific methylation patterns. In support of this, it should be noted that DNMT1 and DNMT3b methyltransferases have a greater *in vivo* binding avidity to dAzaC-containing DNA compared to DNMT2 and 3a (Liu et al., 2003). Perhaps in response to decreasing availability of DNMT1 and 3b, amounts of DNMT2 and 3a are up-regulated as a result of azacytidine treatment and the GC-rich regions shown to be hypermethylated as a result of dAzaC treatment are methylated by these methyltransferases. Even if methylation is affected by a secondary or tertiary mechanism, this finding nonetheless provides more insight into a compound's actions than solely relying on cytotoxicity assessments. Furthermore, mice given phenobarbital exhibit global hypomethylation in liver DNA (Counts et al., 1996), with increased hypermethylation in GC-rich regions (Watson and Goodman, 2002b). Thus, it is informative to look at both global and GC-specific changes.

There are multiple ways in which DNA methylation which may be altered, including perturbing the 1-carbon choline/folate/methionine metabolic pathway required for synthesis of S-adenosyl methionine (SAM), the proximate methyl group donor for DNA (Ziesel and Blusztajn, 1994). For example, arsenic is methylated by SAM, and administration of arsenic is thought to hypomethylate DNA by decreasing the availability of SAM (Okoji et al., 2002). Maintenance of normal DNA methylation may be viewed as a basic homeostatic mechanism. Therefore, detection of alterations of this might enhance early basic toxicity screening by providing a broader picture of a compound's potential toxic effects.

Typically, basic, initial toxicity assessments involve *in vitro* studies aimed at ascertaining the compound of interest's cytolethal and genotoxic affects. In order to illustrate the potential importance of evaluation of DNA methylation for compound prioritization, we will now go through an exercise to illustrate our thought processes as we estimate the relative "toxic" potentials of the model compounds used in this study by employing DNA methylation assessments combined with the cytolethality data presented plus genotoxicity information gleaned from the literature. For the latter, we searched for data concerning four basic in vitro tests, the Ames test, sister chromatid exchange, chromosome aberrations and the mouse lymphoma assay. The model compounds are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action: 'A' represents camptothecin, 'B' represents 5-FU, 'C' represents rotenone, and 'D' represents staurosporine. Using methylation analysis alone, the compounds were ranked according to estimated "toxic" potentials at cytolethal and non-cytolethal concentrations such that a higher number indicates a "safer" compound compared to one ranked with a lower number (Table 2). At cytolethal concentrations, compounds B and D affect multiple changes in DNA methylation while A and C do not. Thus, B and D would be ranked as more toxic than A and C (Table 2a). However, at non-cytolethal concentrations, only compound D affected DNA methylation. We view methylation alterations at non-cytolethal concentrations as being more significant than changes that occur only at cytolethal concentrations. Therefore, D is ranked more toxic than A, B, and C (Table 2b). Thus if all four compounds were nongenotoxic, methylation data plus information concerning cytolethality could be very helpful with regard to an initial prioritization regarding potential toxicity.

Table 2. Estimated "toxic" potential rankings of model compounds based on methylation analysis at cytolethal and non-cytolethal concentrations.

a. Methylation analysis at cytolethal concentrations

Compound [a, b]	Effect on global DNA methylation status	Effect on GC-rich DNA methylation		Estimated "toxic" potential ranking [c]
		Number of hypermethylated sites	Number of hypomethylated sites	
B	Decreased (↓) [d] (Hypo-methylation)	6	0	1
D	Decreased (↓) [e] (Hypo-methylation)	6	5	1
A,C	No change	0	0	2

b. Methylation analysis at non-cytolethal concentrations

Compound [a, b]	Effect on global DNA methylation status	Effect on GC-rich DNA methylation		Estimated "toxic" potential ranking [c]
		Number of hypermethylated sites	Number of hypomethylated sites	
D	Decreased (↓) [d] (Hypo-methylation)	5	2	1
A, B, C	No change	0	0	2

Note. For each compound, the threshold value was estimated to be the concentration below which there was no statistically significant change in cytolethality compared to untreated control cells and above which there was a significant change in at least two cytolethality parameters. A concentration used that was equal to 10-25% of the threshold value was employed. The cytolethal concentration used was selected as the lowest concentration at which the % control values for at least two cytolethality parameters was between 25 and 40%.

[a] The model compounds presented in Table 1 are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action and to emphasize the use of methylation data to derive an estimated "toxic" potential ranking.

[b] A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

[c] Estimated relative potential to elicit a "toxic" reponse, such that a higher number represents a potentially "safer" compound and a lower number represents one more likely to be problematic.

[d] Statistically significant, 2-tailed t-test, $p < 0.05$.

[e] There is a trend toward a decrease in global DNA methylation seen here that is close to reaching statistical significance in a 2-tailed t-test, $p = 0.08$.

Compounds were also ranked based on the concentrations at which a cytolethal effect was obtained (Table 3). This is a crude method of ranking, likely to be important only if a compound is toxic at very low (pM) concentrations, or if there is a extreme range of potencies of the compounds of interest. In this case, the cytolethal concentrations of the model compounds are within a 100-fold range. The genotoxicity data are presented in Table 4. We then used the genotoxicity data with and without the cytolethality data (Table 3) to rank the compounds based on estimated "toxic" potential. Genotoxicity data alone indicated that since A and B are genotoxic and C is not, A and B would be more likely to pose a "toxic" response than compound C (Table 5a). Cytolethality data indicated that a lower dose of A was needed to elicit a cytolethal effect than B, thus permitting these two compounds to be separated in ranking (Table 5b). Finally, methylation data at non-cytolethal concentrations was combined with genotoxicity and cytolethality data to rank the compounds (Table 6). Though compound C was most cytolethal, it ranked least potentially toxic when genotoxicity and methylation data were also considered (Table 6a). If data were available indicating that compound D was equal to or more genotoxic than A and B, then D would be considered the most potentially "toxic" of the group (Figure 6b). The overall contribution of DNA methylation assessments in this initial toxic potential evaluation exercise was threefold: 1) it both supported and strengthened the ranking based upon genotoxicity indicating that compound C was less potentially toxic than compounds A and B in this example, 2) it provided an indication that compound D was more potentially problematic than indicated by cytolethality and genotoxicity assessments, and 3) for nongenotoxic compounds,

Table 3. Toxic potential ranking of the model compounds based on concentration needed to elicit a cytolethal response

Compound [a, b]	Cytolethal concentration (μM)	Estimated "toxic" potential rank [c]
A, C [d]	0.07, 0.10	1
D	0.75	2
B	5.0	3

Note. For each compound, the threshold value was estimated to be the concentration below which there was no statistically significant change in cytolethality compared to untreated control cells and above which there was a significant change in at least two cytolethality parameters. A concentration equal to 10-25% of the threshold value was used as the non-cytolethal concentration. The cytolethal concentration was selected as the first concentration at which the % control values for at least two cytolethality parameters was between 25 and 40%.

[a] The model compounds presented in Table 1 are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action and to emphasize the use of methylation data to derive an estimated "toxic" potential ranking.

[b] A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

[c] Estimated relative potential to elicit a "toxic" response, such that a higher number represents a potentially "safer" compound and a lower number represents one more likely to be problematic.

[d] These compounds were assigned the same rank because the cytolethal concentrations were nearly equivalent.

Table 4. Summary of *in vitro* genotoxicity data for the model compounds

Compound [a, b]	*In vitro* genotoxicity data			
	Ames test	Sister chromatid exchange test	Chromosomal aberration test	Mouse lymphoma test
A	Not available	Positive	Positive	Positive
B	Negative	Positive	Positive	Positive
C	Negative	Negative	Negative	Positive
D	Not available	Not available	Not available	Not available

Note. For the purposes of this exercise the search for genotoxicity data was limited to results of the four common tests presented. These data were obtained from references cited in TOXLINE, National Institutes of Health and National Toxicology Program databases, (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE, http://toxnet.nlm.nih.gov/cgi-fin/sis/htmlgen?Multi and http://ntp-server.niehs.nih.gov, respectively).

[a] The model compounds presented in Table 1 are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action and to emphasize the use of methylation data to derive an estimated "toxic" potential ranking.

[b] A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

Table 5. Estimated "toxic" potential rankings based on *in vitro* genotoxicity data (Table 4) without and using cytolethality data (Table 3)

a. Toxic potential ranking using *in vitro* genotoxicity data.

Compound [a, b]	Estimated "toxic" potential rank [c]
A,B	1
C	2
D	[d]

b. Toxic potential ranking using *in vitro* genotoxicity data combined with cytolethality data

Compound [a, b]	Estimated "toxic" potential rank [c]
A	1
B	2
C	3
D	[d]

[a] The model compounds presented in Table 1 are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action and to emphasize the use of methylation data to derive an estimated "toxic" potential ranking.
[b] A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.
[c] Estimated relative potential to elicit a "toxic" response, such that a higher number represents a potentially "safer" compound and a lower number represents one more likely to be problematic.
[d] Due to a lack of *in vitro* genotoxicity data, D could not be ranked.

Table 6. Estimated "toxic" potential rankings based on methylation analysis at non-cytolethal concentrations (Table 2) in combination with cytolethality (Table 3) and *in vitro* genotoxicity data (Table 4)

a. Estimated "toxic" potential ranking assuming compound D [a, b] is less genotoxic than A and B

Compound [a, b]	Estimated "toxic" potential rank [c]
A	1
B	2
D	1 or 2 [d]
C	3

b. Estimated "toxic" potential ranking assuming compound D [a, b] is as or more genotoxic than A and B

Compound [a, b]	Estimated "toxic" potential rank [c]
D	1
A	2
B	3
C	4

[a] The model compounds presented in Table 1 are now indicated by letter to de-emphasize the fact that we have knowledge of their mechanisms of action and to emphasize the use of methylation data to derive an estimated "toxic" potential ranking.

[b] A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

[c] Estimated relative potential to elicit a "toxic" response, such that a higher number represents a potentially "safer" compound and a lower number represents one more likely to be problematic.

[d] The ranking of D relative to A and B was not determined because we cannot compare the relative "toxic" potentials of a compound that elicited changes in methylation (D) with those that elicit positive results in genotoxicity assays (A and B).

those that were more potent with regard to ability to alter methylation, particularly at non-cytolethal concentrations (Table 2b), might be more potentially toxic.

Additionally, it is very important to recognize that an alteration in DNA methylation in an of itself is not necessarily indicative of toxicity; certain changes in methylation might be representative of normal biological processes, and methylation is a reversible mechanism (Ramchandani et al., 1999). Also, one must consider that human cells are more capable of maintaining normal methylation status compared to rodent cells (reviewed in Goodman and Watson, 2002). For these reasons, methylation analysis needs to be viewed as a component of an overall toxicity assessment.

This study serves as a promising first step in assessing the utility of methylation analysis in early stages of toxicity testing. A next step forward should involve *in vitro* and *in vivo* dose-response comparisons, e.g., are the *in vitro* data predictive of *in vivo* toxicity, and is methylation status altered in target organs? Currently, gene array approaches to assess methylation status are too cumbersome with regard to cost and data analysis issues to be used on a routine, initial basis. However, attempts should be made to adapt methylation analysis to high throughput approaches, e.g. separation of random primed PCR products by capillary electrophoresis rather than the use of polyacrylamide gels.

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SUMMARY

My initial research described in Chapter 1 demonstrated how the ability to maintain patterns of methylation in GC-rich regions might underlie differences in tumor susceptibility in three groups of mice. PB-induced hypermethylation at GC-rich regions was more marked in the relatively tumor-sensitive C3H/He and B6C3F1 groups compared to the relatively tumor-resistant C57BL/6 strain. A previous study performed by Counts et al. demonstrated that the same treatment led to a greater amount of global hypomethylation in the tumor-sensitive C3H/He and B6C3F1 mice compared to the tumor-resistant C57BL/6 mice. Though these findings might at first glance appear to be contradictory, both studies supported the notion that tumor susceptibility is related inversely to the capacity to maintain patterns of methylation. The global and GC-rich methylation changes observed could contribute to carcinogenesis in a number of ways: global hypomethylation could contribute to expression of oncogenes and transposable elements, while GC-rich hypermethylation could silence tumor suppressors (Laird, 1997). Thus, the decreased ability of the tumor-sensitive C3H/H3 and B6C3F1 mice to maintain patterns of global and GC-rich methylation is likely to explain, in part, their increased susceptibility to carcinogenesis.

Secondly, in Chapters 2 and 3, I characterized global, GC-rich and gene-specific methylation changes within the context of the multistage model of carcinogenesis using an initiation/promotion SENCAR mouse skin model in which mice were initiated with DMBA and promoted with different doses of CSC for various amounts of time. I found that increases in GC-rich methylation patterns occur in non-tumor and tumor tissue in a dose-and time-dependent, threshold-exhibiting, reversible fashion during the promotion

stage of carcinogenesis. Tumor tissue exhibited global hypomethylation, but no other tissue exhibited a change in global methylation status. Gene array analysis demonstrated that the *HoxA5* tumor suppressor gene was down-regulated in a reversible fashion as a result of CSC promotion, and methylation analysis demonstrated that repression of *HoxA5* was tightly linked to an increased amount of methylation in the *HoxA5* promoter region. This is the first report indicating reversibility of *HoxA5* methylation regulating *HoxA5* expression. Additionally, I found that increased methylation status of either *p16* or *MGMT* was found in 4/8 (50%) tumor samples, compared to none of the controls. Methylation and subsequent down-regulation of the *HoxA5, p16*, and *MGMT* tumor suppressors could contribute to a cascade of expression changes that could facilitate the clonal expansion of increasingly abnormal cells in the promotion stage of carcinogenesis.

In Chapter 4, I analyzed the methylation status of rat hepatoma cells treated with cytolethal and non-cytolethal doses of various model compounds and used this methyation data in combination with genotoxicity and cytotoxicity data to rank the compounds based on their estimated toxic potential. I found that methylation analysis contributed to the overall ranking of the compounds, indicating that its inclusion into initial, basic *in vitro* assessments of toxicity might be valuable.

In the following outline, the specific aims set forth in the introduction are listed below, and addressed in bold.

Specific Aims Addressed

1. To examine the effects of PB on DNA methylation in GC-rich regions of hepatic DNA from groups of mice that exhibit different levels of susceptibility to liver tumorigenesis.

    a) To determine if differences in cancer susceptibility are linked to differences in the ability to maintain normal patterns of methylation in response to PB.

    In response to PB, GC-rich methylation patterns were more markedly altered (mostly increased) in the relatively tumor-sensitive C3H/H3 and B6C3F1 mice compared to the relatively tumor-resistant C57/BL6

    b) To compare methylation status at GC-rich regions with previous measurements of global methylation change.

    Using this same model, previous studies by Counts et al., (1996) demonstrated that the relatively tumor sensitive C3H/He and B6C3F1 mice exhibited a significantly greater decrease in global levels of methylation in response to PB treatment compared to the relatively tumor resistant C57/BL6 mice. These findings appear to contradict mine, but tumor tissues are commonly characterized by a decrease in global methylation with an increase in methylation at GC-rich regions (Ehrlich, 2002) and importantly, both studies indicated that the relatively tumor sensitive mice exhibited a decreased ability to maintain normal patterns of DNA methylation compared to the relatively tumor resistant mice.

2. To assess methylation changes during stages of carcinogenesis using an

initiation/promotion SENCAR mouse model.

Animals used in this study were initiated with DMBA and promoted with different doses of CSC for various amounts of time. Methylation changes in GC-rich regions occurred were observed in non-tumor tumor tissue in a time-and dose-dependent, reversible fashion in the promotion stage of carcinogenesis. Global hypomethylation was observed in the tumor tissue, and global methylation change was not seen in any non-tumor tissue.

a) To determine threshold doses of CSC necessary to detect changes in methylation at particular timepoints.

The threshold dose of CSC necessary to elicit changes in methylation at GC-rich regions at 6 wks is between 18 and 27 mg. At 9 wks, the threshold dose is between 9 and 18 mg, illustrating dose- and time-dependent methylation alterations.

b) To determine whether changes in methylation at GC-rich regions preceded global decreases or vice versa.

In this model GC-rich methylation changes (mostly increases) were observed in non-tumor and tumor tissue, and global methylation was only altered in tumors, suggesting that in this model GC-rich methylation alterations precede changes in global methyation status.

c) To assess the potential for reversibility of altered methylation in precancerous tissue.

Alterations in GC-rich methylation patterns observed in animals promoted with 27 mg CSC for 9 wks and sacrificed immediately afterwards were not as

marked as those seen in animals given the same promotion schedule but sacrificed following a 6 wk recovery period, indicating that GC-rich methylation alterations were reversible in this model.

d) To ascertain whether particular methylation changes correlate to tumor formation.

Global methylation decrease was a methylation alteration unique to tumor tissue. Furthermore, the 18 mg threshold dose of CSC needed to elicit an increase in GC-rich methylation status following 9 wks or promotion mirrored the threshold necessary for a marked induction in tumor formation following 29 wks of promotion.

3. To assess gene-specific methylation patterns and determine how DNA methylation might be correlated to gene expression in an initiation/promotion SENCAR mouse skin model.

I found that methylation of the tumor suppressor *HoxA5* in the promotion stage of carcinogenesis is reversible, and correlates to a decrease in expression. Additionally, hypermethylation of the tumor suppressors *p16* and *MGMT* was assessed in tumor tissue; either *p16* or *MGMT* was hypermethylated in 50% of tumor tissues compared to none of the initiated-only control samples.

a) To identify a tumor suppressor gene that was down-regulated due to treatment with CSC.

***HoxA5* was significantly down-regulated in animals treated with 27 or 36 mg CSC for 9 wks compared to initiated-only controls. The down-regulation in expression was reversible; animals promoted with 27 mg for 9 wks and given a 6 wk recovery period prior to sacrifice (recovery group) did not exhibit *HoxA5* down-regulation.**

b) To determine if methylation status of the promoter region of the down-regulated tumor suppressor identified in a correlated with expression.

Methylation status was significantly increased in groups promoted with 27 and 36 mg CSC and sacrificed immediately afterwards compared to control or recovery group animals, indicating a distinct inverse relationship between methylation of the *HoxA5* promoter region and *HoxA5* expression. Since this increase in methylation was not observed in the recovery group, the increase in methylation can be seen as a reversible effect.

c) To determine the methylation status in the promoter region of known tumor suppressor genes in skin tumor tissue.

Methylation status of the tumor suppressors *MTMT* and *p16* was assessed in tumor tissue from animals promoted with 27 mg CSC for 29 wks. I found that *MGMT* was hypermethylated in 2/8 tumor tissues, and *p16* was hypermethylated in 2/8 tumor samples. The tumors exhibiting hypermethylation of *p16* were not the same samples exhibiting hypermethylation of *MGMT*, such that 4/8 (50%) of the tumors exhibited an increased amount of methylation in the promoter region of either *p16* or *MGMT*.

4. To determine the value of DNA methylation analysis in basic, initial toxicity assessments.

a) To assess global and GC-specific DNA methylation patterns at concentrations of the model compounds (camptothecin, 5-FU, rotenone, and staurosporine) found to be cytolethal and non-cytolethal based on more traditional *in vitro* toxicity assays (measurements of cell number, ATP, GST, and MTT).

Neither cytolethal nor non-cytolethal concentrations of rotenone or camptothecin led to alterations in DNA methylation. Treatment with a cytolethal concentration of 5-FU led to a global decrease and GC-rich increases in DNA methylation. A non-cytolethal concentration of 5-FU did not lead to changes in methylation status. Treatment with a cytolethal concentration of staurosporine led to alterations in GC-rich methylation patterns , and a non-cytolethal concentration of staurosporine led to a global decrease in DNA methylation and alterations in GC-rich methylation patterns.

b) To combine cytolethality, genotoxicity and DNA methylation data in order to determine the estimated toxic potential of the model compounds.

Combining cytolethality, genotoxicity, and DNA methylation data, I ranked the compounds according to their estimated toxic potential. Since genotoxicity data for staurosporine was not available, this was performed twice, based on different assumptions of staurosporine genotoxicity. If staurosporine is more genotoxic than camptothecin and 5-FU, and the estimated toxic ranking of the compounds in order of most to least

potentially toxic would be as follows: staurosporine, camptothecin, 5-fluororuacil, and rotenone. If staurosporine is less genotoxic than camptothecin and 5- FU, the ranking in order of most to least potentially toxic would be: camptothecin, 5-FU, and rotenone. In this case, staurosporine would be ranked as less toxic than rotenone, but its relative toxic potential compared to camptothecin and 5-FU would not be known.

c) To determine how DNA methylation analysis contributes to the assessment of the relative estimated toxic potentials of the model compounds.

Methylation analysis contributed to a more rational ranking of the model compounds. Specifically, the finding that staurosporine elicited changes in methylation allowed me to rank is as more potentially toxic than rotenone, an assessment I could not have made with cytolethality and genotoxicity data alone.

Hypothesis addressed

My hypothesis is that the ability to maintain normal patterns of DNA methylation is related inversely to susceptibility to carcinogenesis and perhaps other toxic outcomes. This overall hypothesis has been addressed for each of the research projects outlined in Chapters 1-4. In the study presented in Chapter 1, this hypothesis is clearly supported by the observation that following a 2 wk treatment of 0.05% PB, the relatively tumor sensitive C3H/He and B6C3F1 mice were less likely to maintain GC-rich patterns of methylation compared to the relatively tumor resistant C57BL/6 strain. In Chapter 2, the inverse relationship between ability to maintain patterns of DNA methylation and tumor susceptibility is illustrated in the finding that changes in GC-rich methylation patterns seen in animals at 9 wks was between 9 and 18 mg CSC, mirroring the threshold dose of CSC needed to elicit a marked increase in tumor number following 29 wks of continuous promotion. Also, the only SENCAR mouse skin tissues exhibiting global decreases in methylation were tumors, further illustrating the failure to maintain methylation pattern-tumor susceptibility link. In the study outlined in Chapter 3, I present data indicating that gene-specific hypermethylation can contribute to the clonal expansion of increasingly abnormal cells, contributing to tumor susceptibility. For instance, methylation of the tumor suppressor *HoxA5* contributes to its down-regulation, which would be expected to contribute to a decreased level *p53*, which would leads to an increased probability that cells with genetic damage will not undergo apoptosis, increasing the susceptibility to carcinogenesis. Also, methylation of either *p16* or *MGMT* was observed in 50% of the papillomas and none of the initiated-only tissues. Methylation of these genes could lead to increased tumor susceptibility by increasing cellular proliferation and a decreasing

DNA repair, respectively. Finally, in the experiments presented in Chapter 4, I indicate that treatment with cytolethal concentrations of staurosporine and 5-FU, both drugs not previously known to alter methylation, decreased global levels of DNA and altered GC-rich methylation patterns. In these cases, there is a probable relationship between DNA methylation change and toxic potential. Additionally, DNA methylation elicited changes as a result of application of a non-cytolethal concentration of staurosporine. In this case, detection of methylation alterations might provide an early indication of toxic potential not detected by cytolethality assessments.

DISCUSSION

Significance of findings

Below, I have outlined what I view as my most significant findings that illustrate novel contributions to the overall body of scientific literature.

1. Differences in the ability to maintain GC-rich patterns of methylation might help, in part, to explain the differences in tumor susceptibility seen in the C3H/He, B6C3F1, and C57BL/6 mice.

An improved understanding of the mechanisms responsible for the differences in tumor susceptibility observed in the relatively tumor-sensitive C3H/He and B6C3F1 mice compared to the relatively tumor-resistant C57BL/6 strain can provide insight into the importance of particular biological processes involved in cancer as well as whether the use of these models is representative of human cancer risk. This is particularly important since the two-year bioassay used by the National Toxicology Program to assess long-term carcinogenicity employs the tumor-resistant B6C3F1 stock (Haseman and Elwell, 1996). If these mice were tumor susceptible due to a process novel to the mouse, one would have less of a basis for using this model to assess human risk than if the mechanism involved in the difference in tumor susceptibility represents a mechanism shown to contribute to rodent and human cancer risk.

I found that in response to a 2 wk treatment with 0.05% PB, the relatively tumor-sensitive C3H/He and B6C3F1 mice were more likely to exhibit increases in GC-rich methylation patterns compared to the resistant C57BL/6 strain. In a study performed by Counts et al. (1996), global hypomethylation was more likely to be seen in the B6C3F1 mice compared to C57BL/6 mice following a 2-wk treatment with 0.05% PB, indicating

that the ability to maintain global patterns of DNA methylation was related inversely to tumor susceptibility. Global hypomethylation can contribute to carcinogenesis by leading to the up-regulation of oncogenes and/or expression of transposable elements typically silenced by methylation (Counts and Goodman, 1995). Conversely, hypermethylation in GC-rich regions can silence tumor suppressor expression and lead to an increased prevalence of C:T mutations due to the propensity of 5-methylcytosine to deaminate (Laird, 1997). Thus, a global decrease in methylation coupled with GC-rich increases constitutes a plausible cause for some of the observed differences in tumor susceptibility.

Though there might be quantitative differences in methylation change between mice and humans, qualitative patterns of methylation change are similar. Both rodent and human tumor tissues are characterized by decreased global methylation and increased GC-rich methylation levels (Ehrlich, 2002). Additionally, hypermethylation of the same tumor suppressor genes, including *p16, MGMT* and *HoxA5* has been reported in rodent and human neoplastic tissue (Patel et al., 2000; Mukai and Sekiguci, 2002; Hersko et al., 2003; Esteller et al., Viswanathan, 2003; Shiraishi, 2002). Also, specific aberrant patterns of DNA methylation in humans have been shown to be prognostic of cancer risk, indicating that humans that are less able to maintain normal patterns of methylation are also more susceptible to cancer (Cui et al., 2003; Frühwald, 2003). Thus, the finding that differences in the ability of mice to maintain normal methylation patterns is a probable factor underlying differences in tumor susceptibility could be used to assess mechanisms involved in human health.

2. Changes in GC-rich methylation patterns at early timepoints might be predictive of tumor formation at later times.

In the study presented in Chapter 2 characterizing dose- and time-dependent, threshold- exhibiting alterations in GC-rich methylation patterns in the SENCAR mouse skin model, I reported that the 18 mg threshold dose needed to elicit changes in GC-rich methylation patterns at 9 wks mirrored the threshold dose necessary for marked increase in tumor number compared to initiated-only controls following 29 wks of promotion, indicating that methylation changes detected at early times was predictive of tumor formation at later times. I think this is a significant finding because the ability to predict tumor formation based on early changes in DNA methylation could provide the basis for short-term methylation-based assays for tumor potential. Such a development would represent a significant savings of time and resources required to test compounds for tumorigenic potential, and it might additionally assist in the dose-selection of compounds. For example, one could identify the threshold dose necessary for a particular compound to elicit GC-rich changes in methylation at early times, and concentrate further studies with doses under that threshold.

The use of methylation analysis as an early indicator of cancer potential has shown some promise in recent years. For instance, hypermethylation of *RASSF1A* increases with a decreased age of starting smoking and poor prognosis in non-small cell lung cancer (Kim et al., 2003). Hypomethylation of the imprint control region involved in the expression of *Igf2* imprinted gene is significantly more prevalent in colon cancer patients as well as in patients with preneoplastic colon tissue and /or a family history of colon cancer. (Cui et al., 2003).

3. Increases in GC-rich and *HoxA5* methylation patterns are reversible during the promotion stage.

In these studies, I have found that GC-rich methylation patterns, as well as methylation status of the *HoxA5* promoter region in animals promoted with 27 mg CSC for 9 wks and sacrificed immediately afterwards is significantly increased compared to animals given the same promotion schedule, but given a 6 wk recovery period prior to sacrifice (recovery group). This represents one of a very few studies examining reversible changes in methylation, and the first study to indicate that methylation status of *HoxA5* is reversible. This is important in part because it indicates that methylation change in and of itself is not necessarily indicative of toxicity. Cessation of promoter treatment might alleviate the selective pressure applied on cells with down-regulated tumor suppressor genes. Thus, if DNA from the sputum of a chronic smoker contains hypermethylated DNA, and he/she stops smoking, the methylation changes observed might eventually subside along with the decreased risk of lung cancer. Therefore, assessments of methylation might eventually provide early indications of cancer risk, but would not indicate that cancer is inevitable. Furthermore, reversibility is a key component of tumor promotion (Pitot and Dragan, 1994), thus, this observation fits in well with the multistage model of carcinogenesis.

There are three possible mechanisms by which the reversibility observed in GC-rich and *HoxA5* methylation status could occur: 1) through "active demethylation" involving the replacement of a 5-methylcytosine base with a cytosine base (Frémont et al.,1997; Ramchandani et al., 1999), through "passive demethylation" involving DNA replication without maintenance methylation, or 3) through cell turnover during the 6 wk

recovery period. Active demethylation is observed in development (Ehrlich, 2003), and hypomethylation in the IL-2 promoter is seen within 20 minutes of T-cell activation (Bruniquel, 2003). Additionally, a human demethylase protein exhibiting activity in methylated oligos has been reported (Ramchandani et al., 1999). Thus, active demethylation is a possible mechanism. "Passive demethylation" could also explain the observed reversibility in methylation status. However, I do not think that the reason for the complete reversal of *HoxA5* methylation and reversal of GC-rich methylation patterns at particular regions in the recovery group can be solely attributed to cell turnover. First of all, in the skin tissue, there would be a certain degree of cell turnover expected within the 6 wk recovery period, however, there might also be stem cells within the epithelium that would probably remain (Alonso and Fuchs, 2003). Secondly, a small degree of GC-rich methylation remained in the recovery group (Watson et al., 2003; Chapter 2, Figure 6), indicating that it is possible for particular methylation patterns to be at least partially retained following the 6 wk recovery period. Regardless of the mechanism(s) involved, methylation of the *HoxA5* promoter, and the average methylation status of GC-rich DNA, was clearly reversible, a finding indicative of a process involved in the promotion stage (Pitot and Dragan, 1994; Goodman, 2001).

4. The progressive, dose-and time-dependent, reversible changes in methylation patterns observed in the SENCAR mouse skin model fit within the context of the initiation-promotion-progression multistage model of carcinogenesis.

The research described in Chapters 2 and 3 represents a thorough characterization of global, GC-rich and gene-specific methylation changes within the context of the 2-stage initiation-promotion SENCAR mouse skin model. This is significant because it

contributes to an enhanced understanding of the nature of methylation changes in during carcinogenesis and illustrates how these changes would be likely to participate in the clonal expansion of increasingly abnormal cells.

In this model, animals were initiated with DMBA and promoted with CSC. Uninitiated animals promoted with 36 mg CSC for 29 wks did not exhibit a marked increase in tumor number compared to initiated-only controls. In contrast, initiated animals promoted with 36 mg CSC developed a large number of tumors at 29 wks. This illustrates the importance of the initiator in this model, and is consistent with the notion that promoting agents facilitate the expansion of initiated cells (Dragan et al., 1993). DMBA would induce a genetic alteration to a cell such that it would contain a growth advantage over the surrounding cells.

In this particular study, global methylation changes were seen only in papillomas, but progressive increases in methylation at GC-rich regions were observed in a dose-and time-dependent manner throughout the promotion stage. CSC seems to have provided an environment that selected for the expansion of cells with hypermethylated GC-rich regions, including the promoter regions of the tumor suppressors *HoxA5, p16 and MGMT*. Since HOXA5 binds to and up-regulates *p53* transcription, *HoxA5* down-regulation could contribute to decreased *p53* activity (Raman et al., 2000) leading to a decreased capacity for apoptosis. Subsequent methylation-mediated down-regulation of *MGMT* and/or *p16* could facilitate the further clonal expansion of increasingly abnormal cells by decreasing DNA repair and increasing proliferation, respectively.

Notably, papillomas exhibited patterns of methylation characteristic of tumor cells; specifically, an increase in methylation at GC-rich regions along with a decrease in

global methylation patterns (Lin et al., 2001; Ehrlich, 2002). A general scheme of the alterations in global and GC-rich methylation patterns is presented in Figure 1. Though the liver tissue seems to be more prone to global hypomethylation change than SENCAR mouse skin, the same overall pattern of methylation change was observed. This pattern appears to be typical of neoplastic tissue (Ehrlich, 2002). The reason for the frequent observation that neoplastic tissue is globally hypomethylated and regionally hypermethylated is not known. It is probable that increased GC-rich DNA methylation in the promotion stage leads to methylation and inactivation of a gene responsible for maintaining global patterns of methylation. For example, hypermethylation-mediated down-regulation of *MGMT* is prevalent in tumor tissues (Brabender et al., 2003). Since *MGMT* repairs 0^6-methylguanine adducts, an increased prevalence of these DNA adducts would ensue. Since these adducts inhibit methylation (Weitzman et al., 1994), a decreased amount of methylation in regions with these lesions would result.

GC-rich DNA and specifically, the promoter region of *HoxA5* was methylated in a reversible manner, indicative of a mechanism involved in promotion. Once the selective pressure from CSC is relieved, *HoxA5* and GC-rich methylation patterns return to control levels. Hypermethylation of *HoxA5*, *MGMT* and *p16* most likely to represent a subset of tumor suppressors methylated and down-regulated throughout promotion. This study is the first report of the reversibility of *HoxA5* methylation patterns regulating expression and describes how *HoxA5*, *p16*, and *MGMT* hypermethylation could contribute to tumorigenesis within the context of the multistage model of carcinogenesis.

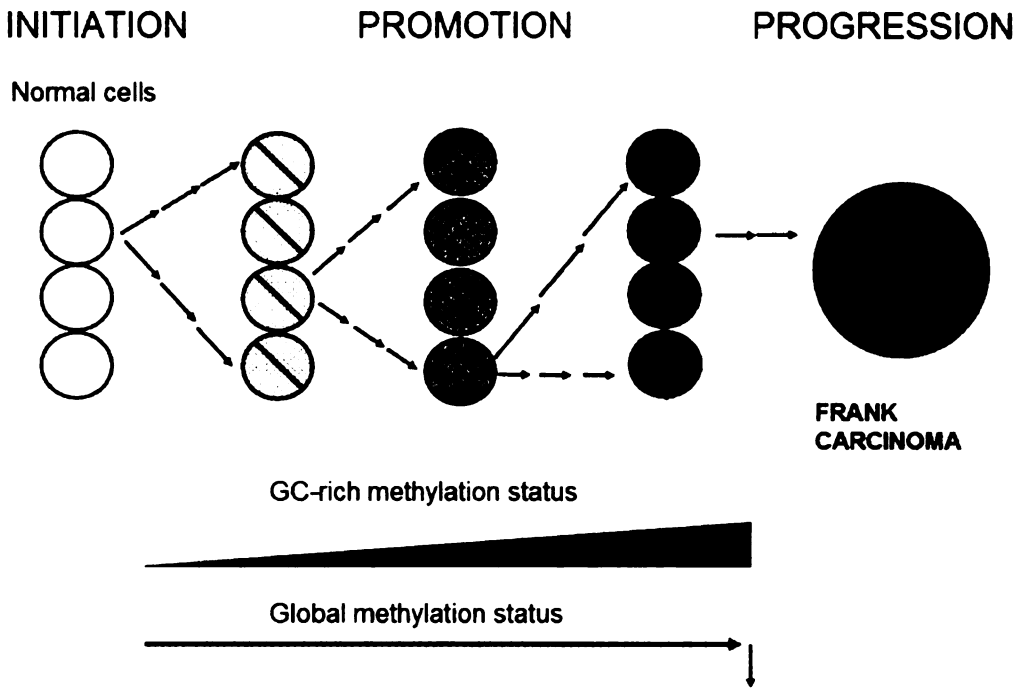

Figure 1. Altered GC-rich methylation might contribute to the clonal expansion of increasingly abnormal cells in the promotion stage of tumorigenesis.
Each line through the cell denotes a critical, heritable change to the genome, caused by a mutation or epigenetic change. Arrows indicate cellular replication. In the SENCAR mouse skin model, progressive increases in GC-rich methylation are seen throughout the promotion stage. This increase in GC-rich methylation is likely to include increases in the methylation status of the promoter regions of tumor suppressor genes, down-regulating their expression and constituting some of the critical changes in the genome. Global hypomethylation was observed in the papilloma tissue; non-tumor tissue maintained patterns of global methylation status. Global hypomethylation might lead to the aberrant expression of transposable elements, or lead to the increased expression of oncogenes. Notably, the pattern of GC-rich methylation increases, and a global methylation decrease is a common theme in neoplastic tissue (Ehlich et al., 2002).

5. When used in conjunction with more classical *in vitro* toxicity assays, methylation analysis provides an enhanced ability to detect toxic potential.

I have found that cytolethal concentrations of staurosporine and 5-FU, 2 of 4 model compounds analyzed that were not previously suspected to affect DNA methylation, led to a global decreases in methylation, and alterations of GC-rich methylation patterns. This finding in and of itself is significant because it indicates that chemically-induced methylation change might be more prevalent than commonly assumed, though an increasing number of reports demonstrate chemically-induced methylation change (Lee et al, 1995; Chen et al., 2001; Detich et al., 2003). Furthermore, treatment with a noncytolethal concentration of staurosporine led to global and GC-rich methylation changes, a finding that suggests that sometimes toxic potential could be detected with methylation analysis before it could be detected using more traditional cytolethality assays. Methylation in and of itself is not necessarily indicative of toxicity, but given a large number of compounds to screen for toxic potential, prioritization for further analysis would be given to a compound that did not affect methylation compared to one that did. In combination with genotoxicity and cytolethality data, DNA methylation data was used to estimate the relative toxic potential of the four model compounds. Without the DNA methylation data, one would not have had a basis for differentiating between the toxic potential of staurosporine and rotenone. Thus, DNA methylation analysis provided a more rational basis for compound prioritization. Also, DNA methylation analysis might be used for dose selection such that a dose high enough to affect methylation would not be used. In light of recent studies indicating that

methylation analysis has shown promise as an early indicator of cancer potential (Palmisano et al., 1999; Cui et al., 2003), and given the increasing number of diseases which might be associated with methylation change including neurological, immunological, and developmental diseases (as reviewed in Watson et al., 2002), the likelihood that DNA methylation change might represent toxic potential seems probable, and this study provides an initial step toward determining if DNA methylation analysis is useful in the assessment of toxic potential.

To further assess the utility of DNA assessment, one would perform *in vivo* experiments complementary to those performed *in vitro* to demonstrate if methylation changes observed *in vitro* are predictive of toxicity *in vivo*. Additionally, in order to further examine mechanisms underlying the effect of particular compounds on DNA methylation status, one could perform additional *in vitro* experiments using chemicals with modes of action similar to those already found to alter methylation. This would help determine if certain drug classes would be more likely to induce methylation change and might provide insight into the mechanisms of how these chemicals induce changes in methylation.

Conclusions

Alterations to normal patterns of DNA methylation can contribute to a number of untoward effects, including, but not limited to, carcinogenesis. Hypomethylation can lead to abnormal increases in gene expression, and the expression of transposable elements is typically silenced by methylation (Counts et al., 1995). On the other hand, hypermethylation can contribute to the silencing of genes, and an increased probability of C-T mutations (Laird, 1997).

The studies I have performed pertaining to the role of DNA methylation in cancer have supported the notion that altered patterns of methylation underlie susceptibility to cancer. Notably, I have demonstrated that differences in the ability to maintain normal patterns of GC-rich methylation patterns are likely to underlie some of the differences in tumor susceptibility seen between C3H/He, B6C3F1 and C57BL/6 mice. In the initiation/promotion SENCAR mouse skin model, increases in GC-rich methylation patterns observed at early times appear to be predictive of tumor formation at later times, further underscoring the relationship between tumor susceptibility and failure to maintain normal patterns of methylation.

Additionally, I have characterized methylation alterations within the context of the multistage model of carcinogenesis and found that GC-rich methylation patterns occur in a dose- and time-dependent, reversible fashion, consistent of a mechanism involved in the promotion stage of carcinogenesis. Also, I have shown that reversible changes in DNA methylation are likely to contribute to regulation of the *HoxA5* tumor suppressor gene. The reversibility observed in *HoxA5* and GC-rich methylation patterns indicates that DNA methylation alterations in and of themselves should not be equated

with toxicity. However, in some cases, it might be appropriate to view them as markers of potential toxicity. For instance, changes in GC-rich methylation patterns observed at 9 wks with the 18 mg CSC dose were reversible, as was the methylation status of the *HoxA5*. However, if promotion continues for 29 wks at that same 18 mg CSC dose, tumor formation is likely to result.

Based on the alterations in methylation patterns we detected, I have suggested concrete, plausible roles for how the DNA methylation alterations observed could lead to the clonal expansion of increasingly abnormal cells in the promotion stage. My findings demonstrated that GC-rich methylation changes were threshold-exhibiting and reversible; key features underlying a mechanism involved in the promotion stage of carcinogenesis (Pitot and Dragan, 1994; Goodman, 2001), and also indications that DNA methylation is a secondary mechanism underlying carcinogenesis (Goodman and Watson, 2002).

I think it is appropriate to consider that DNA methylation might be an important secondary mechanism regulating non-cancer related outcomes, as well. I have described how DNA methylation plays a key role in the regulation of several vital biological processes and how aberrant patterns of DNA methylation are implicated in certain non-cancer related disorders (as reviewed in Watson and Goodman, 2002). It is reasonable to assume that altered DNA methylation could contribute to the abnormal expression of a number of genes which would have the potential to elicit a wide range of toxic effects. Furthermore, there have been an increasing number of studies demonstrating the prevalence of chemically-induced methylation changes, and I have found that methylation levels were altered as a result of treatment from 2/4 model compounds not previously known to affect DNA methylation. In an initial look at the utility of DNA

methylation in assessment of toxicity, I have found that methylation analysis, in combination with cytolethality and genotoxicity data, can provide additional information which was used to rank the model compounds based on estimated toxic potential in a more rational manner. Thus, the use of methylation analysis as a marker of change in genetic expression that underlies a toxic potential might be appropriate, and alterations in DNA methylation should be viewed as disturbances to a homeostatic mechanism governing the overall health of the animal.

REFERENCES FOR INTRODUCTION, SUMMARY, AND DISCUSSION SECTIONS ARE LISTED ON PAGES 212-226.

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