PROGRESSIVE, NON-RANDOM ALTERED PATTERNS OF METHYLATION IN GENE-SPECIFIC AND GC-RICH REGIONS OF DNA UNDERLIE TUMORIGENESIS

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ABSTRACT

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Epigenetics is broadly defined as processes that establish heritable states of gene expression without altering the DNA sequence. DNA methylation, (i.e. 5-methylcytosine content of DNA), is a well characterized epigenetic mark. Altered patterns of DNA methylation can lead to the aberrant expression of genes. My central hypothesis states that the ability to maintain patterns of methylation is inversely related to susceptibility to tumorigenesis. This hypothesis is tested in the context of the central paradigm for explaining events leading to tumorigenesis. The model describes a multi-stage and multi-step (i.e. multi-mechanism) model for the development of precancerous lesions and their evolution into frank carcinomas. The stages defined by this model are initiation, promotion and progression. The promotion stage of tumorigenesis involves the step-wise accumulation of heritable changes which are critical for the selection and clonal expansion of initiated cells. Therefore changes in methylation which accumulate during the promotion stage are not a result of the neoplastic state, but key contributors to the process. With the development of a novel method for measuring changes in methylation in GC-rich regions of the genome with high reproducibility, I have characterized patterns of DNA methylation during the promotion stage of carcinogenesis in three separate

model systems. Specifically I have demonstrated highly similar changes in methylation, predominantly hypomethylation, with three different promoting compounds. In addition, I observed hypomethylation of the promoter region of the Ha-*ras* oncogene with simultaneous stability of patterns of methylation in the promoter region of LINE-1 elements which are retrotransposable elements that comprise ~ 30% of the mouse genome. These findings exemplify the selective nature of promoter-induced (i.e. phenobarbital) disruption and indicate that changes in methylation are not entirely random. Detection of hypomethylation, occurring simultaneously with hypermethylation, in GC-rich regions is demonstrated as a principal contributor to the development and progression of tumors. Consistent with the working model of carcinogenesis, regions of altered methylation are seen to persist from early time points to later precancerous and cancerous time points. This lends strong experimental support for the accumulation of critical changes in DNA methylation, both increases and decreases, during tumor promotion. The extent and frequency (i.e. more changes accumulating in a shorter period of time increases susceptibility) with which changes in methylation accrued seems to strongly relate to the relative susceptibilities of B6C3F1 and C57BL/6 mice to liver tumorigenesis, a key point supporting the overall hypothesis. Further evidence to support the contribution of progressive changes in methylation to the neoplastic state is presented using the SENCAR 2-stage mouse skin initiation/promotion model. Reversibility of altered methylation, a hallmark of promotion, is also observed and discussed. Taken together, my findings from three distinct model systems were complementary and consistent in supporting the notion that progressive, non-random changes (i.e. instability of the epigenome) in methylation underlie tumorigenesis.

This dissertation is dedicated to my husband Todd M. Bachman

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Most importantly, I must thank with all my heart, my parents Jane and Richard Carnell who have supported me in every way imaginable from 500 miles away. They will always be a driving force in my desire to achieve. I must also thank my brother who has always been a source of motivation, competition and brain power when I needed it the most. Finally, my husband deserves all the credit in the world for truly living and understanding what this all means to me.

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INTRODUCTION

Cancer

According to the American Cancer Society, cancer accounts for 23% of all deaths, second only to cardiovascular disease. Understanding the cause and pathogenesis of cancer through research is necessary for treating the millions of people affected worldwide. To do this, research endeavors must build on existing concepts in addition to developing new and different approaches to treatment and diagnosis. Potential and known carcinogens must be logically and rationally assessed in terms of risk posed to humans. This will require a comprehensive look at the underlying molecular mechanisms of cancer with a focus on the mode of action of the chemical(s) of interest. This can enhance the scientific basis for three key aspects of safety assessment: 1) selection of doses for testing, including rational selection of the high dose; 2) evaluation of the dose-response relationship, and 3) rational species-to-species extrapolation.

Cancer is characterized by six fundamental changes in cell physiology including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and the ability to invade and metastasize (Hanahan and Weinberg, 2000). The genetic pathways giving rise to these classic hallmarks have complex origins and can involve the mis-regulation or mutation of critical cell cycle proteins, transcription factors and signal transduction proteins among others. This adds to the difficulty of discerning absolute cause and constrains the notion of one encompassing method to stop or prevent cancer from occurring. However, a working model of carcinogenesis has been proposed and serves as

a unifying factor in determining the steps and targets involved in the progression of precancerous tissue to tumor tissue.

Multi-Stage Carcinogenesis and Epigenetics

A multistage, multi-step (*i.e.* multi-mechanism) process of carcinogenesis provides a framework for explaining events leading to cell proliferation and tumorigenesis (Figure 1). The stages defined by this process are initiation, promotion and progression (Dragan *et al.*, 1993). During initiation, a heritable change occurs in the genome. A heritable change is often associated with direct mutation of the DNA sequence. However, epigenetic modifications (*e.g.* DNA methylation, histone acetylation) which do not affect the base sequence of DNA, can also be heritable alterations. Promotion of the selected cells, or those that were initiated, occurs when an agent (*e.g.* phenobarbital, peroxisome proliferators) allows for preferential growth over neighboring cells. Agents acting through mechanisms not involving direct DNA damage are termed non-genotoxic and can be thought of as acting through a secondary mechanism of carcinogenesis (Goodman and Watson, 2002). This stage is reversible, in that, if the promoting stimulus is withdrawn, the altered cells possessing advantageous growth characteristics stop proliferating and altered foci can "remodel". The promoting stimulus continues to foster the growth of initiated cells and new subsets of cells arising from those that are undergoing clonal expansion. Subsequent to the iterative nature of this process is progression. At this point, cells are clonally expanding even in the absence of the promoting stimulus. In addition, cells at this stage typically exhibit marked

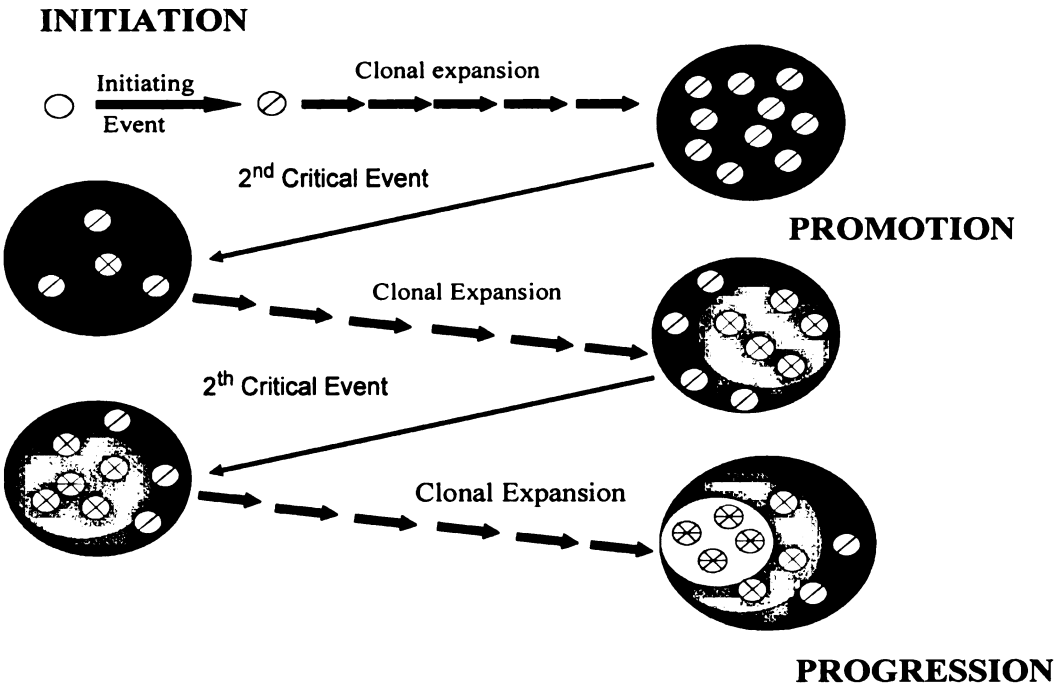


Figure 1 Multistage Carcinogenesis The three stages of carcinogenesis are initiation, promotion, and progression. During initiation a cell (represented by a circle) acquires some heritable change (e.g. mutation or altered DNA methylation) within the genome. Each line through a circle represents a heritable event. In the presence of a promoting stimulus, initiated cells possessing a growth advantage over neighboring cells proliferate. The process repeats until the cells reach a state of autonomous, clonal expansion; this is termed progression.

karyotypic instability including chromosomal damage and changes in ploidy (Dragan *et al.*, 1993).

The focal point during the promotion stage of carcinogenesis is the accumulation of heritable changes within the genome. These are fundamental to the initiation and development of cancer. As mentioned above, mutation is the obvious and standard example of a heritable change which can seed the development of tumorigenesis. Importantly, epigenetics has also taken a parallel role to mutations. Epigenetics is broadly defined as processes that establish heritable states of gene expression without altering the DNA sequence. This includes DNA methylation and histone acetylation each of which alter the regulation of gene expression but do not affect the base sequence of DNA (Feinberg, 2001).

Methylation of cytosines to produce 5-methyl cytosine is a well characterized epigenetic mark. Because both cytosine and 5-methyl cytosine base pair with guanine, this epigenetic modification is not a mutation. The majority of 5-methylation cytosine occurs at cytosines 5' to guanine although methylation of non-CpG dinucleotides such as CpA, and CpT have been reported (Jabbari and Bernardi, 2004) in addition to CpNpG methylation (Jackson *et al.*, 2002). Therefore, altered patterns of methylation can potentially effect a large majority of the genome and evoke widespread consequences. Furthermore, DNA methylation can be a precursor to mutation. Spontaneous deamination of 5-methyl cytosine yields thymine and this can base-pair with adenine resulting in a CG to TA transition mutation (Cooper and Krawczak, 1989). In addition, DNA adduct formation due to oxidative stress or agents such as dimethylsulfate and ethylnitrosourea can result in altered methylation. Under conditions of oxidative stress,

the common DNA adduct, 8-hydroxyl-2'-deoxyguanosine, has been shown to interfere with the ability of the human DNA methyltransferase to methylate target cytosines nearby (Turk *et al.*, 1995). Additionally Tan and Li, 1990, demonstrated that 6-O-methylguanine located 5' to cytosine can affect the maintenance methylation of the opposite strand in a hemimethylated duplex. The presence of this adduct might destabilize the hemi-methylated site and cause the methylase to detach, or the adduct could both enhance or decrease the site as a substrate for the methylase depending on its position within the genome. In this manner, DNA adducts can either increase or decrease the methylation of neighboring cytosines leaving an abnormal, yet heritable, epigenetic pattern.

Histone modifications are also considered reversible epigenetic processes. Nuclear DNA is packaged into nucleosomes. The core histone octamer consists of an H3-H4 tetramer ($H3_2$-$H4_2$) and two H2A-H2B dimers. Around this histone core, approximately 200bp of DNA is wrapped. Each histone has a flexible N-terminal tail which can be reversibly modified by acetylation, methylation, ubiquitination, biotinylatioin, and phosphorylation (Spotswood and Turner, 2002; Petterson and Laniel, 2004). The modification of these histone tails can destabilize higher order chromatin structure. Transcriptionally active chromatin, or euchromatin, is associated with methylation of lysine 4 and 9 in addition to acetylation of lysine 9 and 14 (Espino *et al.*, 2005). Transcriptionally repressed chromatin, or heterochromatin, is associated only with the methylation of lysine 9 (Espino *et al.*, 2005). Each of these mechanistically contribute to the transcriptional regulation of euchromatic genes (Richards and Elgin,

2002). Histone acetylation leads to a more relaxed chromatin conformation while histone deacetylation results in a tighter packaging of the DNA.

There is a tightly regulated relationship between histone modifications, chromatin structure, and DNA methylation (Szyf *et al.*, 2004). The order of events by which chromatin is modified to yield a transcriptionally active or inactive state is not well characterized. However, three routes to epigenetic silencing have been proposed. These include the possibility that DNA methylation dictates histone modification, histone modification mediates DNA methylation, or nucleosome remodeling facilitates DNA methylation (Lund and van Lohuizen, 2004). Evidence supporting aspects of all three of these possibilities has been shown (Fuks *et al.*, 2000; Chaumeil *et al.*, 2004; Dennis *et al.*, 2001)

Although the timeline of events is still being elucidated, the cooperation between histone modifications and DNA methylation is undisputed. A "histone code" hypothesis has been developed to describe the role of histone acetylases and deacetylases in conjunction with ATP-dependent remodeling factors (Strahl and Allis, 2000). These ATP-dependent factors (*e.g.* SWI-SNF, Mi-2, and ISWI families) cause the disruption and sliding of nucleosomes along the helical path of DNA to facilitate transcription (Ballestar and Esteller, 2002). The presence of DNA methylation elicits histone deacetylation and prevents methylation of lysine 4 on histone 3. The removal of 5-methyl cytosine allows for the methylation of lysine 4 on histone 3 and without the underlying repression mechanisms, histones undergo acetylation (Lande-Diner and Cedar, 2005). The link between factors affecting histone conformation and DNA methylation involves methyl-DNA binding proteins (Ballestar and Esteller, 2002). A possible order

of events leading to gene inactivation begins with a low level of DNA methylation at the promoter. This methylation signal recruits the methylated DNA-binding protein MBD2, which recruits histone deacetylases and Dnmt1, one maintenance methylase responsible for regulating the status of DNA methylation. Histone deacetylation and subsequent methylation of a promoter region of a gene by Dnmt1 results in the recruitment of the methyl DNA-binding protein MeCP2. MeCP2 in turn recruits a histone H3, lysine 9 methyltransferase for methylation of lysine 9 and condensation of chromatin (Espino *et al.*, 2005). Deacetylation and methylation reactions coupled to the recruitment of numerous proteins largely prevents transcription factors from gaining access to the DNA. In support of this, a combined administration of 5'-AZA and trichostatin A (histone deacetylase inhibitor) resulted in activation of a cytomegalovirus promoter-driven reporter gene construct (Grassi *et al.*, 2003). Notably, each treatment alone reactivated the reporter gene construct, however, differing enzyme kinetics were reported (Grassi *et al.*, 2003). These reversible reactions clearly cooperate as integrative epigenetic mechanisms for gene regulation and illustrate their significance during the process of tumorigenesis.

The Origin of the Cancer Stem Cell

The origin of the "cancer stem cell" is a highly debated topic and each side of this larger issue deserves mention. The cancer stem cell is, in essence, the cell that acquires the first heritable change to its genome that sets it on a potential path leading to tumor development. These cells that acquire the first critical initiating event(s) could be derived from normal stem cells, early stem cell progenitor cells, or differentiated cells (Bjerkvig *et al.*, 2005). The self- renewing properties of cancer stem cells are characteristic of stem

cells which are an obvious and likely origin. However, de-differentiation of normal cells to a stem-cell like state is also possible. In support of this, differentiated astrocytes in which the epidermal growth factor receptor pathway is activated and tumor suppressors p16 and p19 are inactivated lead to a common high-grade glioma phenotype *in vivo* (Bachoo *et al.*, 2002). The expression of a critical transcription factor, Oct4, is associated with pluripotency and the downregulation of Oct4 has been linked to the differentiation of somatic cell lineages (Tai *et al.*, 2005). Ectopic expression of this transcription factor in certain somatic cells has also been implicated in active dedifferentiation (Shimazaki *et al.*, 1993; Hochedinger *et al.*, 2005). Altered methylation resulting in heritable genomic changes could potentially contribute to the evolution of normal cells into the cancer stem cell-like state. For example, murine embryonic stem cells were induced to differentiate *in vitro* to embryoid bodies and then treated with 5'-aza-2'-deoxycytidine (5'-AZA). During DNA synthesis 5'-AZA is incorporated into newly synthesized DNA in place of deoxycytosine and covalently binds to DNA methyltransferases. This linkage effectively depletes the cell of functional DNMTs and leads to hypomethylation or "demethylation" after successive rounds of replication (Juttermann *et al.*, 1994). The embryoid bodies exhibited stem cell-like characteristics including stem cell like morphology with unclear cell-to-cell boundary and proliferative responsiveness. In addition, increased expression of embryonic stem cell markers such as Oct4, Nanog, and Sox2 were measured suggesting that the differentiated state of these cells was reversed (Ysuji-Takayama *et al.*, 2004). Therefore, both the stem cell theory and dedifferentiation theory for the origin of the cancer-stem cell are not mutually exclusive in the overall context of measuring progressive changes in DNA methylation.

Regulation of Methylation Patterns: DNA methyltransferases

Methylation, as a heritable feature of DNA, is mainly dependent on the maintenance methyltransferase (DNMT1) during DNA replication, however, cycling through various states of methylation is accomplished via de novo and demethylases (Figure 2) (Pradhan and Esteve, 2003). After one round of replication in which a new daughter strand has been synthesized, the DNA exists in a hemi-methylated state where one strand is methylated and the newly synthesized daughter strand is wholly unmethylated. To return to a fully methylated state Dnmt1, a maintenance methylase, recognizes hemimethylated DNA and methylates CpG sites accordingly (Bestor, 2000). Proper maintenance methylation is critical in that a completely hypomethylated state of DNA can occur when two rounds of replication ensues without proper maintenance methylation. Dnmt1 is thought to be essential for the survival of an organism. Mice deficient for Dnmt1 die in mid-gestation with significantly reduced levels of DNA methylation (Bestor and Jaenisch, 1992). Acting as an integral part of cell cycle control, interaction of Dnmt1 with PCNA, p21 WAF1 (inhibitor of cyclin-dependent kinases (CDKs) and the processivity factor of the replication fork has been demonstrated (Chuang *et al.*, 1997). The close association between Dnmt1 and PCNA has also led to the suggestion that Dnmt1 serves as a signal for mis-match repair during replication and methylation of the hemi-methylated state (Wang and Shen, 2002; Mortusewicz *et al.*, 2005) Interestingly, inhibition of Dnmt1 has been shown to negatively affect DNA synthesis and progression through the cell cycle in human non-small cell lung carcinoma, A549 cells (Knox *et al.*, 2000). Furthermore, a network of connections between Dnmt1 and histone modifying enzymes, methyl binding proteins and heterochromatin binding

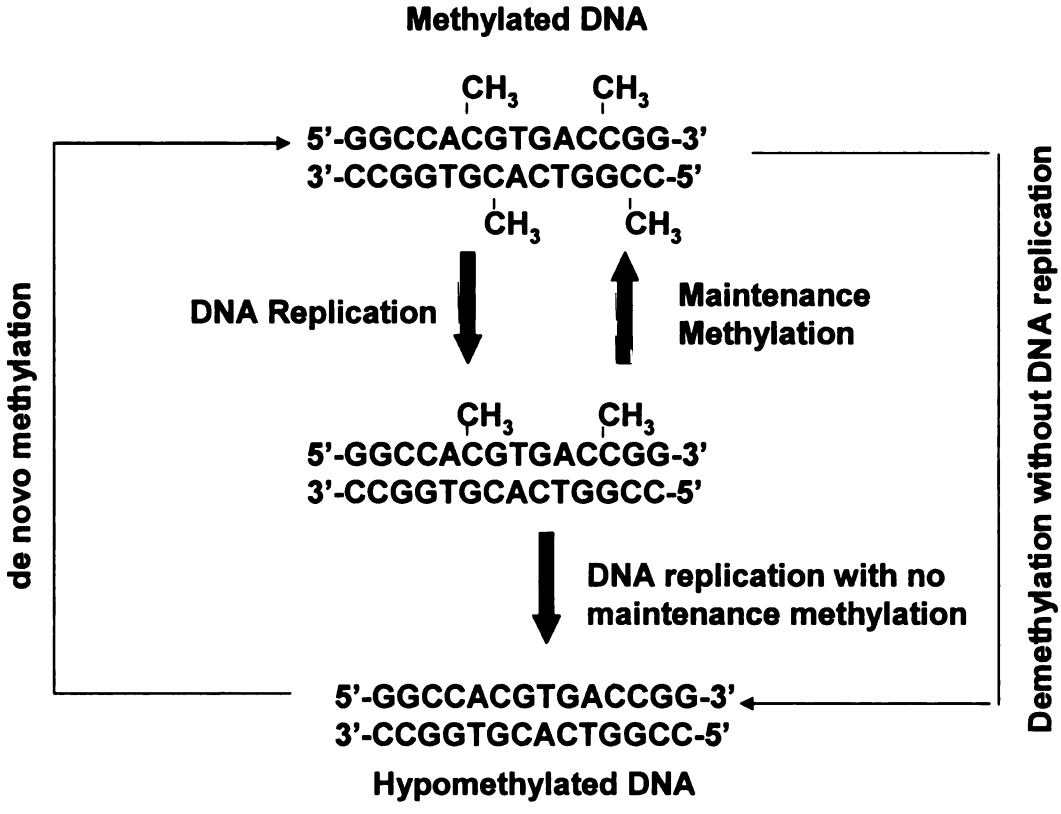


Figure 2 Methylation, A Heritable Feature of DNA Following DNA replication, the DNA exists in a hemi-methylated state; one strand of the DNA is methylated while the newly synthesized strand is not yet methylated. Maintenance methylation will return hemi-methylated DNA to fully methylated DNA. If a second round of replication ensues without proper maintenance methylation, a hypomethylated state of DNA occurs; Both strands of the DNA are unmethylated. In addition, fully methylated DNA can become hypomethylated via demethylation without DNA replication and hypomethylated DNA can be returned to the fully methylated state through *de novo* methylation.
(Adapted from Hergersberg, *Experientia*, 1991)

protein all point to Dnmt1's involvement in gene regulation and epigenetic signaling (Hermann *et al.*, 2004).

Just as failure to methylate hemi-methylated DNA results in hypomethylation, so too can demethylation of fully methylated DNA. Mechanistically, removing a methyl group from cytosine would involve cleavage of a carbon-carbon bond, making this an unlikely reaction due to the high energy requirement (Bhattacharya *et al.*, 1999). Therefore, indirect mechanisms involving base excision and repair have also been proposed (Vairapandi, 2004). Thermodynamically, direct demethylation became feasible with the identification of methanol as the leaving group and water as a possible reactant (Ramchandani *et al.*, 1999). The demethylase might act to stabilize an intermediate state so that a hydroxide ion can then attack the C5 methyl group (Ramchandani *et al.*, 1999). In either case, the main consequence of DNA demethylation is a hypomethylated state of DNA which could have functional consequences. Demethylation-induced hypomethylation has been linked to enhanced transcription of the T-cell growth hormone interleukin-2 gene. This gene is actively demethylated in T lymphocytes and allows for proliferation and the production of other cytokines including interferon γ and IL-4 (Bruniquel and Schwartz, 2003). Demethylation by DNA demethylase has also resulted in the up-regulation of the c-myc oncogene in human gastric cancer (Fang *et al.*, 2004). Therefore removing methyl groups from cytosines within DNA has various implications and raises the question of the specificity of DNA demethylase for DNA in normal cells and cancerous cells. The demethylase activity has been shown to associate with PCNA during replication in normal cells and target hemi-methylated CpG sites. However, in

cancer cell lines, fully methylated CpG islands are the substrate for the DNA demethylase activity (Vairapandi, 2004).

A hypomethylated state of DNA can be returned to the fully methylated state via *de novo* methylation, associated with both Dnmt3a and Dnmt3b enzymes (Pradhan and Esteve, 2003). Both proteins are essential for mouse development. Their close association is demonstrated by the fact that double knockouts in murine embryonic stem cells have a more severe phenotype than each individual deletion mutant indicating there is some compensatory activity by each (Okano *et al.*, 1999). Dnmt3a is ubiquitous while Dnmt3b is normally present at low levels (Xie *et al.*, 1999). Even though Dnmt3a and 3b are highly related , they are encoded by separate genes and do exhibit somewhat specialized roles (Hermann *et al.*, 2004). Dnmt3b is processive supporting its ability to methylate pericentromeric repeats carrying high CG content. One cause of immunodeficiency, centromeric instability, facial abnormalities, (ICF), is mutation of the Dnmt3b gene, however, the Dnmt3a gene is unaffected. Therefore, the characteristic hypomethylation at pericentromeric satellite regions in this rare recessive autosomal disorder is solely attributed to Dnmt3b (Xu *et al.*, 1999). Interestingly, over-expression of a the Dnmt3b4 splice variant has been associated with DNA hypomethylation on pericentromeric satellite region in human hepatocellular carcinomas (Saito *et al.*, 2002). Elevation of the ratio of Dnmt3b4 to a second splice variant, Dnmt3b3, could cause competition for the targeted region upsetting the balance needed to properly maintain methylation of pericentromeric satellite regions (Saito *et al.*, 2002). The higher intrinsic methylation activity of Dnmt3b over Dnmt3a coupled with its frequent over-expression in various tumors, supports a role for Dnmt3b in tumorigenesis (Robertson *et al.*, 1999).

Dnmt3a on the other hand is more specific, showing preference to methylate sites that are flanked by pyrimidines rather than purines and therefore, methylation events are more controlled (Lin *et al.*, 2002). In line with this, the establishment of methylation patterns at single copy genes has been attributed to Dnmt3a, in cooperation with Dmnt3L (Hata *et al.*, 2002). Dnmt3a shows strong interactions with a number of proteins including histone H3, lysine 9 methyltransferase Suv39, Dnmt1, and histone deacetylases (Kim *et al.*, 2002; Fuks *et al.*, 2003). A very detailed network of co-operativity between these enzymes and the methylation machinery including methyl binding proteins and histone acetylases and deacetylases point to compensatory mechanisms which could preserve the cyclic balance of the methylation states of DNA. Therefore, the fidelity of endogenous mechanisms maintaining the proper state of methylation throughout the genome is crucial especially during times when a high percentage of cells are proliferating.

5-Methyl Cytosine Distribution and Abundance

In mammalian genomes, there is a positive correlation between gene density and (G + C) content where 75-80% of genes reside in the (G + C)-richest half of the genome (Waterston, R.H. et al., 2002). Therefore, the distribution of methylated cytosines within CpG dinucleotides has important meaning in understanding the regulation of gene expression. CpG dinucleotides are not evenly distributed throughout the genome (Bird, 2002). An estimated 70% of all CpG sites are methylated; however, completely unmethylated CpG islands regions account for ~1% of the genome and an estimated 15% of the total genomic CpG sites (Roberston and Wolffe, 2000). CpG islands are short

stretches of DNA, at least 200bp in length, possessing 50% or greater GC content and a higher proportion of CpG dinucleotides than expected. In total about 15,500 CpG islands are estimated (Waterson *et al.*, 2002), of which, the majority are mainly found within the promoter regions or first exons of genes (Gardiner-Garden and Frommer, 1987). The normal status of methylation of each individual region varies although the majority of CpG islands are normally unmethylated allowing for transcriptional activity of the respective gene (Antequera, 2003). Many CpG dinucleotides are also located in GC-rich promoter and promoter-like regions of transposable elements (Liang *et al.*, 2002) which comprise approximately 33% of the human and mouse genomes (Yoder *et al.*, 1997) indicating that cytosine methylation could significantly contribute to the regulation of non-coding regions (*i.e.* repetitive regions) as well as the coding regions throughout the genome.

Non-CpG methylation (eg. CpA, CpT, CpC) has also been reported and expands the total proportion of the genome potentially affected by DNA methylation (Dodge *et al.*, 2002). For example, one early report based on the nearest neighbor technique estimated that 55% of all methylation in human spleen DNA could be at dinucleotides other than CpG (Woodcock *et al.*, 1997). Since then, more accurate representations and roles for non-CpG methylation have been proposed. The significance for non-CpG methylation during early development has been questioned due to the fact that 15-20% of total cytosine methylation content of embryonic stem cells is at sequences other than CpG (Ramsahoye *et al.*, 2000). This non-CpG methylation is associated with the activity of the *de novo* methyltransferase Dnmt3a which is highly expressed in embryonic stem cells (Ramsahoye *et al.*, 2000). The functional role of methylated CpA and CpT sites, the

most frequent form of non-CpG methylation identified, during development, is hard to determine due to the fact that Dnmt3a$^{-/-}$ mice die shortly after birth (Okano *et al.*, 1999). However, the CpA methylation was again associated with Dnmt3a and or Dnmt3b in a model of de novo methylation of murine Maloney leukemia virus provirus DNA in virus-infected embryonic stem cells (Dodge *et al.*, 2002). With this model, CpA methylation was detected at ~1.4% of all sites in infected wild-type and ~1.0% in Dnmt1 knockout cells. However, in Dnmt3a and 3b knockout cells, only 0.2% of all sites exhibited CpA methylation demonstrating the relationship between Dnmt3 enzymes and non-CpG methylation.

A large portion of my research involved analysis of the methylation status of the external cytosine within CpCpG sites. Very few studies report methylation at CpNpG sites and even less have proposed a role for CpCpG methylation. In *Arabidopsis*, CpNpG methylation plays a role in gene silencing and is mediated by histone H3 lysine 9 methylation through interaction of the DNA methyltransferase gene with methylated chromatin (Jackson *et al.*, 2002). Evidence for significance in mammalian systems is limited. Methylation at both cytosines with in CpCpG sites has been reported to prevent binding of Sp1, an important transcription factor, to its target cis element (Inoue and Oishi, 2005). However, the functional significance of methylation at solely the external cytosine has not been investigated.

DNA Methylation and Nutrition

The importance of understanding how diet influences carcinogenesis is discussed in the context of altering DNA methylation. Maintaining patterns of methylation is

highly dependent on the availability of methyl groups from S-adenosylmethionine (SAM). This methyl donor is derived from methionine and serves as the main methyl donor in methylation reactions involving DNA, RNA, hormones, neurotransmitters, membrane lipids, and proteins (Ross and Poirier, 2002). SAM is directly synthesized from its precursor methionine, an essential amino acid (Figure 3) (Van den Veyver, 2002). Choline and folate interact with the metabolism of methionine at its precursor homocysteine. Choline is metabolized to betaine which serves as the methyl donor to regenerate methionine (Van den Veyver, 2002). Alternatively, methyl-tetrahydrofolate derived from folate and 1-carbon metabolism can donate a methyl group to homocysteine to form methionine (Van den Veyver, 2002). When SAM donates a methyl group, it is converted to S-adenosylhomocysteine (SAH). The conversion of SAH back to homocysteine leads to the recycling of homocysteine and methionine. The balance of these inter-dependent factors determines the availability and utility of SAM.

Indications for the tight inter-relationships of these factors is apparent when knockout mice are studied. Methylene tetrahydrofolate reductase catalyzes the transfer of a methyl group from methyl-tetrahydrofolate to homocysteine. Mice lacking this enzyme show depleted choline and betaine levels as the maintenance of methionine synthesis is stressed (Niculescu and Zeisel, 2002). In addition, cystathionine beta-synthase knockout mice accumulate homocysteine and must convert it to methionine to remove it. In doing so, choline and betaine pools are depleted (Niculescu and Zeisel, 2002). Importantly, homozygous mutants for cystathionine beta-synthase show a lower genomic DNA methylation status in liver as compared to wild-type. This effect was also observed in kidney tissue but not brain (Choumenkovitch *et al.*, 2002).

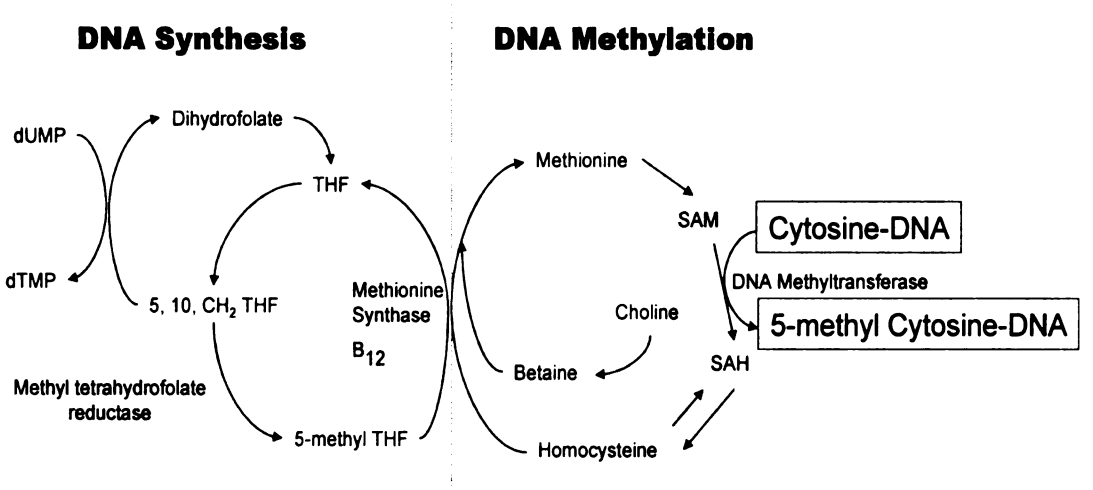


Figure 3 DNA methylation and 1-carbon Metabolism Schematic representation of the cyclic interplay between DNA synthesis and DNA methylation. DNA methyltransferase transfers a methyl group from S-adenosylmethionine (SAM) to cytosine to form 5-methyl cytosine and S-adenosylhomocysteine (SAH). Other dietary factors involved in this process are outlined for their roles in DNA synthesis or DNA methylation cycling. (Adapted from Choi and Mason, 2002)

Forms of methyl deficiency are induced via choline, methionine, choline and methionine, or folate deficient diets. Hypomethylation is frequently observed with methyl-deficient diets. Deficiencies in methionine and choline have been shown to lead to global hypomethylation in the livers of mice (Counts *et al.*, 1996) as well as over expression of oncogenes in the livers of rats (Wainfan and Poirier, 1992). These diets serve as effective promoting agents in multistage hepatocarcinogenesis. A key component to their effects is the induction of a hypomethylated state of DNA. Male F344 rats fed a methyl-deficient diet for 9, 18, 24 and 36wks showed decreased levels of SAM, SAM/SAH ratios, and global DNA hypomethylation (Pogribny *et al.*, 2005). Re-feeding the rats a methyl-adequate diet restored all parameters except the hypomethylated state of DNA and did not prevent the expansion of initiated foci. This suggests that stable DNA hypomethylation induced by methyl-deficiency is a promoting factor in stimulating initiated cells (Pogribny *et al.*, 2005).

Absolute levels of critical factors such as SAM and SAH might not be as important as the ratio between the two (Shivapurkar and Poirier, 1983). The ratio of SAM to SAH has been suggested to contribute in the mis-regulation of DNA methylation reactions. SAH is the intermediate formed during the recycling of SAM to homocysteine, the direct precursor of methionine. In effect, methyl deficiency decreases SAM and increases SAH shifting the proportionality towards SAH which is a feedback inhibitor of DNA methyltransferases. Therefore, the SAM/SAH ratio indirectly serves as a determinant of the extent of methylation (Shivapurkar and Poirier, 1983). SAM, on the other hand, acts as a feedback stimulator for the formation of 5-methyl tetrahydrofolate which donates a methyl group to homocysteine and facilitates the maintenance of

methionine levels (James *et al.*, 2003). This increases the intracellular requirement of folate which when depleted can compromise the *de novo* synthesis of deoxynucleotides in addition to further impairing the synthesis of SAM (James *et al.*, 2003). Therefore, the balance of these factors is critical to keeping up with the demand for maintaining the status of genomic methylation patterns.

Gene-Specific Patterns of Methylation and Regulation of Gene Transcription

Altered methylation as a precursor to toxicity is largely centered on regulating the expression of genes, most importantly, oncogenes, tumor suppressor genes, and transposable elements, which can be either increased or decreased resulting in toxic outcomes. Oncogene expression can be up-regulated via hypomethylation while tumor suppressor genes can be silenced when methylated. Both are classic contributors to the initiation and progression of cancer (Jones and Baylin, 2002). With my research I have consistently identified both increases and decreases in the methylation status of DNA occurring simultaneously in both precancerous and cancerous tissue. Therefore, it is imperative to consider the direct consequences of each distinct type of alteration.

Gene-Specific Methylation: Silencing of Tumor Suppressor Genes

Hypermethylation of promoter regions which are most commonly CpG island regions decrease expression levels of the corresponding gene. This silencing event has important consequences in the context of tumorigenesis and has been well characterized and demonstrated in tumors. Frequently cited genes which are observed to be hypermethylated include, p16, MGMT, CDKN2B, and RASSF1A (Jones and Baylin, 2002). Silencing of the tumor suppressor gene, p16, was demonstrated in gastric

carcinoma tissue (Chong *et al.*, 2003). P16 inhibits the activity of cyclin-dependent kinase 4 (cdk4) or cdk6. When inhibited, cdk4 and cdk6 can not phosphorylate regulatory proteins. For example, the retinoblastoma protein (Rb) must be phosphorylated in order to trigger a series of events transitioning the G_1 to S phase of the cell cycle. Therefore, if p16 expression decreases, cdk4 and cdk6 activity will increase resulting in phosphorylation of the Rb protein and progression of the cell cycle (Byeon *et al.*, 2004). Aberrant promoter methylation of RASSF1A is frequently detected in tumors of bladder, breast, colon, kidney, liver, and lung among others indicating its very common involvement in the progression of cancer (Pfeifer and Dammann, 2005). Knockout mice in which exon 1 of RASSF1A was deleted resulted in a more severe tumor susceptibility phenotype in mice supporting its tumor suppressive role. The biological role of RASSF1A is unknown but is hypothesized to be involved in several growth regulatory and apoptotic pathways (Pfeifer and Dammann, 2005).

In analyzing skin precancerous and cancerous tissue, one of my consistent findings was that hypermethylation is a more frequent occurrence than hypomethylation when promoting with cigarette smoke condensate. This indicates a predominant role for silencing of tumor suppressor genes in advancing skin tumorigenesis, and correlates to the hypermethylation of tumor suppressor promoter regions p16, MGMT, and HOXA5 observed using the same model (Watson *et al.*, 2004). In addition, exposure of B6C3F1 mice to mainstream cigarette smoke has been reported to silence the Death Associated Protein (DAP)-kinase and Retinoic Acid Receptor (RAR)-β genes via promoter hypermethylation and an increase the incidence of primary lung neoplasms (Hutt *et al.*, 2005).

Because DNA methylation is a reversible epigenetic mark, restoring the normal methylation status of these tumor suppressor genes becomes clinically relevant. Experimentally, reversal of a methylated state has been demonstrated. For example, microarray profile analysis revealed the silencing of 30 genes within an analyzed panel of expressed CpG island sequence tags in breast cancer cells, The re-expression of these silenced genes was confirmed by treatment with 5-AZA (Shi *et al.*, 2002). In a clinical setting re-expression of silenced genes via 5-AZA has limited success due to its non-specific effects. However, with proleukemic myelodysplastic syndrome, promising results have been obtained and could be related to the reactivation of the cyclin-dependent kinase inhibitor gene p15 (Herman and Baylin, 2003).

In addition to reactivating silenced tumor suppressor genes, specific methylation profiles of these genes could be used as unique biological and clinical parameters to identify different risk groups among patients (Banelli *et al*, 2005). The CpG island methylator phenotype was originally described in a subset of sporadic colorectal cancer with microsatellite instability (Toyota *et al.*, 1999). This phenotype refers to the simultaneous hypermethylation of multiple genes and this concept has been expanded for the purposes of identifying methylation profiles of silenced genes. On a small scale, gene hypermethylation profiles have been created for neuroblastic tumors (Banelli *et al.*, 2005). On a much larger scale, over 600 primary tumor samples representing 15 major tumors types were categorized and characterized for association with abnormal gene silencing illustrating the potential utility of using tumor suppressor genes as biomarkers for predicting and diagnosing cancer (Estellar *et al.*, 2001).

Gene-Specific Methylation: Activation of Oncogenes

Hypomethylation of promoter regions of genes has been linked to an increase in transcriptional activity. Ha-*ras* is a classic oncogene which plays a central role in signal transduction pathways, specifically the SOS-Ras-Raf-MAP kinase mitogenic cascade which transfers signals from growth factor receptors and integrins to the nucleus, leading to cell proliferation (Hanahan and Weinberg, 2000). It has commonly been implicated in tumorigenesis due to the high rate of mutation observed. Specifically, codons 12 and 61 are frequently mutated in spontaneous and mutagen-induced C3H/HE and B6C3F1mouse liver tumors (Whysner *et al.*, 1996). When phenobarbital, a non-genotoxic rodent liver tumor promoter is administered without previous initiation, findings show that an increase in the number of tumors is observed in susceptible mice. For example, in B6C3F1 mice exposed to 0.05% PB for 1 year, 100% of mice had 3-8 tumors; for those mice not exposed to PB, 29% had only 1-2 tumors per mouse. Additionally, the frequency of mutation in Ha-*ras* is lower than that found in spontaneous tumors (Whysner *et al.*, 1996). To extend the previous example, only 7% of PB-induced liver tumors showed point mutations in codon 61 as compared to 64% of spontaneous tumors (Maronpot *et al.*, 1995). This leads to the possibility that Ha-*ras* is regulated by an epigenetic mechanism such as methylation.

In much the same way, methyl deficiency elicited through diet or via agents such as arsenic can lead to a deficit in available stores of methyl groups resulting in global hypomethylation and gene specific altered methylation as observed with Ha-*ras*. In relatively sensitive and resistant mice, a choline-devoid methionine-deficient diet resulted in hypomethylation of Ha-*ras* in liver after 12wks of administration (Counts *et al.*, 1997).

In 2002, Okoji *et al.*, showed that a methyl deficient diet administered to male C57BL/6J mice in conjunction with arsenic leads to hepatic DNA global hypomethylation and a reduced frequency of methylation at cytosine sites within the promoter region of the Ha-*ras* gene. Therefore, it is plausible that altered DNA methylation by either a deficiency induced state or as a consequence of non-genotoxic agents might result in increased expression of cell cycle control genes and hence aid tumor development.

Although numerous studies have focused on the occurrence of Ha-*ras* hypomethylation and activation in liver, regulation of the methylation status of Ha-*ras*, c-myc, c-jun, cyclin D and r-ras have also been implicated in various other cancers and models. Digestion with MspI, HpaII, and HhaI has shown that Ha-*ras* is hypomethylated in some human colon and lung cancers (Feinberg and Vogelstein, 1983). In addition, site specific hypomethylation of a single CCGG site in the third exon of the c-myc oncogene was correlated to malignancy *in vitro* (Vachtenheim *et al.*, 1994). Hypomethylation and over-expression of c-jun and c-myc protooncogenes was demonstrated in liver tumors initiated by N-methyl-N-nitrosourea and promoted with dichloroacetic and trichloroacetic in female B6C3F1 mice (Toa *et al.*, 2000). Similarly, cyclin D, a protein involved in triggering the onset of the S phase in the cell cycle is over-expressed in a subset of gastric carcinoma. Hypomethylation of the promoter region of this gene was observed in 71% of gastric carcinomas analyzed and was correlated to an increase in expression of that gene (Oshimo *et al.*, 2003). Also associated with gastric cancer, r-ras is thought to inhibit Bcl-2 mediated rescue of apoptosis. This gene was seen to be silenced in normal gastric mucosa but activated via hypomethylation in more than half of gastric cancers (Nishigaki *et al.*, 2005). These studies demonstrate that methylation as an epigenetic mechanism

contributes to the activation of oncogenes during the process of tumorigenesis in both murine and human models.

Gene-Specific Methylation: Activation of Transposable Elements

This section is a brief synopsis of the more extensive review which was published in Toxicological Sciences in 2003. Please refer to the Carnell and Goodman, 2003 reference to obtain this review.

Evidence for methylation as a contributor to transcriptional control has been implicated in oncogenes and tumor suppressor genes as well as transposable elements (Jones and Baylin, 2002; Yoder *et al.*, 1997). Transposable elements account for approximately one third of the human genome and are distributed in a non-random fashion (Yoder *et al.*, 1997). The term transposable element encompasses both transposons and retrotransposons (Figure 4). Transposons have inverted terminal repeats, encode a transposase activity, and move from one site to another through a "cut and paste" mechanism (Smit and Riggs, 1996). Retrotransposons (*e.g.* LINE elements), which move by a "copy and paste" mechanism, proceed through an RNA intermediate largely dependent on their encoded reverse transcriptase activity. However, they might utilize the host's reverse transcriptase (Ostertag and Kazazian Jr., 2001). In this manner, a copy of the original can be integrated into a new genomic location. Therefore, stability of the genome depends upon keeping these movable and amplifiable elements transcriptionally repressed.

It is instructive to consider the role of altered methylation as an epigenetic mechanism for the activation of retrotransposable elements leading to their expression

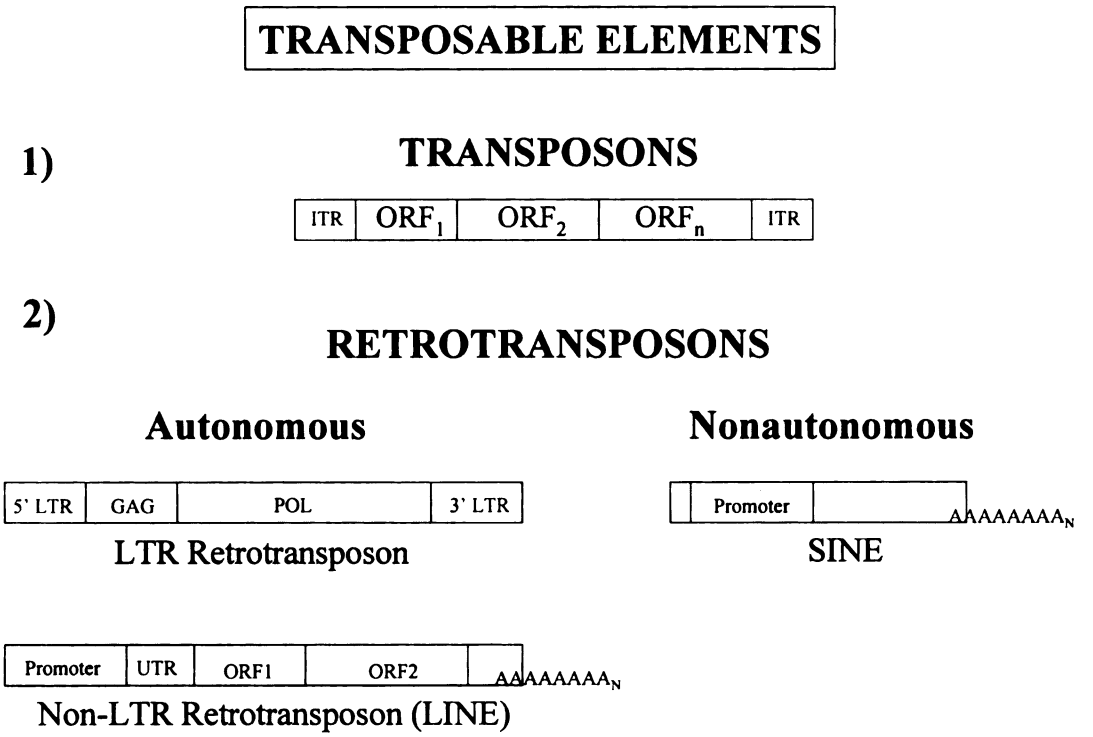


Figure 4 Transposable elements encompass both transposons and retrotransposons
(1) Transposons have inverted terminal repeats (ITR), which act as cis elements in the integration process, and two (or more) open reading frames (ORF), one of which encodes a transposase activity. These elements move by a "cut and paste" mechanism. (2) Retrotransposons are divided into autonomous and nonautonomous elements. Autonomous elements include long terminal repeat (LTR) and non-LTR subgroups. LTR-containing elements are structurally similar to retroviruses although they lack a functional *env* gene. Non-LTR elements contain an internal promoter for RNA polymerase II, a 5' untranslated region (UTR) and a 3' deoxyadenosine (A)-rich tract. Nonautonomous elements (SINEs) contain an internal promoter for RNA polymerase III and a 3' A-rich tract (Carnell and Goodman, 2003).

and possible retrotransposition (Figure 5) (Carnell and Goodman, 2003). Given the sheer number and distribution of these elements, both their movement and expression can lead to unstable conditions within the genome. Insertional mutagenesis, being the obvious end result of integration can be linked to chromosomal rearrangements leading to numerous diseases (Ostertag and Kazazian, 2001). Along with colon cancer and leukemia, other human diseases associated with insertional mutagenesis include hemophilia A, Duchenne's muscular dystrophy, and Huntingdon's disease. In addition to this, deletions and duplications can arise from unequal crossing over and mis-pairing of homologous sequences (Kazazian and Goodier, 2002). Furthermore, altered expression of genes harboring the integrant have been cited (Britten, 1997).

Aberrant transposable element activity presents a clear risk to the stability and integrity of the genome. Therefore, maintenance of these elements in a transcriptionally silent state is essential. Methylation has been suggested as a likely source of regulation. Hence, the mutagenicity and altered stability created by these elements might be a product of disturbed methylation patterns. Global hypomethylation, a common feature of tumor cells, has been associated with hypomethylation of LINE elements (Kaneda *et al.*, 2004). Here again, the interplay between epigenetics and mutagenesis takes shape. In this manner, altered DNA methylation might lead to the aberrant transcriptional activation of retrotransposons which could occur by a secondary, threshold exhibiting mechanism (Goodman and Watson, 2002). Further research into the timing and mechanism of altered methylation as a precursor to toxicity must then include oncogenes and tumor suppressor genes as classic contributors, the mis-regulation of retrotransposons,

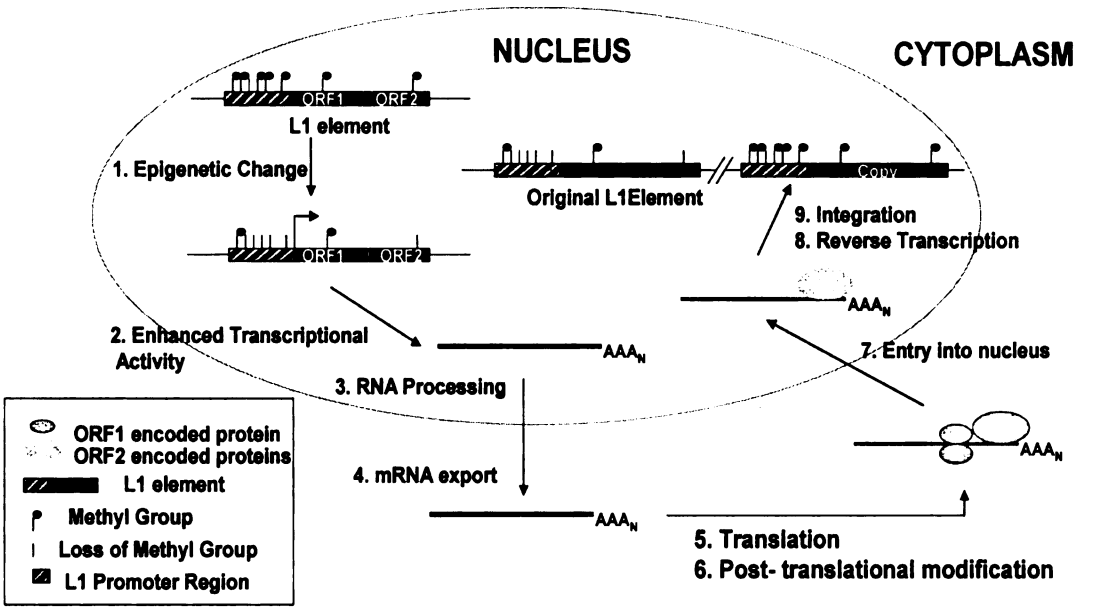


Figure 5 Altered Methylation and LINE-1 Elements Schematic representation of an epigenetic change as a precursor to expression and movement of retrotransposable elements. (1) An epigenetic change, e.g., hypomethylation of the retrotransposable elements allows for (2) enhanced transcriptional activity. (3) RNA processing and (4) mRNA export ensue. (5) Translation and (6) posttranslational modification precede the formation of a ribonucleoprotein particle in which ORF1 and ORF2 encoded proteins are associated with the original mRNA. (7) Once entry into the nucleus has occurred, (8 and 9) reverse transcription and integration are achieved via the encoded reverse transcriptase and endonuclease through a mechanism termed target primed reverse transcription (TPRT) (Carnell and Goodman, 2003).

and the confounding interaction of both working together to create genomic instability and disrupt homeostatic mechanisms.

Altered Methylation As A Precursor To Disease:

Although the intense focus of my research was on the role of DNA methylation in cancer, it is important to put into context the power of altered DNA methylation in non-cancer outcomes. Numerous disorders have been associated with aberrant regulation of DNA methylation patterns. These include, but are not limited to, imprinting disorders, repeat instability diseases, mental disorders and syndromes resulting from defects of the methylation machinery. Imprinting is the variable phenotypic expression of a gene which is dependent on whether it is of paternal or maternal origin. Approximately 80 genes are known to be imprinted and loss of this programming has been linked to human diseases (Roberston, 2005). An important regulator of imprinted gene expression is the CCCTC-binding factor (CTCF) which regulates the ability of distant enhancers to access promoters and has been shown to only bind to the unmethylated parental allele (Roberston, 2005). Beckwith-Wiedemann Syndrome (BWS) is a maternally transmitted disorder resulting in a predisposition to embryonic tumors (Robertson, 2005). BWS arises from a loss of imprinting at two imprinting control regions. Hypermethylation of the maternal allele is commonly seen at the first imprinting control region and loss of DNA methylation occurs within region 2 to produce the characteristic anatomical malformations (Robertson, 2005). Similarly, Prader-Willi syndrome results from the loss of paternally expressed genes and is characterized by hyperphagia, obesity during childhood and mental retardation. Other documented imprinting disorders include

Angelman syndrome, Albright hereditary osteodytrophy and transient neonatal diabetes mellitus.

Repeat instability diseases arise from the expansion of trinucleotide repeats which leads to mutation or silencing of genes (Roberston, 2005). Fragile X syndrome is the most common example and is a form of inherited mental retardation. Normally, the fragile X mental retardation-1 (FMR1) gene contains a highly polymorphic CGG repeat, between 6 and 52 repeats within its 5'-untranslated region of exon 1. In fragile X patients, the copy number of the repeats increase dramatically to 200-600 which is also accompanied by *de novo* methylation and histone deacetylation of the CpG island upstream of the gene. This aberrant methylation and histone modification result in silencing of the gene (Robertson and Wolffe, 2000). Aberrant hypomethylation is the causal factor in facioscapulohumeral muscular dystrophy (FSHD). Affected individuals show repeat contraction 11-150 copies down to 1-10. This contraction results in loss of methylation and the aberrant expression of proximal genes (Robertson, 2005).

Altered methylation might be a contributing factor in neurological and developmental disorders. Mutations in the DNMT3b gene which codes for a de novo methylase, leads to ICF or immunodeficiency, centromeric instability and facial anomalies (Jones and Baylin, 2002). Here brain development is disrupted when methylation patterns are not maintained due to improper gene expression patterns and chromosomal structure. Aberrant methylation resulting from folate deficiency or vitamin B12 deficiency is important in regards to neuropsychiatric symptoms. Methylation changes seen in the CNS might be linked to low levels of SAM (Singh *et al.*, 2003). In light of this, SAM supplementation has been used as an antidepressant (Bottiglieri *et al.*,

1994). Alterations in the folate and methyl-group metabolism including increased methionine adenosyltransferase activity, increased incidence of methyl-tetrahydrofolate reductase mutations and increased homocysteine levels have been reported in patients with schizophrenia. All these factors indicate that schizophrenia might in part arise from deficiencies in methylation and therefore altered methylation which would begin to explain the various nonmendelian irregularities of schizophrenia (Petronis, 2004). Mutations in MeCP2, are the primary cause of the neurodevelopmental disorder Rett syndrome which affects approximately 1 out of every 15,000 females worldwide and is associated with mental retardation and autism (Van den Veyver, 2002). Missense mutations have been identified in the MeCP2 gene which cluster in the methyl binding domain resulting in a decreased affinity of the MeCP2 for its target methylated CpG dinucleotide (Shahbazian and Zoghi, 2002). Similarly, mice lacking MBD2 have a neurobehavioral phenotype (Van den Veyver, 2002).

Cardiovascular disease has also been explored for connections with altered patterns of DNA methylation. Patients with vascular disease showed increased plasma total homocysteine and S-adenosylhomocyteine (SAH) and lower SAM/SAH ratios. Altered global DNA methylation status in white blood cells from male atherosclerotic vascular patients was correlated with increases in total homocysteine and SAH providing a cursory link between the two (Castro *et al.*, 2003). Because of cellular proliferation and monoclonality of some cells within atherosclerotic lesions, atherosclerosis has been compared to benign vascular tumors (Hiltunene and Yla-Herttuala, 2003). Therefore, changes in DNA methylation observed during atherogenesis might contribute to lesion development in a similar way to tumor development (Hiltunene and Yla-Herttuala, 2003)

Interestingly, global DNA methylation, a common feature of tumor DNA, has been measured in murine, human and rabbits with advanced atherosclerosis (Zaina *et al.*, 2005). Genes at least partially regulated by DNA methylation that play a role in atherosclerosis include IFN-γ, PDGF, and the human estrogen receptor (Hiltunene and Yla-Herttuala, 2003). Studies points to the hypermethylation of specifically the human estrogen receptor as an early predisposing factor (Zaina *et al.*, 2005). Therefore, the role of altered DNA methylation extends far beyond cancer and disrupts various homeostatic mechanisms leading to a range of human disorders.

DNA Methylation and Promotion of Tumorigenesis: Rodent Models

Rodent models of carcinogenesis are normally the first line of experimentation in trying to understand the underlying molecular mechanisms so that ultimately, concepts can be applied to prediction, diagnosis and clinical treatment of human cancer. In addition, deducing the relative risk to humans through the use of murine models is commonplace in reproductive and carcinogenicity testing. However, inducing malignant transformation in human and mouse cells bears some basic differences (Hahn and Weinberg, 2002). These differences should not diminish the value of mouse models but serve as a cautionary reminder of the need for critical assessment of the significance of the results. Both the murine liver tumorigenesis model and SENCAR 2-stage mouse skin model were employed in testing the potential of three very different compounds to act as tumor promoting agents capable of disrupting DNA methylation patterns.

Phenobarbital and Liver Tumorigenesis

Differences in the susceptibility of strains and stocks of mice to develop liver tumors is extremely valuable in that it allows for the comparison of molecular events occurring in highly susceptible and more resistant mice which might shed light on specific critical events involved in tumorigenesis. Theoretically, use of these models could provide insight regarding the basis for variable levels of human susceptibility to the formation of cancer. In addition, the liver is the primary target site of carcinogenesis for more than 200 chemicals as identified by the National Toxicology Program data (Haseman *et al.*, 1984). B6C3F1 are particularly sensitive to the formation of liver tumors as they are derived from a cross between the relatively resistant maternal strain, C57BL/6J, and the highly susceptible paternal strain, C3H/He. The incidence of spontaneous liver tumors in B6C3F1 mice over an 18month period was reported to be 29%, with 1-2 tumors per mouse (Becker, 1982). This relative sensitivity seems to be enhanced during chemically induced hepatocarcinogenesis. B6C3F1 mice administered 0.05% PB in their drinking water for 18 months exhibited a 100% liver tumor incidence with 3-8 tumors observed per mouse. (Becker, 1982). Owing to the high sensitivity of these mice, promoting agents have been tested alone or following exposure to an initiating agent. The fact that tumor formation is induced in mice only exposed to a promoting agent suggests that a few "spontaneously" initiated hepatocytes are present in these mice. In addition, due to the heritability of changes in methylation, epigenetic initiation is also possible (Goodman and Watson, 2002). Initiation and promotion events in the mouse liver tumor model correlate to the formation of altered hepatic foci. These foci represent the clonal expansion of a single initiated cell in response to a tumor

promoting agent and supports the framework of multi-stage carcinogenesis (Klaunig *et al.*, 1990).

The sensitivity of the B6C3F1 mouse to liver tumorigenesis is a good experimental model for assessing mechanisms of carcinogenicity, however, in using the relative resistant C57BL/6 mouse strain as a parallel experiment additional value is added to the results obtained. C57BL/6 mice are considered relatively resistant to liver tumorigenesis because they rarely develop spontaneous liver tumors even when exposed to the promoting agent PB (Becker, 1982). Emphasis has been placed on discerning the genetic and molecular differences between these mice concerning their relative sensitivities to hepatocarcinogenesis. The hepatocarcinogen sensitive locus (hcs) has been implicated in the variable sensitivities of these mice (Drinkwater *et al.*, 1989). The intermediate susceptibility of B6C3F1 are due to the semi-dominant alleles of the highly susceptible C3H/He and resistant C57BL/6 parents of origin. This locus appears to affect the growth rate of pre-neoplastic foci during promotion (Manenti *et al.*, 1994). The growth and development of preneoplastic foci in response to initiation with N,N-diethylnitrosamine (DEN) and promotion with PB was mouse strain-dependent (C3H/He < B6C3F1 < C57BL/6) (Goldsworthy and Fransson-Stern, 2002). The time to conversion of foci to masses also correlated with strain susceptibility (Goldsworthy and Fransson-Stern, 2002).

The majority of cellular and molecular differences between the tumor-prone and tumor-resistant mice are generally seen during the promoting stage of tumorigenesis. For example, initiation of C3H/He and C57BL/6 mice with DEN did not reveal any differences in the persistence of hepatic DNA adducts or DNA repair (Drinkwater and

Ginsler, 1986). However, differences in the rate and induction of apoptosis during promotion, once thought to play a major role in strain susceptibilities, has recently been contested. Hepatocarcinogenesis was induced by a single dose of DEN followed by promotion with PB for 90 wks. Growth rates of preneoplastic foci and tumors were largely determined by the relative rates of cell proliferation in C3H/He and C57BL/6 mice. Importantly, apoptotic activity in preneoplastic foci was low in both mouse strains and appears to play only a minor role in susceptibility differences (Bursch *et al.*, 2005a). In support of this, a follow-up study showed that apoptotic activity in both strains is comparable and does not increase following cessation of the promotion stimulus (*i.e.* PB) (Bursch *et al.*, 2005b). Therefore, the focus for susceptibility differences remains on rates of cell proliferation. In line with this theory, parenchymal cells were tested for responsiveness to signals inducing replication or apoptosis. Hepatocytes from C57BL/6 mice possess a low basal rate of DNA synthesis and low inducibility by epidermal growth factor, but a higher sensitivity to induction of apoptosis by TGF-β1 than hepatocytes of the C3H/He strain (Parzefall *et al.*, 2002). In addition, based on work with chimeric mice, Lee (1991) provided convincing evidence that the susceptibility differences lie within the hepatocytes themselves and not within the micro-environment. This indicates that growth potential and possibly clues to susceptibility are manifested at the cellular and molecular level.

Phenobarbital, as demonstrated, has somewhat served as the compound of choice for the investigation of multistage carcinogenesis in the context of liver tumorigenesis in mice. Fundamentally, PB is used as a sedative and anticonvulsant and is known for its ability to induce expression of P450 genes, specifically CYB2B1 (Whysner, 1996). In

addition PB has been shown to block gap junctional intracellular communication (GJIC), a frequent finding in cancer cells, with interesting strain specific differences (Ito *et al.*, 1998; Warner *et al.*, 2003; Trosko and Chang, 2001). However, the most relevant finding to my research involving PB has been the observed differences of strains and stocks of mice to maintain patterns of methylation (Ray *et al.*, 1994; Counts *et al.*, 1996; Watson and Goodman, 2002). Previous studies in our lab have shown that PB induces a greater extent of global hypomethylation at 1, 2, and 4 weeks in the B6C3F1 mice as compared to C57BL/6 (Counts et al., 1996). Critical to this finding was the fact that cell proliferation was enhanced to a greater extent in the C57BL/6 mice as compared to the B6C3F1 mice. Therefore, global hypomethylation levels in the B6C3F1 mice can not be solely attributed to decreased fidelity of the maintenance methyltransferase in the face of PB-induced increased cellular proliferation (Counts *et al.*, 1996). Refined analysis of changes in methylation has revealed that GC-rich hypermethylation exists concurrently with global hypomethylation (Jones and Laird, 1999). This was demonstrated using a global approach to specifically measuring changes in GC-rich regions of DNA which are normally associated with the promoter regions of genes. A 2wk exposure of PB induced a greater degree of altered methylation, specifically hypermethylation, in GC-rich regions in B6C3F1 mice than C57BL/6 mice (Watson and Goodman, 2002).

Hypomethylation of the promoter regions of oncogenes has also been a result of PB promotion. Increased levels of *raf* and Ha-*ras* expression in liver were observed following a 2wk promoting dose of PB. Increased expression of *raf* was associated with hypomethylation and was only observed in B6C3F1 (Ray *et al.*, 1994). With a lower dose of PB, only increases in Ha-*ras* mRNA were observed indicating that lower doses of

PB promote hepatocytes with increased Ha-*ras* expression while higher doses select for cells exhibiting increased expression of both Ha-*ras* and *raf* (Counts *et al.*, 1997). Collectively, these studies indicate a reduced capacity of the B6C3F1 mouse to maintain hepatic patterns of methylation and hence shows enhanced sensitivity to tumorigenesis. However, there is still a need to refine and specifically identify which changes in methylation are in common or are different between B6C3F1 and C57BL/6 mice in order to assess more accurately the importance of increases and decreases in methylation. In addition, the specificity of PB to alter methylation in its target tissue needs to be tested. Thus I have extended previous studies by employing an advanced and highly sensitive technique for measuring increases, decreases, and new methylations in GC-rich regions in response to 2 or 4 wk, 0.05% dose of PB in B6C3F1 and C57BL/6 mice. In addition transposable elements as well as Ha-*ras* were examined for altered methylation in response to PB promotion. Chapter Two focuses on the design and experimental results of this study.

SENCAR Mouse Skin Initiation-Promotion Model

One of the best defined experimental *in vivo* models for epithelial cancer development is the chemically induced tumor model of mouse skin. The outbred SENCAR (acronym for SENsitive to skin CARcinogenesis) mouse was developed in the 1960's and 1970's when mice, sensitive to papilloma formation in response to administration of initiating and promoting agents were selected for via breeding (Stern and Conti, 1996; Boutwell, 1964). These mice also have a rather high spontaneous incidence of tumors (♀65.6% and ♂69.4%) of which the majority are papillary tumors of the lung (Melchionne *et al.*, 1986). However, it is the sensitivity to initiating and

promoting agents which is highly advantageous to discerning the role of altered DNA methylation in tumorigenesis during defined stages of carcinogenesis, particularly initiation and promotion Treatment of SENCAR mouse skin with a single application of an initiating agent followed by repeated application of a promoter results in the formation of benign papillomas and malignant carcinomas. Initiation by 7, 12-dimethylbenz[α]anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA) is a standard regimen for a consistent response to the induction of skin tumors (Hennings *et al.*, 1997; Coghlan *et al.*, 2000). DMBA is a polycyclic aromatic hydrocarbon that requires metabolism by the mouse epidermal aryl hydrocarbon hydroxylase enzyme system (AHH) of which the highest activity is found in the epidermal layer of mouse skin (DiGiovanni, 1992). DMBA is genotoxic and it is known to bind extensively to DNA creating DNA adducts (DiGiovanni, 1992). In addition, an important part of initiation is thought to be associated with somatic mutation of c-Ha-*ras* as a high percentage of DMBA-induced mouse skin carcinomas and papillomas have activated c-Ha-*ras* (Balmain *et al.*, 1984).

In using this model, the timing and appearance of regions of altered methylation in both GC-rich regions and in gene-specific promoter regions in response to skin promotion can be studied in addition to altered patterns of methylation in tumor tissue. Altered patterns of methylation have been identified in response to initiation with DMBA and promotion with cigarette smoke condensate (CSC). Both increases and, less frequently, decreases were observed (Watson *et al.*, 2003). Gene promoter hypermethylation was also observed for the tumor suppressors p16, MGMT, and HoxA5 (Watson *et al.*, 2004). Importantly, repression of HoxA5 was linked to an increased

amount of methylation in the HoxA5 promoter region. Methylation of the promoter region was reversible and correlated to restoration of normal expression. The fundamental theory of multistage carcinogenesis during the promotion stage is that heritable, "critical" changes in the genome progressively accumulate, but are reversible upon the cessation of the promoting agent. A clear demonstration of progressive, non-random changes in methylation was accomplished by combining the novel technique to measure GC-rich changes in methylation (arbitrarily primed PCR and capillary electrophoresis) with the multistage model of carcinogenesis. In doing so I was able to track changes in methylation from very early time points through to neoplastic stages. Details are provided in Chapter 3.

A Novel Approach to Measuring Global Changes in DNA Methylation

My experimental results concerning altered methylation in GC-Rich regions have been obtained via methylation sensitive restriction digestion, arbitrarily primed PCR and capillary electrophoretic separation of PCR products. Development of this method has provided a more refined and quantitative approach to assessing altered methylation in response to treatment. Experimental details of this methodology are found in Chapters 1, 2 and 3. However, outlining the advantages over earlier versions of this technique in addition to explaining the theoretical "proof of concept" is necessary in light of its central importance to my experimental findings.

The precursor version of this technique as described in Watson and Goodman, 2002 and Watson *et al.*, 2003, was successfully employed to measure changes in GC-rich regions. The technique involved PCR of digested genomic DNA in which PCR

products were radiolabeled with α-P^{33}-dATP nucleotides. Therefore, the higher the adenine content of the PCR product, the more label that was incorporated. PCR products were separated on a 6% polyacrylamide sequencing gel. Following electrophoresis the gel was stored with a phosphoimage screen and exposure of that screen was detected with the phosphorimager. The intensities of each separate band were determined using the NIH image analysis program and statistical differences between band intensities identified changes in methylation when comparing control and treated samples.

Numerous limitations of this method have been overcome with the optimization of capillary electrophoretic separation of PCR products. This newly developed method provides multiple advantages. One of the most valuable features is the quantitative data that are produced. The ability to perform statistical calculations adds credibility and confidence to the results. A higher level of reproducibility was gained in addition to a 10 fold expansion of the number of analyzable PCR products. The volume of information obtained from a single experiment created an in depth means of answering questions concerning treatment related disruption of control methylation patterns in various model systems and organs.

The "proof of concept" for this methodology was based on the well documented observation that the majority of 5-methyl cytosine occurs at cytosines which are 5' to guanine. Both MspI and HpaII restriction enzymes recognize the 5' CCGG 3' sequence and cut between the internal cytosine and guanine. HpaII will not cut the DNA if the internal cytosine is methylated, however MspI will. Therefore, based on the above statement, MspI should digest the DNA more thoroughly than HpaII. In order to test this, a sample of control DNA from 3 animals was digested with MspI and HpaII separately.

PCR was performed on these digested samples. An average peak area of the three animals from each digest was calculated for each PCR product size formed. The average peak area of each MspI PCR product was compared to the area peak area of the corresponding HpaII PCR product (*i.e.* the PCR products were of the same size in base pairs). Therefore, the amount of PCR product of a particular size formed following digestion with MspI was compared to the amount of PCR product formed of the same size following digestion with HpaII. The MspI digest was calculated as a percent of the HpaII digest. With this calculation, all negative numbers indicate more restriction by MspI whereas all positive numbers indicate less restriction by MspI. Three control mouse liver DNA samples from 3 animals and 3 DNA samples from mice treated with 0.05% PB for 2wks were used in two separate tests (Figures 6 and 7). Importantly, comparison of both control and treated samples from each digest shows a greater amount of restriction by MspI (*i.e.* the majority of calculated values fell below the x-axis) indicating that the method is credible. Fundamentally, capillary electrophoresis as a method of detection of PCR product following arbitrarily primed PCR shows conceptual validity.

With this "proof of concept" in hand, control patterns of methylation could be compared to treatment-induced altered methylation patterns. Four types of altered methylation can be detected: 1) complete hypomethylation, a 100% loss of methylation within a region of DNA 2) partial hypomethylation, a statistically significant decrease as compared to control 3) hypermethylation, a statistically significant increase as compared to control and 4) new methylation, a gain of methylation within a completely unmethylated region following treatment. The simultaneous detection of increases,

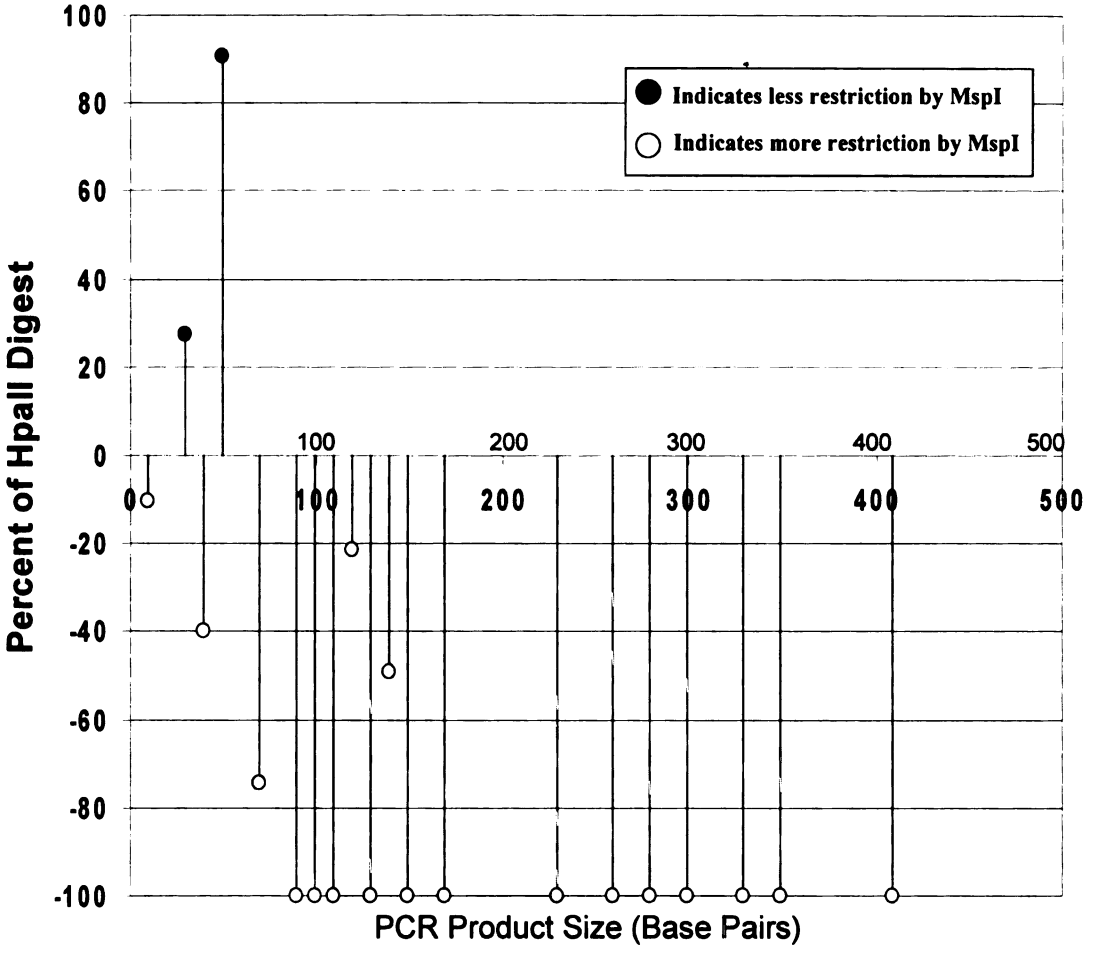


Figure 6 B6C3F1 Control Animals: Peak Areas of PCR products generated following digestion with MspI as a Percent of those generated following the HpaII Digest Liver DNA from 3 Control B6C3F1 animals was digested with MspI and HpaII separately. PCR was performed on the digested samples and the average peak area of the 3 animals from each digest was calculated for each PCR product size. The average peak area formed following digestion with MspI was calculated as a percent of the average peak area of the same PCR product formed following digestion with HpaII. All negative numbers indicate more restriction by MspI and all positive numbers indicate less restriction by MspI.

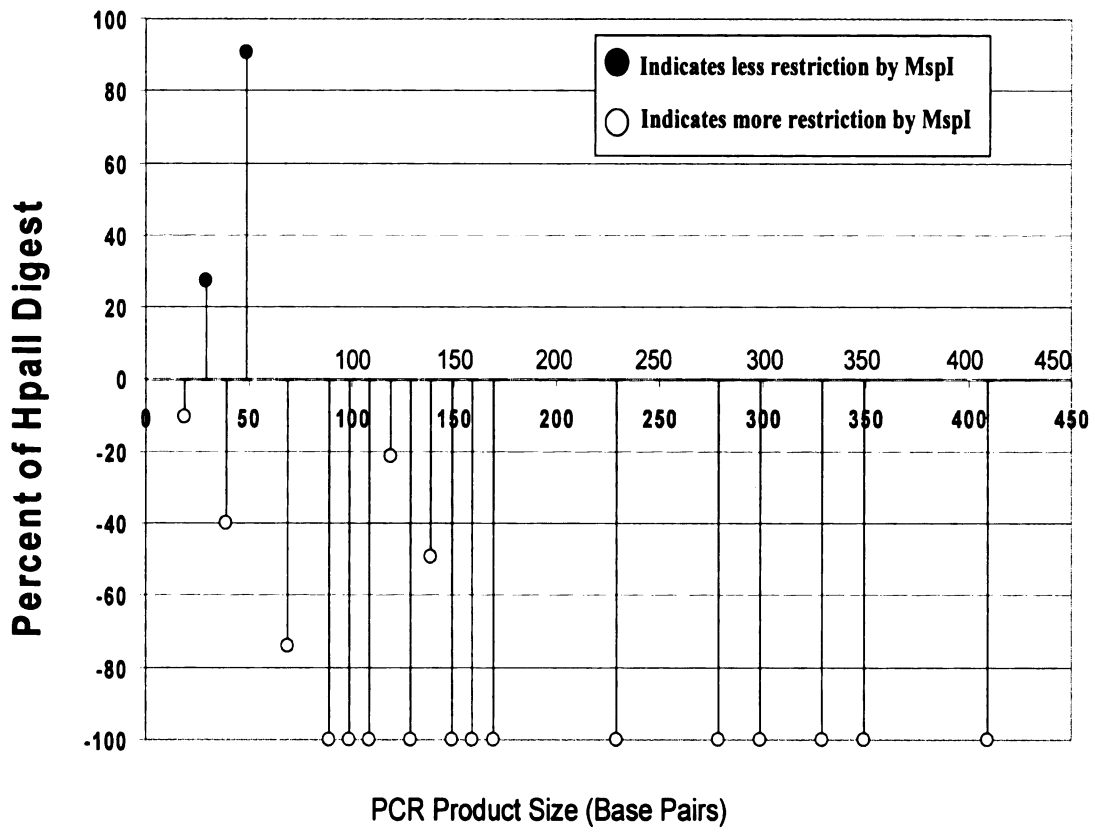


Figure 7 B6C3F1 PB Treated Animals: Peak Areas of PCR products generated following digestion with MspI as a Percent of those generated following the HpaII Digest Liver DNA from 3 B6C3F1 animals treated with 0.05% PB for 2wks was digested with MspI and HpaII separately. PCR was performed on the digested samples and the average peak area of the 3 animals from each digest was calculated for each PCR product size. The average peak area formed following digestion with MspI was calculated as a percent of the average peak area of the same PCR product formed following digestion with HpaII. All negative numbers indicate more restriction by MspI and all positive numbers indicate less restriction by MspI.

decreases and new methylations is a very important to assessing genome wide altered methylation. This methodology was applied in three different model systems including hepatocytes in culture, mouse liver and kidney and mouse skin. The data obtained were reproducible and highly consistent across models. This methodology is further discussed in Chapters 1, 2, and 3.

Hypothesis and Objectives

Research projects were designed and developed to test aspects directly and indirectly related to my overall hypothesis. **Susceptibility to carcinogenesis is related inversely to the capacity to maintain normal DNA methylation patterns.** This broad hypothesis includes the possibility of altered methylation as a precursor to toxicity and allows for the interpretation that susceptibility can largely denote physiological states and events that are conducive to fostering the process of carcinogenesis. One extension of this overall hypothesis is that altered DNA methylation is a cause and not an effect of tumorigenesis. The following three sub-hypotheses lend strong support for this concept.

1. Changes in methylation are progressive during the promotion stage of tumorigenesis
2. Changes in methylation are non-random during the promotion stage of tumorigenesis
3. Changes in the promoter regions of genes correlate to changes in gene expression.

Diethanolamine (DEA) is an alkanolamine found in many consumer products and is widely used in industry. In 1999, the National Toxicology Program applied DEA in 95% ethanol to the skin of mice and rats for 2yrs which led to significant increases in the incidence and multiplicity of liver tumors in mice but not rats. In a follow up study using B6C3F1 mice, dermal application of DEA resulted in decreased levels of SAM, increased levels of SAH, and a reduction in phosphocholine (McKeeman *et al.*, 2002). All of these factors are indicative of choline deficiency which has also shown to disrupt cellular growth and division, induce a methyl-deficient state and cause liver tumors in B6C3F1 mice (Zeisel, 1996; Newberne, 1982). In addition, a methyl-deficient diet alters methylation of DNA in target tissue (Counts *et al.*, 1996). Given that DEA is not DNA reactive (Knaak *et al.*, 1997), it possibly acts by disrupting choline homeostasis to induce

choline deficiency and consequently alters DNA methylation patterning which facilitates tumorigenesis. By using B6C3F1 mouse hepatocytes in culture I was interested in measuring DEA-induced changes in methylation to establish that DEA is working, at least in part, through an epigenetic mechanism. Importantly, patterns of altered methylation induced by choline deficiency are compared to DEA to determine the extent of similarity between them. Similarities would support the notion that changes in methylation are not simply a random process. In addition, treatment with phenobarbital acted as a positive control for inducing altered patterns of methylation in mouse hepatocytes.

The extent of altered methylation in response to PB has previously been investigated in mice which are considered susceptible (B6C3F1) or resistant (C57BL/6) to liver tumorigenesis (Counts *et al.*, 1996, Watson and Goodman, 2002). The findings strongly indicate that when compared to the relatively resistant C57BL/6 mouse, the tumor-prone B6C3F1 mouse is less able to maintain its normal methylation patterns, and therefore exhibits a greater degree of hypomethylation throughout the genome (*i.e.* global hypomethylation) in addition to more extensive disruption of patterns of methylation in GC-rich regions. In order to further characterize the patterns of altered methylation induced in the B6C3F1 and C57BL/6 mice, I examined PB-induced changes in methylation on a region by region basis for similarities and, more importantly, differences between them. Specifically PB-induced regions of altered methylation unique to B6C3F1 liver were identified and hypothesized to be important for the development of hepatocarcinogenesis. Kidney, a non-target tissue, was used to assess the specificity of PB for altered methylation in liver. Progressive, non-random changes in

methylation were identified and tested for reversibility. In addition, methylation of the promoter regions of the Ha-*ras* oncogene and LINE-1 elements were examined. Activation of the Ha-*ras* oncogene and or LINE-1 elements via hypomethylation could lead to disruption of cell cycle regulation and genomic instability.

The progressive accumulation of heritable changes is fundamental to the process of carcinogenesis. Increasingly aberrant subclone populations arise from the clonal expansion of initiated cells during promotion. The SENCAR mouse 2-stage initiation/promotion model of carcinogenesis has been successfully used to observe the contribution of epigenetic mechanisms to this process. Specifically, altered patterns of methylation in GC-rich regions in addition to gene-specific promoter regions of genes in precancerous and cancerous tissue were measured in response to promotion with cigarette smoke condensate (CSC). Dose and time dependent changes were observed and were largely reversible, a hallmark of tumor promotion (Watson *et al.*, 2003; Dragan *et al.*, 1993). In addition hypermethylation of three tumor suppressor genes was demonstrated (Watson *et al.*, 2004). I extended these initial studies to investigate more clearly the step-wise accumulation of changes in methylation over time which then "carry forward" to tumor tissue. These specific and non-random methylation changes could be critical to regulating the expression of oncogenes, tumor suppressors, and possibly retrotransposable elements. Therefore the methylation status of the Ha-*ras* oncogene and the LINE-1 element were analyzed and compared to their expression. Finally, reversibility of changes in methylation were assessed to fully evaluate the stability of altered DNA methylation following cessation of the promoting stimulus.

Each of the three chapters in this dissertation focus on testing the outlined hypotheses and objectives by addressing the following specific aims.

A) Examination of DEA-induced altered methylation in GC-rich regions as a contributor to the development of liver tumors in B6C3F1 mice.

1) To assess global and GC rich methylation alterations in response to DEA, CD, and PB in B6C3F1 mice.

2) Compare changes in methylation in GC-rich regions following DEA and PB treatment to those observed after choline deficiency.

B) Effects of phenobarbital (PB) on gene specific and GC rich regional methylation status of hepatic DNA in tumor prone and tumor-resistant mice.

1) To determine if cancer susceptibility in mice is related to differences in the ability to maintain normal patterns of methylation in response to PB

2) Characterize progressive changes in methylation by specifically identifying regions of altered methylation that carry forward with time in the B6C3F1 and C57BL/6 mice.

3) Identify changes in methylation which are unique to B6C3F1 liver when compared to altered DNA methylation observed in C57BL/6 liver or B6C3F1 kidney.

4) To determine the effects of PB on the methylation status of the promoter regions of Ha-*ras* and LINE-1 elements and subsequently analyze changes in their gene expression.

5) To test the reversibility of PB-induced altered methylation in GC-Rich regions and gene-specific promoter regions (*i.e.* Ha-*ras* and LINE-1 elements).

C) Characterization of GC-rich and gene specific methylation changes in tumor and precancerous skin tissue during the promotion stage of the 2-stage, initiation/promotion SENCAR mouse model.

1) Evaluate the methylation status of GC-rich regions following 8wk promotion with increasing doses (3, 9, 18, 27mg) of the promoting agent, CSC.

2) Evaluate the methylation status of GC-rich regions following 4wk and 8wk promotion with 27mg of CSC.

3) Evaluate the methylation status of GC-rich regions in tumor tissue (29wk) and compare to precancerous tissue.

4) Characterize progressive changes in methylation by specifically identifying regions of altered methylation that carry forward with time from 4wk to 8wk and from 8wk to tumor tissue (29wk)

5) Assess the reversibility of changes in methylation in GC-rich regions upon cessation of CSC application.

6) Evaluate changes in the methylation status of the promoter regions of Ha-*ras* and LINE-1 elements to changes in gene expression in both precancerous and tumor tissue.

REFERENCES FOR INTRODUCTION, SUMMARY, AND DISCUSSION SECTIONS ARE LISTED ON PAGES 231-241.

CHAPTER 1

DIETHANOLAMINE AND PHENOBARBITAL PRODUCE AN ALTERED PATTERN OF METHYLATION IN GC-RICH REGIONS OF DNA IN B6C3F1 MOUSE HEPATOCYTES SIMILAR TO THAT RESULTING FROM CHOLINE DEFICIENCY

This chapter represents a manuscript that was submitted to Toxicological Sciences in November, 2005. Authors include: Bachman, Ammie N. Kamendulis, Lisa M. and Goodman , Jay I.

ABSTRACT

DNA methylation is an epigenetic mechanism regulating transcription, which when disrupted, can alter gene expression and contribute to carcinogenesis. Diethanolamine (DEA), a non-genotoxic alkanolamine, produces liver tumors in mice. Studies suggest DEA inhibits choline uptake and causes biochemical changes consistent with choline deficiency (CD). Rodents fed methyl-deficient diets exhibit altered methylation of hepatic DNA and an increase in liver tumors, e.g., CD causes liver tumors in B6C3F1 mice. We hypothesize that DEA-induced CD leads to altered methylation patterns which facilitates tumorigenesis. B6C3F1 hepatocytes in primary culture were grown in the presence of either 4.5mM DEA, 3mM Phenobarbital (PB) or CD media for 48hrs. These concentrations induced comparable increases in DNA synthesis. PB, a nongenotoxic rodent liver carcinogen known to alter methylation in mouse liver, was included as a positive control. Global, average, DNA methylation status was not affected. The methylation status of GC-rich regions of DNA, which are often associated with promoter regions, were assessed via methylation-sensitive restriction digestion and arbitrarily primed PCR with capillary electrophoretic separation and detection of PCR products. DEA, PB and CD treatments resulted in 54, 63, and 54 regions of altered methylation (RAMs), respectively, and the majority were hypomethylations. A high proportion of RAMs (72%) were identical when DEA was compared to CD. Similarly, 70% were identical between PB and CD. Altered patterns of methylation in GC-rich regions induced by DEA and PB resemble that of CD and indicate that altered DNA methylation is an epigenetic mechanism involved in the facilitation of mouse liver tumorigenesis.

INTRODUCTION

Diethanolamine (DEA), an alkanolamine, is used in industrial applications such as textile processing, industrial gas purification, and preparation of agricultural chemicals. In addition, fatty acid condensates synthesized from DEA are found in numerous consumer products such as cosmetics, soaps and detergents (Knaak *et al.*, 1997). Widespread human exposure to DEA prompted the National Toxicology Program (NTP) to examine its carcinogenic potential. Dermal applications of DEA in 95% ethanol for 2 years led to significant increases in the incidence and multiplicity of liver tumors in male and female B6C3F1 mice, but not F344 rats. Recently, DEA- induced increases in liver cell proliferation were observed *in vitro*. Importantly, this effect was specific to F344 rats and B6C3F1 mice and not observed with human hepatocytes (Kamendulis and Klaunig, 2005). Based on *in vitro* genetic toxicity studies DEA and/or its metabolites are not mutagenic (NTP, 1999), suggesting that it induces a tumorigenic response via a secondary, non-genotoxic mechanism(s).

Similar in structure to ethanolamine and choline, two essential precursors for the synthesis of phospholipids, DEA is incorporated into hepatic phospholipids, perhaps disrupting regulation of choline and 1-carbon metabolism. Furthermore, DEA can inhibit the uptake of choline leading to intracellular deficiency, even if there is an adequate amount of choline in the diet (Lehman-McKeeman and Gamsky, 1999). Deficiencies in the major dietary sources of methyl groups, specifically, choline and methionine, lead to hepatocarcinogenesis in rodents (Poirier, 1994, Henning and Swendseid, 1996). Choline deficiency (CD) causes hepatocyte proliferation and apoptosis (Albright *et al.*, 1996, Ziesel, 1996). In particular, CD in rodents, including

B6C3F1 mice, in the absence of known carcinogens, increases liver tumor development (Newberne *et al.*, 1982, Newberne and Rodgers, 1986.)

Diets lacking in choline and methionine result in altered levels of S-adenosyl methionine (SAM), and S-adenosyl homocysteine (SAH). SAM is the main methyl donor for a variety of methylation reactions including DNA methylation (Ziesel, 1996). In effect, methyl deficiency decreases SAM and increases SAH shifting the proportionality towards SAH which is a feedback inhibitor of DNA methyltransferases and, therefore, the SAM/SAH ratio is a determinant of the extent of methylation (Shivapukar and Poirier, 1983). In B6C3F1 mice, dermal application of DEA resulted in decreased levels of SAM, increased levels of SAH, and a reduction in phosphocholine, the intracellular storage form of choline, which are all consistent with previous reports of biochemical changes associated with CD which leads to methyl deficiency (Lehman-McKeeman *et al.*, 2002). Indeed, deficiencies in methionine and choline have been shown to lead to global, average hypomethylation of DNA in the livers of B6C3F1 mice (Counts *et al.*, 1996).

It has been hypothesized that alteration of the epigenome, specifically DNA methylation is a mechanism underlying DEA-induced tumorigenesis in B6C3F1 mouse liver (Lehman-McKeeman and Gamsky, 1999; Kammendulis and Klaunig, 2005). Methylation of cytosines to produce 5-methyl cytosine is a well characterized, heritable, epigenetic mark (Feinberg, 2001). The majority of 5-methyl cytosine occurs at cytosines 5' to guanine. These CpG dinucleotides are not evenly distributed throughout the genome (Bird, 2002), but are concentrated in GC-rich promoter regions of genes and transposable elements typically being located within CpG islands which are stretches of DNA, at least

200bp in length that possess a 50% or greater GC content and a higher proportion of CpG dinucleotides than expected (Gardiner-Garden and Frommer, 1987). Decreases in methylation are associated with increases in gene transcription while increases in methylation are associated with decreases in gene transcription (Jones and Laird, 1999).

Phenobarbital (PB) is a non-genotoxic promoter of rodent liver tumors (Whysner *et al.*, 1996). Increased cell proliferation and altered DNA methylation are likely involved in tumor promotion (Goodman and Watson, 2002). Following PB administration increases in DNA synthesis occur in B6C3F1 liver, indicating enhanced cell proliferation, as early as 1-2 weeks (Klaunig, 1993). Additionally, PB induces more global hypomethylation in the liver tumor-prone B6C3F1 mouse, as compared to the relatively resistant C57BL/6, mouse (Counts *et al.*, 1996). A more critical look at this has shown that PB induces hypermethylation in selected GC-rich regions of DNA in addition to global hypomethylation demonstrating a non-random disruption of the epigenome (Watson and Goodman, 2002).

Using B6C3F1 mouse hepatocytes in primary culture, we have examined GC-rich regions of the genome for changes in methylation in response to treatment with DEA, choline deficient media or PB. The hypothesis being tested is that DEA-induced CD leads to altered methylation patterns which facilitate mouse liver tumorigenesis. The effects of DEA and PB on DNA methylation status was ascertained and compared with changes produced by CD. Specifically, we have assessed global (average) methylation and evaluated the methylation status of GC-rich regions of the genome using an arbitrarily primed PCR approach.

MATERIALS AND METHODS

Mouse Hepatocytes

Male B6C3F1 mice, 6-8 weeks old, obtained from Harlan Sprague-Dawley were housed in a facility at Indiana University School of Medicine (IUSM) and cared for in accordance with the University's animal use and care guidelines. Hepatocytes were isolated by a 2-step in situ collagenase perfusion (Klaunig *et al.*, 1981), cultured, and treated with 4.5mM DEA, 0.0898 mg/l choline, or 3mM PB for 48hrs at IUSM. Isolated hepatocytes from each of 3 animals per dosing group were divided and cultured in two plates. DNA was obtained from 8-10x10^6 cells using TRIzol Reagent, following the manufacturer's guidelines.

DNA Synthesis

Replicative DNA synthesis was measured according to the method of James and Roberts, (1996). BrdU (20mM final concentration) was added to cell cultures during the last 16 hours of culture. Cells,1 x 10^6 hepatocytes/60mm culture dish, were washed and fixed with methanol. Incorporated BrdU was localized using an anti-BrdU antibody followed by a peroxidase linked secondary antibody and a DAB substrate. Replicative DNA synthesis was measured by scoring the percentage of BrdU positive nuclei in a minimum of 1000 hepatocytes. Statistical significance was determined via a Randomized Complete Block Design ANOVA, post-hoc test, Tukey's, $p<0.05$.

SssI Global (Average) Methylation Assay

This assay allows for methylation at the 5' position of cytosine at every unmethylated CpG site in DNA via the enzyme *SssI* methylase using [Methyl-^{3}H] S-adenosyl

methionine (SAM) as the methyl donor, as described previously (Counts *et al.*, 1996). Global DNA methylation can be determined by the amount of ^{3}H-methyl groups incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the degree of methylation. Each DNA sample was incubated with 0.75µg of DNA per 5 replicates with 2.25 units SssI Methylase, 1.5µCi [^{3}H-methyl] SAM and reaction buffer (10mM Tris, 120mM NaCl, 10mM EDTA, 1mM DTT, pH 7.9) to volume. Reactions were spotted onto DE81 ion exchange filters and washed with 25ml 0.5M phosphate buffer, 2ml 70% ethanol and 2ml 100% ethanol and allowed to dry before scintillation counting. All results are expressed as cpm/µg DNA.

Arbitrarily Primed PCR and Capillary Electrophoresis

We have developed an arbitrarily primed PCR procedure (AP-PCR) that provides a thorough, overall evaluation of the methylation status of GC-rich regions of DNA. Importantly, a comparison of data obtained from DNA isolated from control and treated tissue permits the simultaneous detection of treatment-related increased methylation (hypermethylation, more methylation in a region that was methylated in control), decreased methylation (hypomethylation, less methylation in a region that was methylated in control) and new methylations (methylation in regions that were not methylated in control). Therefore, an in depth picture of treatment related altered methylation is provided. This technique employs methylation sensitive restriction digestion, arbitrarily primed PCR and capillary electrophoretic separation of PCR products.

Restriction Digests:

DNA samples are subjected to double digests with restriction enzymes: a) a methylation insensitive enzyme, and b) a methylation sensitive enzyme. *RsaI* is the methylation insensitive enzyme which is used initially to cut DNA into fragments in order to facilitate complete digestion by the second enzyme, a methylation-sensitive restriction enzyme. The methylation sensitive enzymes used in this study were *MspI* and *HpaII*. Both recognize 5'CCGG 3' sites, and cut between the cytosine and guanine. *MspI* will not restrict DNA if the external cytosine is methylated, while *HpaII* will not restrict DNA if the internal cytosine is methylated. Both *RsaI/MspI* and *RsaI/HpaII* double digests were employed.

Arbitrarily Primed PCR (AP-PCR) and Capillary Electrophoresis:

PCR is performed on restriction digests using a single arbitrary primer 5' AACCCTCACCCTAACCCCGG 3' (Gonzalgo *et al.*, 1997), that was modified by having it fluorescently labeled at the 5' end with HEX™ (purchased from Integrated DNA Technologies). This primer was designed to bind well to GC-rich regions and the 5'CCGG 3' sequence at its 3' end increases the probability of primer annealing to the HpaII and MspI restriction site. This allows for detection of methylation at the site of primer annealing and between sites of primer annealing. Each PCR product is viewed as representing a GC-rich region of the genome. PCR products were purified, using a sephadex G50 superfine matrix, and separated via capillary electrophoresis, using a ABI 3700 Genetic Analyzer (Genomics Technology Support Facility (GTSF) at Michigan State University). Base pair markers are run simultaneously with the samples in order to accurately size the PCR products. The results represented as size of PCR products, in

base pairs, and their corresponding peak areas are analyzed using the Excel® program. A consensus, average, peak area for each PCR product reporting in control and treated groups is prepared, and the consensus control and treated peak areas at a specific PCR product are compared. This permits us to detect treatment-related: a) hypomethylations which include both 100% decreases and decreases which are statistically significant when compared to control, b) hypermethylations which are increases which are statistically significant when compared to control, and c) new methylations which are indicated by the formation of a PCR product following treatment which was not formed under control conditions. Significance is determined via a Student's *t*-test, $p < 0.05$. Analysis of the data includes the following assumptions: 1) each separate PCR product of a defined size represents a distinct region of the genome, 2) a region can include one or more recognition sequences for the specific methylation-sensitive restriction enzyme employed located between the annealing sites of the up- and down-stream primers; thus, the amount of each PCR product formed can be viewed as representing an "average" of the methylation status of the particular recognition sequences located between the up- and down-stream primers, and 3) changes in the amount of each PCR product represents the altered methylation status of a particular GC-rich region of DNA. A detailed account of the AP-PCR, capillary electrophoresis method, including the data analysis steps are provided as supplementary data in Appendix 1.

RESULTS

In order to provide an equivalent baseline from which we could compare the effects of DEA, PB or CD on the methylation status of DNA in B6C3F1 hepatocytes, we selected concentrations (4.5 mM, 3 mM and 0.098 mg/l for DEA, PB and CD media, respectively) that produced equivalent increases in DNA synthesis during the 48 hr culture period (Table 1).

DEA or PB treatment as well as culture in CD media did not affect global, average methylation status (Figure 1).

Analysis of GC-rich regions of DNA provided a more detailed picture of altered methylation patterns than simply evaluating global, average methylation. DEA treatment resulted in 43 regions of hypomethylation, which composed 80% of the total aberrant regions detected within GC-rich areas of DNA (Figure 2a). Of these, 26 (60%) exhibited a 100% decrease (i.e. a complete loss of methylation) at those regions. The large degree of significant decreases in methylation (both partial and complete hypomethylation) was approximately equal in number at both the external and internal cytosine of 5'-CCGG-3' regions based upon the results of the RsaI/MspI and RsaI/HpaII digests. In comparison, relatively few regions of methylation increased with only 1 hypermethylation and 10 new regions of methylation (Figure 2a and 2d). Here increases were mainly detected via the RsaI/HpaII digest indicating a preference for altered methylation of the internal cytosine within the recognition sequence.

PB produced a pattern of altered methylation similar to DEA. The largest proportion of altered regions, 75%, were hypomethylations (Figure 2b) with 49% of the total decreases exhibiting a complete loss of methylation. Increases in methylation

Table 1. Summary of Replicative DNA Synthesis

Treatment	Labeling Index[a,b]
Control	1.84±0.09
Diethanolamine	7.51±0.21[c]
Choline Deficiency	7.14±0.17[c]
Phenobarbital	7.61±0.24[c]

[a] Labeling Index: percentage of BrdU positive nuclei in a minimum of 1000 hepatocytes
[b] Labeling index is expressed as mean (n=3) percent ± standard error
[c] Statistically different from control. Statistical significance was determined via ANOVA, Tukey's $p<0.05$

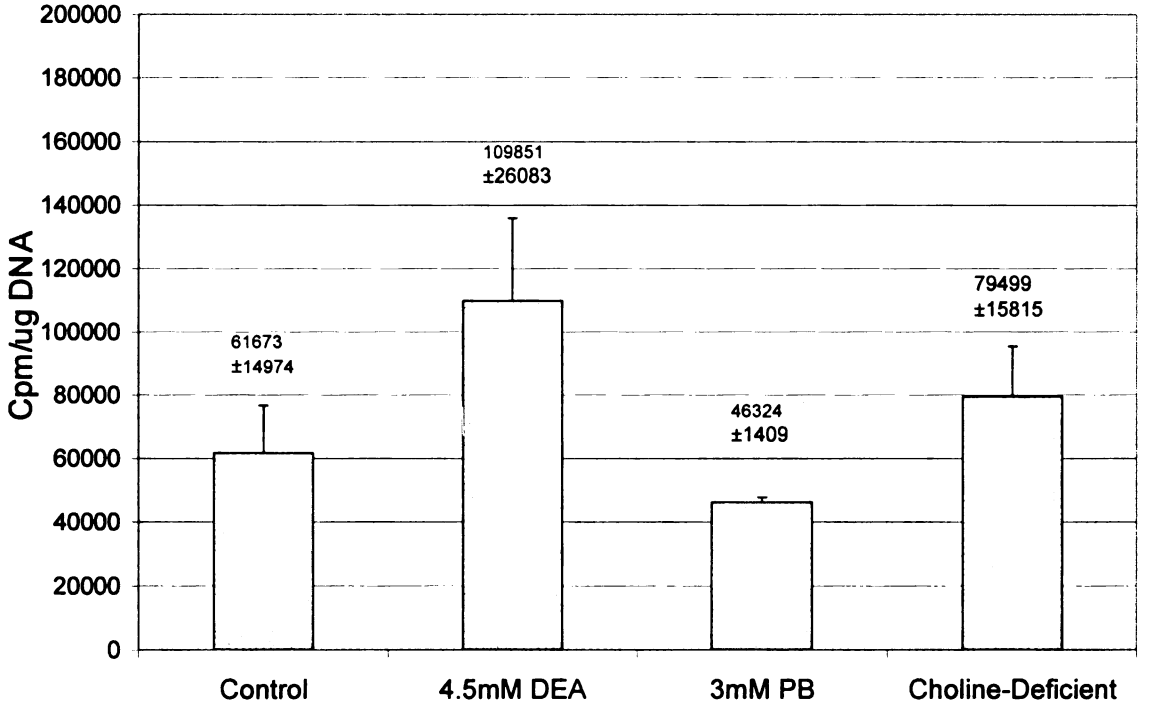


Figure 1: Global Methylation Status in DNA from Primary Mouse Hepatocytes. Global methylation of DNA isolated from primary mouse hepatocytes treated for 48hrs. with 4.5mM DEA, choline deficient media or 3mM phenobarbital is presented. Each bar represents the mean CPM/ug DNA of 3 animals, +/- standard error. DEA, choline deficiency, and phenobarbital treatment were statistically ($p<0.05$) no different from control.

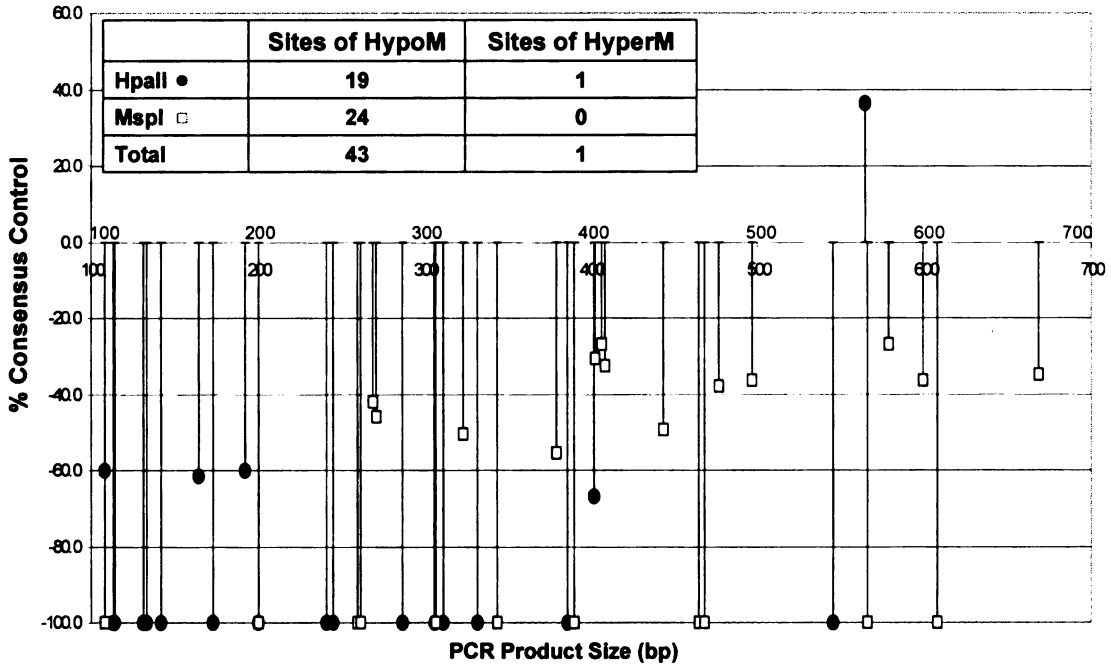
included 3 regions of hypermethylation and 13 regions of new methylation (Figure 2b and 2c). Similar to the results obtained from DEA treatment, there was a bias towards increased methylation at the internal cytosine within the 5'CCGG 3' recognition sequence.

DNA isolated from hepatocytes maintained in CD media exhibited the greatest number of regions where 5'Me-C content was either partially or completely decreased (Figure 2c). Methylation was lost completely in 37 of the 49 (76%) total hypomethylated regions. Very few increases in methylation were observed; 1 site of hypermethylation was identified via the RsaI/HpaII digest and 4 regions of new methylation were identified via the RsaI/MspI digest (Figure 2c and 2d) indicating that of the small number of increases, most occurred at the external cytosine in contrast to increases induced by DEA or PB which occurred mainly at the internal cytosine. The predominate alteration in methylation patterns was a decrease in methylation at multiple regions within GC-rich regions. PB produced the greatest degree of altered methylation with 63 total altered regions. DEA and CD treatment were strikingly similar with 54 total altered regions (Table 2).

Due to the overall similarity in patterns of altered methylation among the different treatments, a more refined approach to analyzing and comparing the data was employed. Changes occurring at identical PCR product sizes between two treatments were considered common regions of altered methylation. Figure 3 depicts the 39 regions of altered methylation in common between DEA and CD treatments. The magnitudes of change at only 2 regions of the 39 total regions were statistically different (Figure 3 and Table 3). Of the 44 common regions of altered methylation between PB and CD

Figure 2. GC-Rich DNA Methylation Status in Primary B6C3F1 Mouse Hepatocytes
RsaI/HpaII (closed symbols) and RsaI/MspI (open symbols) digestion and subsequent AP-PCR was performed on DNA isolated from B6C3F1 mouse hepatocytes treated with either DEA (A), phenobarbital (B), or choline deficient media (C) for 48hrs. Regions of hypomethylation were prevalent across all treatments. (D) Regions of new methylation resulting from treatment are shown in terms of the peak area for each PCR product size. Four regions of new methylation whose peak areas exceeded the scale of the chart were labeled above the chart with their corresponding peak area values.
Tables tallying the regions of altered methylation for each treatment are shown as an inset in each chart. Regions of hypo-, hyper-, and new methylation determined by the data are expressed in terms of the treated mean for each PCR product size as a percent of the control mean for each PCR product size. All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's *t*-test, $p<0.05$) are depicted.

A. Diethanolamine



B. Phenobarbital

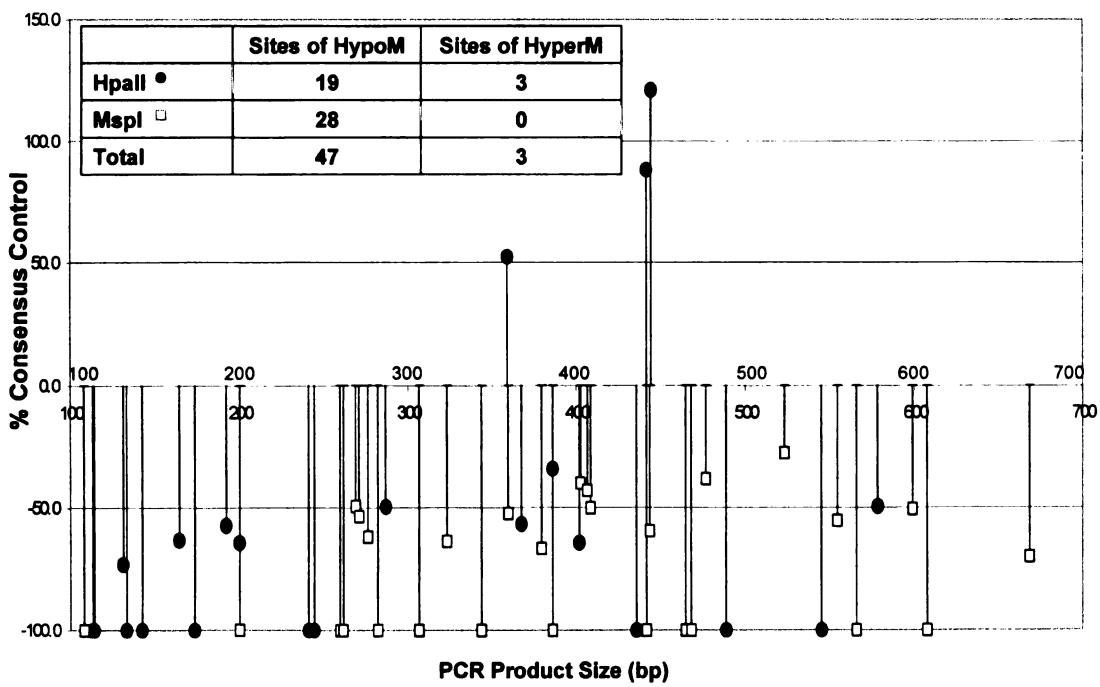
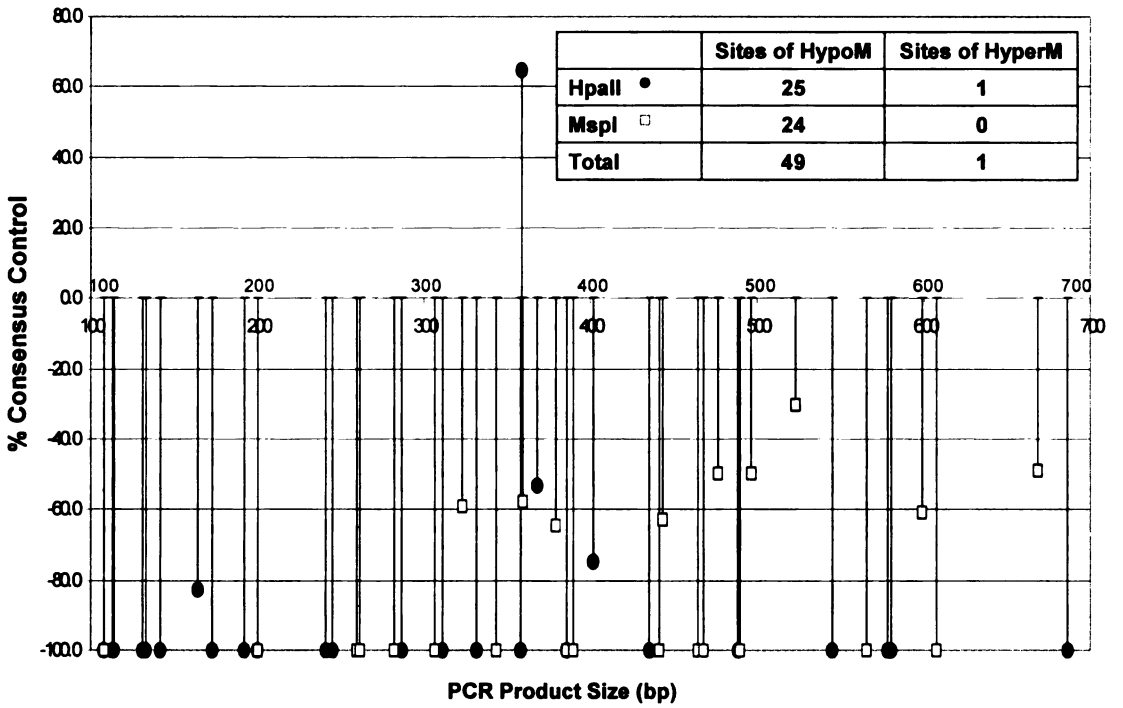


Figure 2 (cont'd)

C. Choline Deficiency



D. Sites of New Methylation

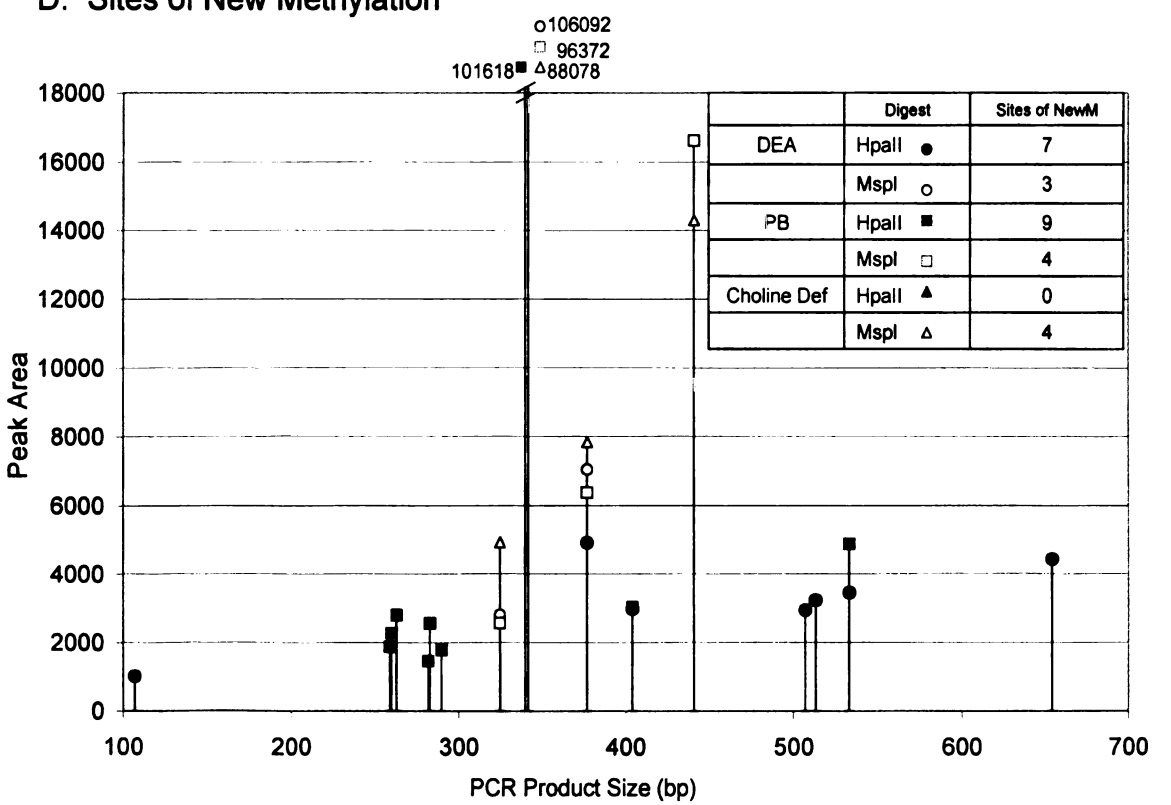


Table 2. Summary of GC-Rich Regions of Altered Methylation (RAMs)

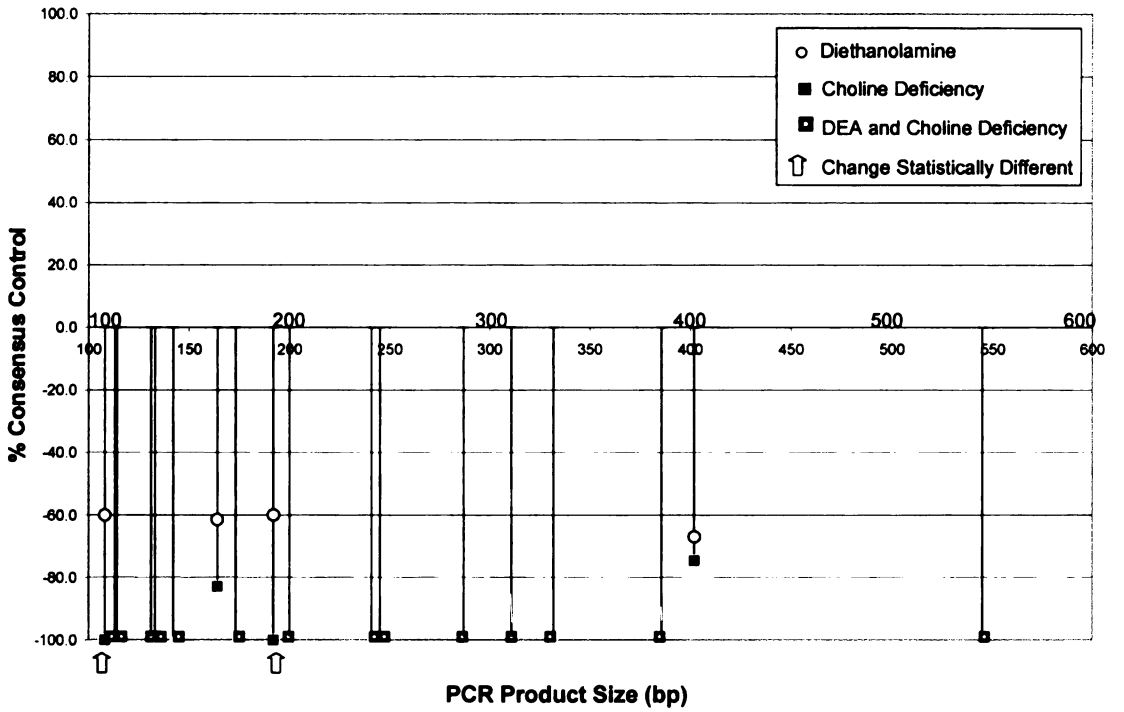
Treatment	Digest	RAMs Hypomethylation[a]	RAMs Hypermethylation[b]	RAMs "New" Methylation[c]	TOTAL
Diethanolamine	**HpaII**	19	1	7	
	MspI	24	0	3	
Total[d]		43	1	10	54
Phenobarbital	**HpaII**	19	3	9	
	MspI	28	0	4	
Total[d]		47	3	13	63
Choline Deficiency	**HpaII**	25	1	0	
	MspI	24	0	4	
Total[d]		49	1	4	54

[a] Hypomethylated RAMs include both statistically significant ($p<0.05$) decreases and 100% decreases.
[b] Hypermethylated RAMs are only those increases which are statistically significant ($p<0.05$).
[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.
[d] Total RAMs including hypomethylations, hypermethylation, and new methylations for the combined digests are reported for each treatment

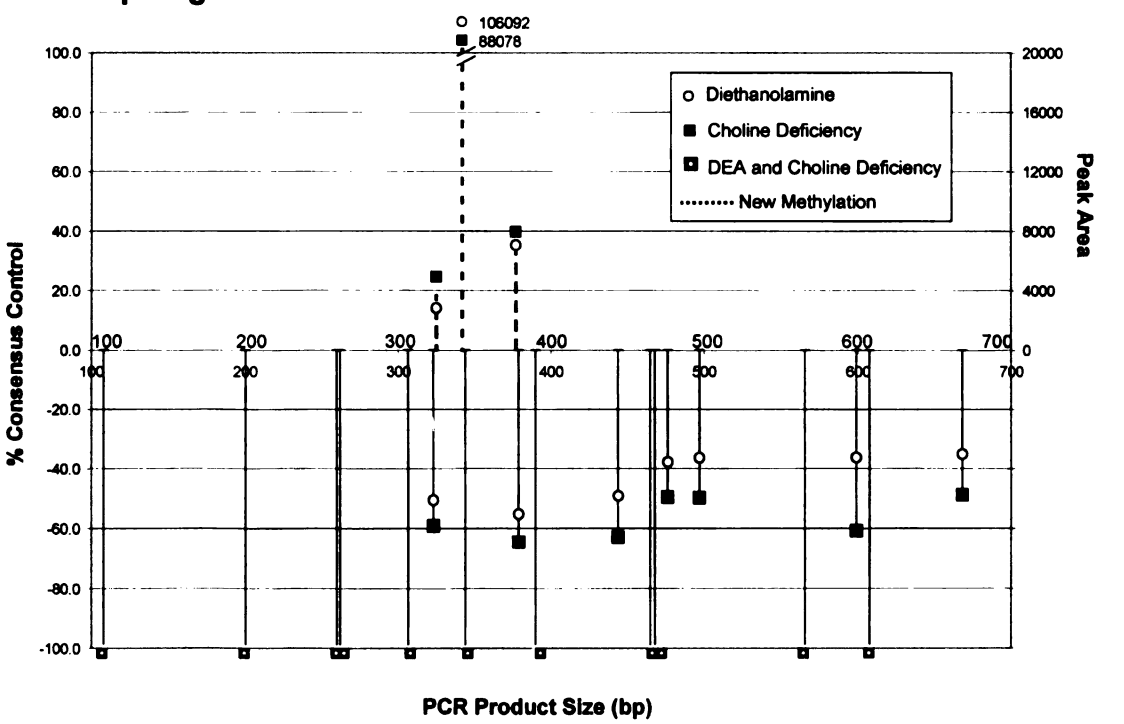
Figure 3. Comparison of Diethanolamine and Choline deficiency Induced Aberrant GC-rich Methylation Patterns Regions of altered methylation induced by DEA and choline deficiency are compared for the RsaI/HpaII (A) and RsaI/MspI (B) digestion. PCR products of identical size formed in both the control and treatment groups were considered to be common regions of altered methylation. These common regions of hypo- hyper- and new methylations are represented. For the majority of common regions of aberrant methylation, the magnitude and direction of change induced by DEA and choline deficiency were statistically no different (One-Way ANOVA, $p<0.05$). At only two common regions identified by the RsaI/HpaII digest, were the magnitudes of change statistically different. In each case, choline deficiency induced a greater loss of methylation than DEA.

All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's *t*-test, $p<0.05$) are depicted

A. HpaII Digest



B. MspI Digest



treatments, only 5 regions differed statistically in magnitude (Figure 4, and Table 3). The patterns of altered methylation produced by DEA and PB were 72% and 70% similar, respectively, to that of CD demonstrating the high degree of similarity (Table 3). Unique changes elicited by DEA and PB were few in numbers (Table 4).

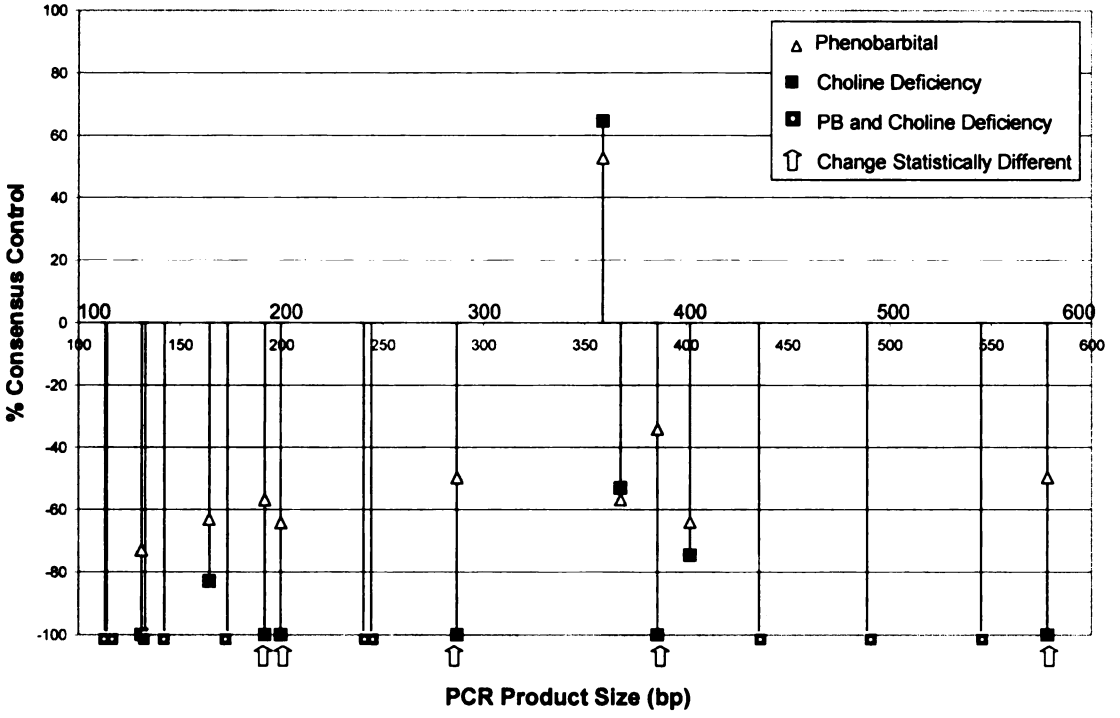
Table 3. Common Regions of Altered Methylation (RAMs): Comparison of Diethanolamine (DEA) or Phenobarbital (PB) with Choline Deficient (Choline Def) Treatment[a]

DEA vs. Choline Def	Common RAMs With Decreased Methylation[b]	Common RAMs With Increased Methylation[c]	Total RAMs In Common	Percent Similarity
Complete Hypomethylation[d]	25	-	39/54[j]	72%
Partial Hypomethylation[e]	9	-		
New Methylation[f]	-	3		
Magnitude of Change Statistically Different[h,i]	2	-		
PB vs. Choline Def	Common RAMs With Decreased Methylation[b]	Common RAMs With Increased Methylation[c]	Total RAMs In Common	Percent Similarity
Complete Hypomethylation[d]	23	-	44/63[j]	70%
Partial Hypomethylation[e]	12	-		
New Methylation[f]	-	3		
Hypermethylation[g]	-	1		
Magnitude of Change Statistically Different[h,i]	5	-		

[a] Data are summarized from Figures 2 and 3.
[b] Total RAMs exhibiting decreased methylation (i.e. complete hypomethylations and partial hypomethylations) that are in common between DEA and Choline Def or PB and Choline Def treatments
[c] Total RAMs exhibiting increased methylation (i.e. hypermethylations and new methylations) that are in common between DEA and Choline Def or PB and Choline Def.
[d] Complete hypomethylation indicates a complete or 100% loss of methylation.
[e] Partial Hypomethylations are statistically significant ($p<0.05$) decreases as compared to control
[f] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.
[g] Hypermethylations are statistically significant ($p<0.05$) increases as compared to control.
[h] Significance was based on a One Way ANOVA, $p<0.05$.
[i] Choline Def showed a significantly greater extent of hypomethylation than DEA and PB treatment at the number of RAM indicated.
[j] Total RAMs are reported from Table 2.

Figure 4. Comparison of Phenobarbital and Choline deficiency induced aberrant GC-rich methylation patterns Regions of altered methylation induced by PB and choline deficiency are compared for the RsaI/HpaII (A) and RsaI/MspI (B) digestion. PCR products of identical size formed in both the control and treatment groups were considered to be common regions of altered methylation. These common regions of hypo- hyper- and new methylations are represented. For most regions, the magnitude and direction of change induced by PB and choline deficiency were statistically no different (One-Way ANOVA, $p<0.05$). The magnitudes of decrease for five common regions, identified by the RsaI/HpaII digest, were statistically different. In each case, choline deficiency induced a greater loss of methylation than PB.
All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's *t*-test, $p<0.05$) are depicted.

A. HpaII Digest



B. MspI Digest

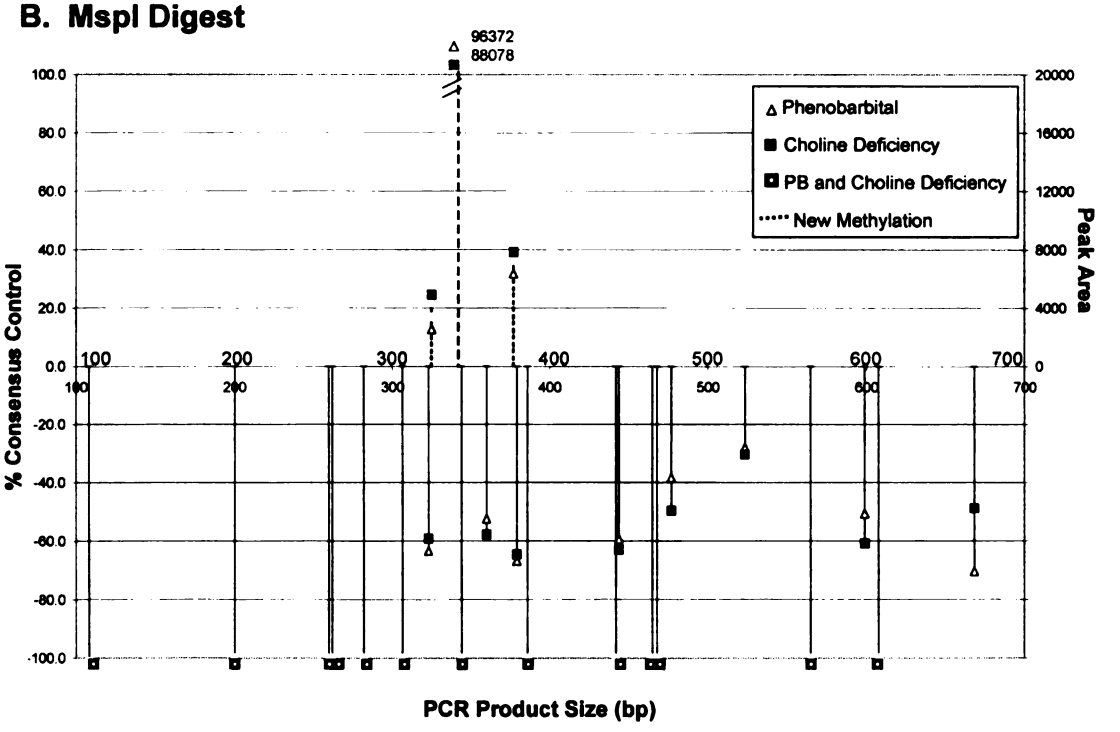


Table 4. Unique[a] Regions of Altered Methylation (RAMs): Diethanolamine (DEA) or Phenobarbital (PB) as Compared to Choline Deficient Treatment

	DEA	PB
Complete Hypomethylation[b]	1	1
Partial Hypomethylation[c]	6	7
Hypermethylation[d]	1	2
New Methylation[e]	7	9
Total RAM NOT in Common	**15/54[f]**	**19/63[f]**
Percent Difference	**28%**	**30%**

[a] Unique RAMs denotes all RAM which were not in common between DEA and choline deficiency or PB and choline deficiency.
[b] Complete hypomethylation indicates a complete or 100% loss of methylation.
[c] Partial Hypomethylations are statistically significant ($p<0.05$) decreases as compared to control
[d] Hypermethylations are statistically significant ($p<0.05$) increases as compared to control.
[e] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.
[f] Total RAMs are reported from Table 2.

DISCUSSION

We have developed and applied a novel procedure for analyzing altered methylation in GC-rich regions of the genome, including CpG islands. Simple in design, this technique employs methylation sensitive restriction digestion of DNA, arbitrarily primed PCR amplification, and electrophoretic separation of PCR products to provide a detailed, quantitative overview of the extent of treatment-related disruption of methylation throughout the genome. Comparably, the strength and utility of our technique lies in its ability to simultaneously identify increases, decreases and new methylations within multiple, distinct regions of the genome. This provides a sensitive, quantitative method which reproducibly detects the extent of treatment-related altered patterns of methylation.

There are a variety of techniques for analyzing changes in methylation within a particular gene. Methylation specific PCR, including variations such as MethyLight and HM Methyl Light can be effectively employed for these applications (Cottrell and Laird, 2003). Other procedures include combined bisulfite restriction analysis (COBRA) which assesses the methylation status of particular CpG sites (Xiong and Laird, 1997) and the enzymatic regional methylation assay for determining changes in methylation between two primers designed for a targeted region (Galm *et al.*, 2002). These are excellent methods for evaluating specific genes. However, their utility is limited when one wants to discern the extent to which a particular treatment might disrupt normal methylation patterns, e.g., in this situation a gene-by-gene approach would be too cumbersome.

There are other approaches for assessing methylation status of the genome. For example, the combination of sodium bisulfite conversion of DNA followed by PCR

amplification of alu and LINE elements can be used to estimate methylation changes (Yang *et al.*, 2004); however, the focus is only on repetitive elements, i.e., "junk DNA" and not genes. Also, the methylation-sensitive amplified fragment length polymorphism (AFLP) technique allows for comparative genome wide scanning of methylation status via fingerprinting techniques and has recently been adapted to a DNA microarray hybridization technique (Yamamoto and Yamamoto, 2004). This procedure requires a custom microarray panel and a complex approach to data analysis. Global, average methylation analysis via SssI methyltransferase (Balaghi and Wagner, 1993) is straightforward, but limited in scope; increases in methylation in one portion of the genome may balance out decreases in other areas. The combined AP-PCR capillary electrophoresis technique described in this paper affords the ability to assess altered DNA methylation (increases, decreases and new methylations) in multiple GC-rich regions of the genome simultaneously and quantitatively. Furthermore, it is highly appropriate under situations when the research question being asked is, "Does a particular treatment cause disruption of normal patterns of DNA methylation and to what extent does this occur?" With this methodology we have assessed DEA, CD, and PB induced alteration of methylation in B6C3F1 mouse hepatocytes.

The ability of DEA to alter methylation *in vitro* in B6C3F1 mouse hepatocytes was investigated as a proposed non-genotoxic mode of action of the compound's ability to cause carcinogenesis in mouse liver. Treatment-induced increases in cell proliferation, as measured by increases in DNA synthesis, have been reported and proposed to facilitate tumorigenesis in B6C3F1 mice (Klaunig, 1993, Zeisel, 1996). Specifically, genetic and epigenetic alterations of the DNA are possible contributors (Counts *et al.*, 1996). Cell

proliferation fosters the occurrence and accumulation of spontaneous mutations (Schulte-Hermann, 1987). More importantly, high rates of DNA synthesis might compromise the capacity to maintain normal methylation patterns leading to mis-regulated gene expression patterns. Therefore, by selecting doses based on induction of comparable increases in cell proliferation, we were able to directly compare and analyze changes in methylation.

Several factors work in concert to sustain normal methylation levels. These include the maintenance and *de novo* DNA methyltransferases (e.g. Dnmt1, Dnmt3a and 3b), demethylases and the availability of both SAM and methyl groups. For example, Dnmt1 is the maintenance methyltransferase responsible for methylating newly synthesized daughter strands of DNA; this ensures the heritability of the methylation pattern (Hermann et al., 2004). Altered patterns of methylation, specifically hypomethylation, may arise when the activity of Dnmt1 does not increase with enhanced rates of DNA synthesis. Alternatively, the same effect could be observed if SAM does not provide a sufficient supply of methyl groups, (i.e. methyl deficiency depletes the availability of methyl groups), to maintain the up regulated Dnmt1 activity. DNA methylation patterns are also under the influence of demethylases (e.g., MBD2) which can decrease the level of 5-methyl cytosine when cells are not synthesizing DNA (Detich *et al.*, 2003). Thus, indicating that DNA methylation is reversible. Importantly, SAM directly inhibits MBD2 and, therefore, diminished formation of SAM during a state of methyl deficiency could relieve the inhibition of demethylase activity and facilitate hypomethylation of DNA (Detich *et al.*, 2003). As hypothesized, DEA, by inducing cellular choline depletion, contributes to perturbation of 1-carbon metabolism, leading to

decreased availability of methyl groups, impaired formation of SAM, and disruption of normal DNA methylation patterns.

Assessment of global (average) methylation status and methylation of GC-rich regions of DNA were performed. Global, average levels of methylation following treatment with DEA, CD media and PB were comparable to control (Figure 1). This could indicate that, 1) global methylation levels are unaffected by DEA, CD, and PB or 2) approximately equal levels of methylation increases and decreases are occurring simultaneously in multiple regions of the genome. This second possibility underlies the importance of specifically examining GC-rich regions for a more detailed picture of overall altered methylation.

Within GC-rich regions of DNA, hypomethylation was the predominant alteration induced by DEA, PB and CD. Hypomethylation in the promoter regions of genes is associated with increased gene expression (Jones and Baylin, 2002). Critical losses of methylation in the promoter regions of oncogenes such as c-jun and c-myc, CDNK3 (cyclin-dependent kinase inhibitor 3), and c-Ha-*ras*, have been demonstrated (Niculescu *et al.*, 2004; Tao *et al.*, 2000). Hypomethylation associated overexpression of c-jun and c-myc was observed in livers promoted with dichloroacetic and trichloroacetic acid, both of which are considered non-genotoxic carcinogens (Tao *et al.*, 2000). Human neuroblastoma cells cultured in CD media showed loss of methylation in the promoter region of the CDNK3 gene, an important regulator of cell cycle progression, and up-regulation of expression. In addition, genetic instability via activation of transposable elements (Roman-Gomez, 2005), elevated mutation rates (Chen *et al.*, 1998) and chromosomal instability (Eden *et al.*, 2003) have all been associated with

hypomethylated DNA. Methyl deficiency in rats induced irreversible global DNA hypomethylation in rat liver which supported a role for loss of methylation during the cancer initiation and or promotion stages of hepatocarcinogenesis (Pogribny *et al.*, 2005). These studies emphasize and support our view that DNA hypomethylation is a mechanism involved in tumor promotion (Counts and Goodman, 1994) and the data presented in the current paper support the hypothesis that DEA, CD and PB treatment act by this mechanism to produce mouse liver tumors.

Altered methylation status of cytosines within the CpG dinucleotide is most commonly investigated; however, methylation of CpNpG and non-CpG sites also exists. In particular, the role of altered methylation at CpCpG sites has not been thoroughly investigated. There are three possible states of methylation of the CpCpG sites analyzed. These include: 1) ^{m}CpCpG, methylation of the external cytosine, 2) Cp^{m}CpG, methylation of the internal cytosine and 3) ^{m}Cp^{m}CpG, methylation of both the internal and external cytosine. Our results show that loss of methylation status at both ^{m}CpG and ^{m}CpCpG sites occurs with approximately equal frequency suggesting that factors affecting the methylation status of ^{m}CpG sites also act on ^{m}CpCpG sites. Studies evaluating non-CpG methylation have mainly focused on CpA, CpT, and CpC methylation. However, one particular study proposed a biological role for methylation of both cytosines within CpCpG sites. Methylation of both cytosines within CpCpG sites has been reported to prevent binding of Sp1, an important transcription factor, to its target cis element thereby contributing to abnormal regulation of gene expression (Clark *et al.*, 1997; Inoue and Oishi, 2005). Effects due to methylation of only the external cytosine

were not reported. This stresses the importance of a broad and critical analysis of both CpG and non-CpG methylation during the promotion stage of tumorigenesis.

We have demonstrated remarkable similarities between the DEA, CD, and PB treatment related disruption of methylation patterns in B6C3F1 mouse hepatocytes grown *in vitro* during a short 48 hour exposure. This indicates that a common mechanism is shared by all three treatments. The extreme similarity between patterns of altered methylation in GC-rich regions due to DEA and CD supports the notion that DEA indirectly depletes the pool of methyl groups needed for methylation of cytosine by inhibiting choline uptake into cells (Lehman-McKeeman and Gamsky, 1999). The resulting hypomethylation mimics that of dietary CD. Dietary PB has been shown to cause global hypomethylation (Counts *et al.*, 1996), and hypermethylation, along with some decreased methylation, in GC-rich regions of DNA (Watson and Goodman, 2002) in the livers of B6C3F1 mice after 2 and 4 wk of administration. Therefore, continued exposure to the promoting stimuli, may lead to progressive changes in methylation including hypomethylations, hypermethylations, and new methylations which accrue in a stepwise manner to contribute to tumorigenesis. This is consistent with the view that a variety of alterations in methylation contribute to carcinogenesis (Counts and Goodman, 1995), and that there are progressive alterations of methylation during the transformation process (Watson *et al.*, 2003). Hence, altered methylation, initially hypomethylation, is a likely epigenetic, non-genotoxic, mode of action underlying the abilities of DEA, PB and CD to promote the development of mouse liver tumors.

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CHAPTER 1 APPENDIX

This appendix is a detailed description of the materials and methods for the arbitrarily primed PCR and capillary electrophoretic approach employed to assess methylation status in GC-rich regions throughout the genome. In addition, data organization and analysis, including statistical calculations performed using the Excel® program, are explained in detail. This appendix can also serve as supplementary information on the materials and methods for Chapters 2 and 3.

ARBITRARILY PRIMED PCR AND CAPILLARY ELECTROPHORESIS

Restriction Digests:

DNA samples, of which duplicates are prepared, are subjected to double digests with restriction enzymes: a) a methylation insensitive enzyme, and b) a methylation sensitive enzyme. *RsaI,* is the methylation insensitive enzyme which is used initially to cut DNA into fragments in order to facilitate complete digestion by the second enzyme, a methylation-sensitive restriction enzyme. DNA samples are subjected to double digests with restriction enzymes: a) a methylation insensitive enzyme, and b) a methylation sensitive enzyme. *RsaI,* is the methylation insensitive enzyme which is used initially to cut DNA into fragments in order to facilitate complete digestion by the second enzyme, a methylation-sensitive restriction enzyme. The methylation sensitive enzymes used in this study were *MspI* and *HpaII.* Both recognize 5'CCGG 3' sites, and cut between the cytosine and guanine. *MspI* will not restrict DNA if the external cytosine is methylated, while *HpaII* will not restrict DNA if the internal cytosine is methylated. Both *RsaI/MspI* and *RsaI/HpaII* double digests were employed.

The MspI and HpaII restriction enzymes allow for analysis of methylation at both CpG sites and CpCpG sites. Methylation of cytosines, not 5'to guanine is less commonly examined. Restriction digests contain 1μg DNA and 5.0 units RsaI in Roche Buffer L. Samples are incubated for 1hr. at 37^0C before addition of 2.5 units of either MspI or HpaII. A second 2.5 unit aliquot of the respective enzyme is added after an additional 2hr, and in order to insure complete digestion, total incubation time is 18hr. The enzymes were inactivated by incubating at 65^0C for 10min. Samples were stored at 4^0C until analyzed.

Arbitrarily Primed PCR (AP-PCR):

AP-PCR is performed on restriction digests using a single arbitrary primer, 5' AAC CCT CAC CCT AAC CCC GG 3' (modified from Gonzalgo *et al.*, 1997), fluorescently labeled at the 5' end with HEX™ (purchased from Integrated DNA Technologies). This primer was designed to bind well to GC-rich regions and the 5'CCGG 3' sequence at its 3' end increases the probability of primer annealing to the *MspI* and *HpaII* restriction site, allowing for the detection of methylation at the site of primer annealing and between sites of primer annealing. Each PCR reaction was composed of 5.0μl of the restriction digest, 0.8μM primer, 1.0 unit Taq polymerase, 1X MasterAmp™ PCR PreMix L, and glass distilled water (GDW) to 10ul. In order to obtain a sufficient quantity of PCR product for capillary electrophoresis, duplicate PCR reactions were prepared for every one restriction digest and are combined prior to purification. The Taq polymerase was added to each reaction following a 5min incubation at 80^0C. Cycling conditions were as follows: 94^0C for 2 min, 5 cycles of 94^0C for 30 s, 40^0C for 1 min, and 72^0C for 1 min 30 s, 40 cycles of 94^0C for 15 s, 55^0C for 15 s, and 72^0C for 1 min, and a single time delay of 5 min at 72^0C followed by a 4^0C soak. Combined PCR reactions were desalted and purified at the Genomics Technology Support Facility (GTSF) at Michigan State University, using a sephadex G50 superfine matrix.

Capillary Electrophoretic Separation of Products:

Ten nanograms of each purified, desalted PCR product were added to a solution of formamide and a carboxy-X-rodamine (ROX™)-labeled size marker for sizing and

normalization of the results. Size marker fragments increased incrementally by 200bp up to 1000bp. Using a 10sec injection time, a 2ul aliquot was injected into the GTSF Applied Biosystems 3700 Genetic analyzer. Data were collected using Genescan 3.7 which compiles the results as size of PCR product in base pairs with a corresponding peak area representative of the amount of PCR product generated. Only fragments greater than 100 bp and peak areas with corresponding peak heights greater than 100 units were analyzed to minimize incorporating background noise and/or primer-dimers into the data set.

Raw Data Organization

The data are organized into rows and columns within the excel workbook where a list of all PCR product sizes (base pairs) from every analyzed sample are placed in one column. The peak areas with their corresponding PCR product size from each sample are placed into their corresponding row. The excel file will appear as PCR product sizes in column A and across the rows of each size will be all the samples reporting a peak area for that size PCR product as shown in Table 1.

Following this raw data organization, the RsaI/HpaII data are placed in a separate workbook from RsaI/MspI data.

Removal of Background Data

The following data analysis steps are performed on each digest separately. Using the following rules, the data are aligned across treatment groups and "background" data, (i.e.,

Appendix Table 1. Excel workbook illustrating the Organization of Raw Data into PCR Product Size and Corresponding Peak Area

PCR Product Size (base pairs)	Control 1	Control 1	Treatment 1	Treatment 1
56[a]		2185[b]	4321	
75	3018	1466	3274	
79	330			
99			1705	
108				
114	34695			1528
126				1683
148				

[a] PCR product sizes in base pairs are reported.
[b] Peak area values representing the amount of PCR product are reported for each corresponding PCR product size.

reported peak areas that do not "fit" within our rules and are deemed to be spurious) are removed from futher calculations.

A. Data that have a corresponding PCR product size of 99bp or less are omitted. For example, in Table 1, the 4 rows containing 56, 75, 79, and 99bp would be deleted. This is to ensure that the final data analysis does not include PCR product sizes and peak areas that may be attributed to background fluorescence or primer dimers.

B. Minimum Data Requirement for each PCR Product Size. Each row (each PCR product size) is examined individually within a defined control/treatment group, and the peak area data points that do not meet the following criteria are deleted. In order for the data to be considered valid, peak area data points must be reported for at least 50% of the number of animals and at least 50% of the total number of replicates. For example if there are 4 control animals, each with 2 replicates, 2 of the 4 animals and 4 of the 8 replicates must report a peak area for the given PCR product size. For odd numbers of animals such as 5 animals with 2 replicates each, 3 animals and 5 replicates must report a peak area.

C. Due to the possibility of occasional slight variation with regard to determining the size of PCR products, peak area points which are within 2 base pairs are, under the circumstances outlined below, combined to complete (or add to) a row.

The goal of these occasional adjustments is to move selected peak area data points only if the move is one or two base pairs away and there is a clear fit with other replicates. Extensive manipulation is avoided.

An example of this is illustrated in Table 2. With the presented data, a complete row of peak area points must contain at least 4 peak area points because there are 4 animals (#1-4) and 2 replicates of each animal. The numbers with strikethrough font would be deleted and those peak area data points within 2 bp of neighboring data would be moved to fill in the data set for that PCR product size.

Some of these data point adjustments are somewhat subjective, thus two people organizing the same data set might end up with slightly different alignments, however, their calculated outcomes should be virtually identical.

Alignment of the Control and Treated Data

Once data are aligned between control/treatment groups, the results of two groups, experimental and control, are compared at each PCR product size. Without altering the control data, the treatment data are aligned with the control, ONLY if the PCR product sizes are within 1bp of the corresponding control PCR product size.

Before moving the treated data, the raw data are taken into account. If all the peak area points that were moved originally are within 2bp of the **control** PCR product size it is being aligned to then a move is deemed to be appropriate.

Table 3 demonstrates the alignment of a row of treated data with the control data.

At this point the data adjustments are complete.

Appendix Table 2. Excel workbook illustrating the removal of background and adjustment of data according to the minimum data requirements and the outlined guidelines

PCR Product (base pairs)	Sample 1 Replicate 1	Sample 1 Replicate 2	Sample 2 Replicate 1	Sample 2 Replicate 2	Sample 3 Replicate 1	Sample 3 Replicate 2	Sample 4 Replicate 1	Sample 4 Replicate 2
263						~~1930~~[b]		
266						~~1349~~[b]		
268						~~2916~~[b]		
270		~~5930~~[b]						
271								
272	5874[a]	7868	6679	7351	9824		6222	5837
273						14059[c]		
274								
275			~~845~~[b]					
276		65145[d]						
277	68353		16983	29863	72469	58139		38949

[a] Peak area values representing the amount of PCR product are reported for each corresponding PCR product size.

[b] With a total of 4 samples, 2 replicates per samples, at least 2 of the 4 animals and 4 of the eight replicates must be reporting a peak area. A single peak area for the corresponding size does not meet the requirements and is deleted (represented by the strikethrough font).

[c] A peak area data point that is within 2 base pairs of a row of data points that meet all requirements, can be added to that row to complete the row. The 14059 data point would be added to the 272bp PCR product size row.

[d] A peak are data point that is within 2 base pairs of a row of data points that meet all requirements, can be added to that row even though it will not complete the row. The 65145 data point would be added to the 277bp PCR product size row.

Appendix Table 3. Excel Workbook demonstrating the alignment of a Treated peak area data points to the control PCR product size reporting peak area data points

PCR Product Size (bp)	Control Sample 1 Replicate 1	Control Sample 1 Replicate 2	Control Sample 2 Replicate 1	Control Sample 2 Replicate 2	PCR Product Size (bp)	Treated Sample 1 Replicate 1	Treated Sample 1 Replicate 2	Treated Sample 2 Replicate 1	Treated Sample 2 Replicate 2
Data Arrangement Prior to Aligning									
343	252585[a]	166902	156223	286118	343				
344					344	315897	274255	35888	80682
Aligned Data[b]									
343	252585	166902	156223	286118	343	315897	274255	35888	80682
344					344				

[a] Peak area values representing the amount of PCR product are reported for each corresponding PCR product size.

[b] Performing this alignment is appropriate only if in the raw data (from the treated group) all the peak areas fell within the 342, 343, 344 OR 343, 344, 345 base pair range.

Visualization of Data Consistency: Creation of Individual Animal Average Peak Area Charts

In order to visualize the consistency of the data across the animals within a particular experimental group, the average peak areas for each animal at each PCR product size are plotted (Figure 1).

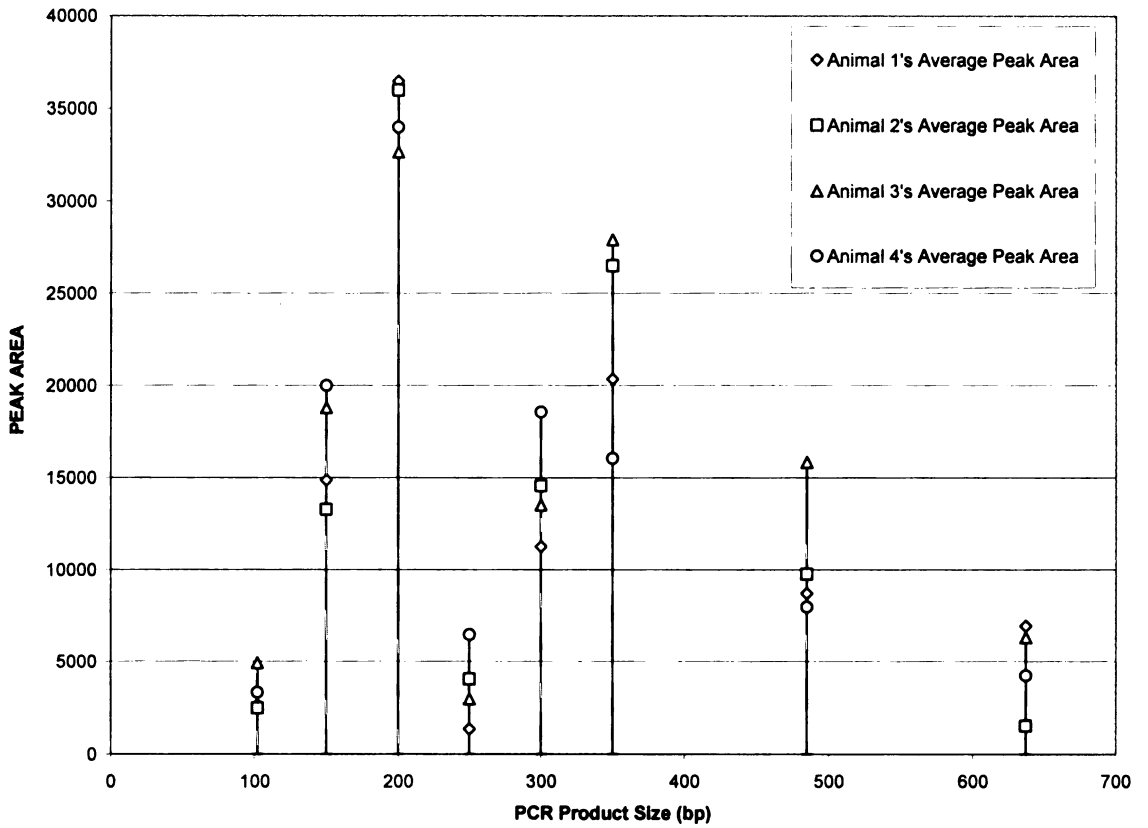
Data Calculations and Analysis

The results as size of PCR products, in base pairs, and their corresponding peak areas are analyzed using the Excel® program. To compare changes between treated and control groups within a digest, first, a consensus control is created. For each distinct PCR product, an average peak area is determined across all the control samples. The standard deviation, standard error, and 95% confidence interval are also calculated. A consensus treated is created using the same analysis steps indicated for creating a consensus control. The following equations are used to calculated the consensus treated peak areas for each PCR product size as a percent of the corresponding consensus control peak areas:

% MspI consensus control = ((MspI consensus treated – MspI consensus control)/MspI consensus control) x 100

% HpaII consensus control = ((HpaII consensus treated – HpaII consensus control)/HpaII consensus control) x 100

These results are plotted using the Excel® program as size of PCR product in base pairs, on the x-axis vs. percent consensus control (as calculated above). In this manner, positive values indicate regions where treatment resulted in increased methylation (hypermethylation), i.e., more of a specific size PCR product formed as compared to control. Negative values represent regions where treatment resulted in decreased



Appendix Figure 1. Individual Animal Average Peak Area Chart For Visualization of Data Reproducibility and Consistency. Peak areas are compared across all the animals for each individual PCR product size within a treatment group. Each animal's average peak area is plotted on the y-axis and the PCR product size (bp) is plotted on the x-axis. Peak area is representative of the amount of PCR product amplified.

methylation (hypomethylation), i.e., less of a specific size PCR product formed as compared to control. Individual *t*-tests ($p<0.05$) were performed to compare the average peak area for a given PCR product size for the treated group with the average peak area for the same PCR product size for the control group. In this fashion we are able to discern three distinct types of changes in methylation: hypermethylation, hypomethylation and new methylation. Hypermethylations are increases which are statistically significant when compared to control. Hypomethylations include both 100% decreases and decreases which are statistically significant when compared to control. The formation of a PCR product following treatment which was not observed under control conditions is deemed a new methylation. Each PCR product of a distinct size is assumed to represent a distinct region of the genome.

The extent of similarity of changes across treatment groups was compared on a region by region basis. PCR products of identical size between the control and treatment groups were considered to be common regions of altered methylation. At each of these corresponding regions, a One-Way ANOVA ($p<0.05$) was used to determine statistical significance.

CHAPTER 2

PHENOBARBITAL INDUCES PROGRESSIVE PATTERNS OF GC-RICH AND GENE-SPECIFIC ALTERED METHYLATION IN THE LIVER OF TUMOR-PRONE B6C3F1 MICE

This chapter represents a draft version of a manuscript that will be submitted to Toxicological Sciences in December, 2005. Authors include: Bachman, Ammie N., Phillips, Jennifer, M. and Goodman, Jay I.

ABSTRACT

Altered DNA methylation contributes to tumorigenesis in that changes in methylation can alter gene expression in a heritable fashion. Phenobarbital (PB) is a non-genotoxic rodent carcinogen which induces global hypomethylation and regions of hypermethylation in mouse liver. Liver tumor sensitive (B6C3F1) and resistant (C57BL/6) male mice were administered 0.05% (w/w) PB in drinking water for 2 or 4wks. In addition, 2wk recovery groups were included to assess reversibility. DNA was isolated from both liver (target) and kidney (non-target) tissue. The methylation status of GC-rich regions of DNA was quantitatively assessed via methylation-sensitive restriction digestion, arbitrarily primed PCR and capillary electrophoretic separation of PCR products in order to discern PB-induced regions of altered methylation (RAMs). Those PB-induced RAMs which carry forward from an early to a later time point are more likely to be mechanistically relevant as compared to those that do not. In this context, it is important to note that 12 of the 69 RAMs (17%) present in B6C3F1 liver at 2 wk were also seen at 4 wk while only 1 of the 123 RAMs (<1%) present in C57BL/6 liver was seen at this later time point. In the B6C3F1 mice, 57 unique (as compared to the C57BL/6) regions of altered hepatic methylation (RAMs), predominantly hypomethylation, were observed after 2 wk of treatment with PB and this increased to 86 at 4wk. Changes in methylation were largely reversible. Comparatively, altered methylation in the liver was highly dissimilar to kidney. Following 4 wk of treatment with PB, bisulfite sequencing revealed hypomethylation of the 5' promoter region of Ha-*ras* in B6C3F1, but not C57BL/6, which correlated with an increase in gene expression. These data support the 3 key points: 1) progressive, non-random changes in methylation

play an important role in tumorigenesis; 2) altered DNA methylation is an epigenetic mechanism underlying the ability of PB to cause liver tumorigenesis; and 3) susceptibility to tumorigenesis is related inversely to the capacity to maintain normal patterns of methylation.

INTRODUCTION

Phenobarbital (PB) is a rodent liver carcinogen which lacks the ability to damage DNA in a direct fashion (Whysner et al., 1996 and 1998). Therefore, it is considered to be a nongenotoxic compound. PB is frequently used as a model compound in carcinogenesis studies. Susceptibility to liver cancer varies widely with genetic background in mice. B6C3F1 mice are particularly sensitive to the formation of liver tumors. They are derived from a cross between the relatively resistant maternal strain, C57BL/6, and the highly susceptible paternal strain, C3H/He. Spontaneous rates of liver tumor formation are high in the sensitive B6C3F1 mice (29%) as compared to the resistant C57BL/6 mice (0%) and administration of PB (0.05% w/w in drinking water) results in a 100% hepatic tumor incidence in B6C3F1 mice while the incidence in the C57BL/6 strain is 0% (Becker, 1982). Additionally, N-nitrosodiethylamine (DEN), a classic genotoxic carcinogen, is far more potent with regard to producing liver tumors in B6C3F1 as compared to C57BL/6 mice (Drinkwater and Ginsler, 1986; Buchmann *et al.*, 1991). Interestingly, the activation of the Ha-*ras* proto-oncogene via a point mutation in codon 61 occurs at a different frequency in spontaneous, DEN-induced and PB-induced B6C3F1 liver tumors. The incidence is approximately 50-60% in spontaneous (Stowers *et al.*, 1988; Fox et al., 1990), and 30-35% in DEN-induced (Stowers *et al.*, 1988; Buchmann *et al.*, 1991) while it is only 7% in the PB-induced tumors (Fox et al., 1990). This indicates that the mechanism by which PB causes these tumors might be somewhat different than that of DEN and is not simply a "magnification" of what occurs spontaneously.

Operationally, carcinogenesis can be described experimentally as having three defined stages: initiation, promotion and progression (Dragan *et al.*, 1993). Cells possessing heritable changes in the genome, acquired during initiation, are selected for and proliferate, given an appropriate promoting stimulus, and eventually are able to progress to frank carcinomas. Exceedingly aberrant subclone populations arise with the progressive accumulation of either mutations or, importantly, epigenetic changes. Specifically, DNA methylation is an epigenetic modification which, when altered, can affect the normal expression of genes in a heritable fashion (Goodman and Watson, 2002). Therefore, altered patterns of methylation are thought to play a causative role in all stages of tumorigenesis, and this is not incompatible with a role for mutations, too (Goodman and Watson, 2002).

Increased DNA methylation (hypermethylation) might silence the expression of tumor suppressor genes (Goodman and Watson, 2002; Jones and Baylin, 2002) while decreased methylation (hypomethylation) might facilitate the expression of oncogenes (Costello and Plass, 2001; Goodman and Watson, 2002). While most attention was previously focused on hypermethylation, the contribution of hypomethylation is gaining more interest. Retrotransposable elements, e.g., LINE-1 elements, are normally kept in a highly methylated state in order to keep them transcriptionally silenced. Hypomethylation may lead to their expression resulting in genomic instability which could play a role in tumorigenesis (Carnell and Goodman, 2003). Mice with decreased Dnmt1 expression exhibit genome-wide DNA hypomethylation, chromosomal instability, and activation of oncogenes. Additionally, they develop aggressive T cell lymphomas (Gaudet *et al.*, 2003). Fibroblasts, derived from embryonic stem cells (ES) which were

transiently demethylated in order to cause loss of imprinting, were tumorigenic when placed into immunosuppressed mice (Holm *et al.*, 2005). Furthermore, chimeric mice derived from the ES which lacked imprinting developed tumors in multiple tissues which arose from the ES cells (Holm *et al.*, 2005). Actually, there are multiple roles for altered DNA methylation in carcinogenesis and this likely involves a combination of selected hypo- and hypermethylations of key genes (Counts and Goodman, Cell, 1995).

A global decrease in hepatic DNA methylation which occurs simultaneously with hypermethylation of GC-rich regions is more pronounced in the liver tumor-prone B6C3F1 mouse as compared to the resistant C57BL/6 mice treated with PB (Counts *et al.*, 1996; Watson and Goodman, 2002). Thus, it has been hypothesized that, sensitivity to tumorigenesis might be related inversely to the capacity to maintain normal patterns of DNA methylation (Counts *et al.*, 1996; Goodman and Watson, 2002; Watson and Goodman, 2002).

Altered patterns of DNA methylation in GC-rich regions of DNA were measured in response to a tumor promoting dose of PB in target (i.e. liver) and non-target (i.e. kidney) tissues. Reversibility, a hallmark of tumor promotion was assessed for the observed changes in liver. The methylation status of the promoter regions of Ha-*ras* and LINE-1 elements were measured and correlated to changes in gene expression. Relatively resistant, C57BL/6 mice are used as a "control" comparison to the tumor prone B6C3F1 mice in testing the hypothesis that progressive, non-random changes in methylation underlie susceptibility to tumorigenesis.

MATERIALS AND METHODS

Animals

Male B6C3F1 (C57BL/6 X C3H/He) and C57BL/6 mice (ages 29-32 days) were obtained from Charles River Laboratories (Wilmington, MA). Animals were allowed to acclimate for 7 days prior to being randomly assigned to treatment groups. B6C3F1 mice were house 5/cage and C57BL/6 mice were housed individually, in a temperature controlled environment and given food and water *ad libitum*. Care was given in accordance with the All Use and Animal Care Guidelines of Michigan State University. Mice, 6-7 animals per group, were administered PB at a concentration of either 0.05% (w/w) or 0.002% (w/w) in the drinking water for 2 or 4 weeks. Recovery groups were given control water for two weeks subsequent to dosing. In this manner, reversibility of alterations in methylation induced by phenobarbital can be assessed. Mice were euthanized by CO_2 asphyxiation, and the livers and kidneys were snap-frozen at -80^0C.

DNA and RNA Isolation

In order to isolate DNA, 1ml of TRIzol Reagent (Invitrogen) per 100mg frozen sample, was added to a dounce homogenizer. Frozen liver or kidney tissue was added to the TRIzol Reagent and thoroughly homogenized. RNA and DNA was isolated according to the manufacturer's protocol.

Arbitrarily Primed PCR and Capillary Electrophoresis

We have developed an arbitrarily primed PCR procedure (AP-PCR) that provides a thorough, overall evaluation of the methylation status of GC-rich regions of DNA.

Importantly, a comparison of data obtained from DNA isolated from control and treated tissue permits the simultaneous detection of treatment-related increased methylation (more methylation in a region that was methylated in control), decreased methylation (less methylation in a region that was methylated in control) and new methylations (methylation in regions that were not methylated in control). Therefore, an in depth picture of treatment related altered methylation is provided. This technique employs methylation sensitive restriction digestion, arbitrarily primed PCR and capillary electrophoretic separation of PCR products. Please refer to Chapter 1 for details concerning basic experimental methods and data calculations. All new information related to the method follows.

Restriction Digestion – BfaI/BssHII Double Digest

BssHII recognizes GCGCGC and cuts between the 5' guanine and cytosine. These sequences are predominantly found within CpG islands and therefore cutting is less frequent (Shiraishi *et al.*, 1995). Generally, BssHII restricts its target sequence only if all cytosines are unmethylated. Restriction digests contain 1μg DNA and 5.0 units BfaI in 1X New England Biolabs Buffer 4. Samples are incubated for 1hr. at 37^{0}C before addition of 2.5 units BssHII. Following addition of BssHII, the incubation temperature is raised to 50^{0}C. A second 2.5 unit aliquot of BssHII is added after an additional 2hr, and in order to insure complete digestion, total incubation time is 18hr. The enzymes were inactivated by incubating at 80^{0}C for 10min. Samples were stored at 4^{0}C until analyzed.

Reversibility of altered methylation: Calculations

The extent to which altered methylation observed following specific periods of promotion was reversible was calculated by using each 2 or 4wk 0.05% PB promotion

group as the working "control" pattern of methylation. The recovery data are calculated as a % of this "control". The 2wk, 0.05% PB promotion group served as the "control" for the 2wk 0.05% PB promotion, 2wk recovery group. The 4wk, 0.05% PB promotion group served as the "control" for the 4wk 0.05% PB promotion, 2wk recovery group.

A consensus, average, peak area for each PCR product reporting in 2 or 4wk, 0.05% PB promotion groups is prepared and designated as the "control" consensus. In addition a consensus, average, peak area for each PCR product reporting in 2 or 4wk 0.05% PB promotion, 2wk recovery is prepared and designated as the "reversal" consensus. "Control" consensus and "reversal" consensus peak areas at each specific PCR product are compared. The following equations are used to calculate the "reversal" consensus peak areas for each PCR product size as a percent of the corresponding "control" consensus peak areas.

% MspI "control" consensus = ((MspI "reversal" consensus – MspI "control" consensus)/MspI "control" consensus)) x 100

% HpaII "control" consensus = ((HpaII "reversal" consensus – HpaII "control" consensus)/HpaII "control" consensus)) x 100

As described previously, hypomethylations, hypermethylations and new methylations are tallied.

Steps to determining whether a CSC-induced change in methylation reversed following the recovery period

1. A list of all the PCR product sizes exhibiting 2 or 4wk 0.05% PB-induced hypo-, hyper-, and new methylations are compiled.

2. A list of the PCR product sizes exhibiting changes in methylation that occurred during recovery as calculated above, are compiled.

3. The two lists are aligned by PCR product size so that changes in methylation due to promotion and changes in methylation which occur during recovery are directly comparable. An example of this appears in Table 1.

4. Changes in methylation induced by PB promotion are only considered recoverable if a change in methylation with opposite direction (i.e. hypomethylations are opposite to hypermethylations and new methylations) is observed following recovery.

Assumptions the AP-PCR Data Analysis

Analysis of the data includes the following assumptions: 1) each separate PCR product of a defined size represents a distinct region of the genome, 2) a region can include one or more recognition sequences for the specific methylation-sensitive restriction enzyme employed located between the annealing sites of the up- and down-stream primers; thus, the amount of each PCR product formed can be viewed as representing an "average" of the methylation status of the particular recognition sequences located between the up- and down-stream primers, and 3) changes in the amount of each PCR product represents the altered methylation status of a particular GC-rich region of DNA.

Table 1. Example of Comparisons for Determining whether a PB-induced Change in Methylation Reversed Following the Recovery Period

4wk Promotion Group		4wk, Promotion, 2wk Recovery Group			
PCR product size (base pairs)	Change in Methylation	PCR product size	Change in Methylation	Changes in methylation are Opposite in Direction?	Promotion induced change in methylation is reversed?[a]
245	New Meth	245	HypoM	Yes	Yes
315	HyperM	315	HyperM	No	No
456	HypoM	456	New Meth	Yes	Yes
505	New Meth	505	HypoM	Yes	Yes
515	HyperM	515	HypoM	Yes	Yes

[a] Changes in methylation induced by CSC promotion are only considered recoverable if a change in methylation with opposite direction (i.e. hypomethylations (HypoM) are opposite to hypermethylations (HyperM) and new methylations (NewMeth)) are observed following recovery.

Common and Unique Regions of Altered Methylation

PCR products of identical size that occur in two treatment groups (e.g. 2wk and 4wk 0.05% PB) were considered to be common regions of altered methylation (RAMs). The methylation changes associated with RAMs in common between 2 treatment groups are considered equivalent and persistent if the changes in methylation are in the same direction and the extents of change are statistically no different as determined by 2-way ANOVA, p=0.05. RAMs in common between 2 treatment groups are considered unique RAMs if, 1) the changes in methylation are opposite in direction (i.e. a hypomethylation is elicited by one treatment and a new methylation is elicited in the same region by the comparison treatment) or 2) the changes in methylation are in the same direction but the extents of change are statistically different as determined by 2-way ANOVA, p=0.05. RAMs are deemed to be unique if they are only observed in a particular treatment group.

Evaluating Total RAMs

The RsaI/MspI and RsaI/HpaII digests were considered separate experiments to determine regions of the genome which exhibit altered methylation in response to treatment. Although HpaII and MspI both restrict CCGG sites, HpaII identifies altered methylation at the internal cytosine while MspI identifies altered methylation at the external cytosine. When we look at the total number of RAMs identified by HpaII and MspI there is the unavoidable possibility that a slight amount of double counting might occur. This would be the case if, methylation were altered at both the internal and external cytosine within CCGG sites of particular genomic region. Therefore, we would

be considering differences in methylation within a given region even if there were some "double counting" of RAMS.

% Dissimilarity Calculations

Control vs. Treatment

The dissimilarity between control patterns of methylation and altered patterns resulting from PB administration was calculated. The total number of PCR products reporting in control was added to the total number of unique PCR products (i.e. those PCR product sizes that were not formed under control conditions) reporting from treatment to get the total number of combined PCR products. This represents the total number of regions (PCR products) analyzed between the two groups. The total number of combined PCR products divided by the total number of regions (PCR products) exhibiting a statistically significant change (hypomethylations, hypermethylations, and new methylations) times 100 equals the percent dissimilarity from control.

PB Treated B6C3F1 vs. PB Treated C57BL/6

The dissimilarity between the extent of altered methylation due to PB in C57 in comparison to B6 at 2 and 4 wks was calculated. Unique RAMs were identified for the B6 mouse. These include 1) RAMs in common with C57, but the methylation change is opposite in direction, 2) RAMs in common with C57, but the extent of change is statistically greater (2-way ANOVA, $p<0.05$) in B6, and 3) RAMs which are only observed in the B6 mouse. The unique RAMs were divided by the total RAMs (i.e. all hypomethylations, hypermethylations, and new methylations in B6 mouse) and

multiplied by 100 to get the percent dissimilarity. In addition, the dissimilarity between the extent of altered methylation due to PB in liver in comparison to kidney for B6 and C57 was calculated. Unique RAMs were identified for the liver. These include: 1) RAMs in common with kidney, but the methylation change is opposite in direction, 2) RAMs in common with kidney, but the extent of change is statistically greater (2-way ANOVA, $p<0.05$) in liver, and 3) RAMs which are only observed in the liver. The unique RAMs were divided by the total RAMs (i.e. all hypomethylations, hypermethylations, and new methylations in liver) and multiplied by 100 to get the percent dissimilarity.

Gene-Specific Methylation Analysis: Bisulfite Sequencing of 5' Promoter Region of Ha-ras

Bisulfite Conversion and PCR Amplification

Bisulfite conversion of DNA effectively deaminates all un-methylated cytosines to uracil leaving methylated cytosines unaffected. 2μg DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). PCR is performed with bisulfite converted DNA which allows for the replacement of uracil with thymine and 5-methylcytosine with cytosine. Consequently, only cytosines that were originally methylated remain in the DNA sequence. PCR is carried out using primers specific for bisulfite converted DNA and containing no CpG sites. The two Ha-*ras* primers, 5' GGT GGG TTA GAG TGT TTA AGA TTT G 3' and 5' CTC TTA CTC TAA AAA ACA TTT CCA C 3' were used to amplify the -950nt to -1232nt (283bp) region of the Ha-*ras* promoter relative to the transcriptional start site. Primers were designed based on

sequence information obtained from Brown *et al.*, 1988 and Neades *et al.*, 1991. Each PCR reaction contained 0.5µg bisulfite converted DNA, 1X Failsafe™ Buffer G (Epicentre®; Madison, WI) 0.3µM each primer, 1.5 units Taq Polymerase (Invitrogen™) and GDW to a final volume of 25ul. Cycling conditions were: 95^0C for 3 min, 38 cycles of 95^0C for 45 s, 58^0C for 45 s, and 72^0C for 1 min, followed by 1 time delay cycle of 72^0C for 3min and a 4^0C soak. Amplification of the target region was verified by gel electrophoresis on a 3% agarose gel. Duplicate Ha-*ras* PCR reactions were combined and purified using the Qiagen Qiaquick PCR Purification Kit. Samples were quantified fluorometrically.

Sequencing

Automated sequencing of purified Ha-*ras* PCR products was carried out at the Genomics Technology Support Facility at Michigan State University using an ABI PRISM®3100 Genetic Analyzer. Two separate sequencing reactions are performed for each gene. Sequencing reactions are composed of 20ng PCR product, 30 pmol of either the forward primer or reverse primer, and

Reverse Transcription of RNA

RNA samples were treated with DNaseI (Invitrogen) to purify the RNA from contaminating DNA remaining after isolation. Each reaction contained 2ug RNA, 1X DNaseI reaction buffer, 2 units DNaseI, and DEPC-treated GDW to a final volume of 20ul. Samples were incubated at room temp. for 15min followed by addition of $MgCl_2$ to a final concentration of 2.27mM. RNA was heated to 65^0C for 10min to inactivate the

DNaseI enzyme. The TaqMan Reverse Transcription Kit (Applied Biosystems; Foster City, CA) was used to reverse transcribed the DNaseI treated RNA. Each reverse transcription reaction contained, 1X Reverse Transcription Reaction Buffer, 5.5mM MgCl2, 200uM of each dNTP, 2.5uM random hexamer, 20 units RNase Inhibitor, 62.5 units Multiscribe Reverse Transcriptase and DEPC-treated GDW to a final volume of 50ul. The reactions are incubated at 25^0C for 10min, 42^0C for 1hr, and 95^0C for 5 min. All samples were stored at 4^0C until needed.

Expression of Ha-ras and LINE-1

Real Time PCR: Primers

A custom TaqMan assay including primers (For 5' TGG TGG GCA ACA AGT GTG A3' and Rev 5' GGC CTG CCG AGA CTC A 3') and probe (5' FAM CTG GCT GCT CGC ACT GT 3') specific for Exon 3 of the Ha-*ras* gene was purchased from Applied Biosystems. The assay was designed based on sequence information obtained from Brown *et al.*, 1988 and Neades *et al.*, 1991. A custom TaqMan assay, including primers (For 5' GGT CAA ATC TAA GTG GAT CAA GGA ACT 3' and Rev 5' GCT TTT CCC CAC TTT CTC CTC TAT 3') and probe (5' FAM CAG AGA CAC TGA AAC TT 3'), specific for ORF2 of the LINE-1 element (Accession M13002) was purchased from Applied Biosystems. In addition an Applied Biosystems custom TaqMan assay, including primers (For 5' CTA CTA CCG ATT GGA TGG TTT AGT GA 3' and Rev 5' GTC AAG TTC GAC CGT CTT CTC A 3' and probe (FAM 5' CCG TGG GCC GAC CC3'), was used for the control gene, 18S rRNA (Accession X00686).

Real Time PCR

Triplicate reactions for both the gene of interest (Ha-*ras* or LINE-1) and the control gene (18S) were prepared per sample. Standards were also prepared for 18S, Ha-*ras* and LINE-1 and ranged from 5×10^1 copies/ul to 5×10^7 copies/ul. Each Ha-*ras* or LINE reaction contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 8ul cDNA and GDW to a final volume of 25ul. Each 18S reaction contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 2ul 1:100 diluted cDNA and GDW to a final volume of 25ul. Reactions for each standard contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 2ul standard and GDW to a final volume of 25ul. Cycling conditions were as follows: 50^0C for 2 min, 95^0C for 10min, and 40 cycles of 95^0C for 15sec. and 60^0C for 1 min. The absolute standard curve method for quantifying fold change over control was employed.

RESULTS

Genome-wide analysis of altered methylation in GC-rich regions in response to 2 or 4wk PB, 0.05% (w/w) in the diet, discerned numerous regions of altered methylation (RAMs) and established the occurrence of hypomethylations, hypermethylations, and new methylations in B6C3F1 and C57BL/6 mice. B6C3F1 mice exhibited 69 total RAMs, primarily hypomethylations at 2wks (Table 2). With 4wks treatment, hypomethylated, hypermethylated, and new RAMs in the B6C3F1 mice increased to 98, a 42% increase in total RAMs. In contrast, while a large numbers of RAMs (123 total) were observed in the liver of C57BL/6 mice at 2wks, the total decreased to 88, a 28% decrease, primarily due to a lower number of hypermethylations and new methylations at the later time point (Table2).

Quantifying hypomethylated, hypermethylated and newly methylated RAMs allows for the identification and comparison of patterns and trends of PB-induced altered methylation between the liver tumor-prone B6C3F1 mice and the relatively resistant C57BL/6 mice. Additionally, assessing the extent to which 2 or 4 week PB disrupted methylation patterns as compared to controls is important, too. An evaluation of percent dissimilarity provided an initial, overall assessment of PB's effects in B6C3F1 and C57BL/6 mice. Methylation patterns arising from 2 wks PB were 45% and 67% dissimilar to methylation patterns of controls in B6C3F1 and C57BL/6 mice, respectively (Table 2). At 4 wks, dissimilarity increased to 61% and 79% (Table 3).

Overall dissimilarities revealed that PB-induced patterns of methylation in C57BL/6 deviated from controls slightly more than in B6C3F1 emphasizing the need for a more refined approach to identify, evaluate and prioritize changes in methylation which

Table 2. Methylation Sensitive Digestion with RsaI/MspI or RsaI/HpaII: Summary of GC-Rich Regions of Altered Methylation (RAMs) in the Liver of B6C3F1 and C57BL/6 Mice in Response to 2 or 4wk 0.05% PB

Treatment	Digest	RAMs HypoM[a]	RAMs HyperM[b]	RAMs "New" Methylation[c]	TOTAL
2 wk 0.05% PB					
B6C3F1	**HpaII**	20	1	6	27
	MspI	26	6	10	42
Total[d]		**46**	**7**	**16**	**69**
C57BL/6	**HpaII**	19	6	17	42
	MspI	18	33	30	81
Total[d]		**37**	**39**	**47**	**123**
4 wk 0.05% PB					
B6C3F1	**HpaII**	13	4	21	38
	MspI	46	7	7	60
Total[d]		**59**	**11**	**28**	**98**
C57BL/6	**HpaII**	36	8	9	53
	MspI	35	0	0	35
Total[d]		**71**	**8**	**9**	**88**

[a] Hypomethylated (HypoM) RAMs include both statistically significant ($p<0.05$) decreases and 100% decreases.

[b] Hypermethylated (HyperM) RAMs are only those increases which are statistically significant ($p<0.05$).

[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not present under control conditions.

[d] Total RAMs including hypomethylations, hypermethylation, and new methylations for the combined digests are reported for each treatment

Table 3. Dissimilarity Between PB-Induced Methylation Changes and Control Methylation Patterns in B6C3F1 and C57BL/6 Mouse Liver

Dissimilarity Between:		Total Regions[a]	Total RAM as Compared to Control[b]	Percent Dissimilarity To Control	
B6C3F1					
Control	2 weeks 0.05% PB	153	69	69/153	**45%**
Control	4 weeks 0.05% PB	202	123	123/202	**61%**
C57BL/6					
Control	2 weeks 0.05% PB	147	98	98/147	**67%**
Control	4 weeks 0.05% PB	111	88	88/111	**79%**

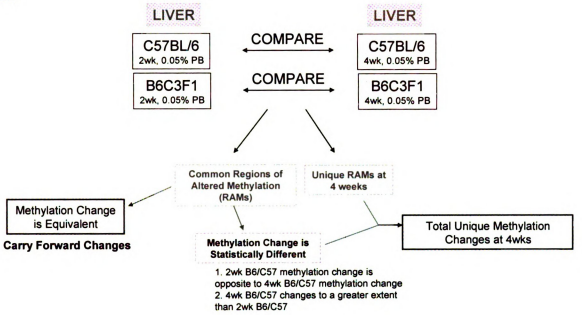
[a] Total regions includes every PCR product size reporting between control and treated groups. Each PCR product represents a region of the genome.
[b] Total RAMs represents the number of regions exhibiting a statistically significant ($p<0.05$) change in methylation including complete hypomethylations (100%), partial hypomethylations, hypermethylations, or new methylations.

might mechanistically be important regarding promotion of tumorigenesis. An important feature of the promotion stage of tumorigenesis is the progressive accumulation of heritable alterations to the genome. Therefore, we evaluated the number of changes in methylation which "carry forward" over time during PB-treatment. "Carry forward" RAMs, identified in the B6C3F1 and C57BL/6 mice, included all RAMs that were in common between the 2 and 4 week time points which exhibited equivalent changes in methylation (Figure 1a). RAMs identified only at 4 weeks plus those RAMs which were in common with those identified at 2 weeks but the methylation change was either 1) opposite in direction or 2) changing to a greater extent at 4 weeks were classified as unique RAMs (Figure 1a). A total of 12 PB-induced RAMs (17% of the total RAMs observed at 2 wk) including 9 hypomethylations, 1 hypermethylation, and 2 new methylations, carried forward from 2 to 4 wks in B6C3F1 liver (Figure 1b). Strikingly, only one hypomethylation out of 123 total RAMs (<1%) in C57BL/6 liver was seen to carry forward to 4 wk (Figure 1b).

Two-and four-week periods of promotion with PB were followed by 2 wk recovery periods. Figure 2 illustrates that with 2 wks PB exposure and 2wks recovery in B6C3F1 mouse liver 100% (7/7) of the hypermethylations and 75% (12/16) of the new methylations reversed. In addition, a large number of hypermethylations (73%, 8/11) and new methylations (61%, 17/28) which included two of the persistent RAMs were seen to reverse during the recovery period following 4wk PB administration. Recovery was similar in C57BL/6 mice in that 31% (12/39) and 100% (8/8) of hypermethylations and 51% (24/47) and 89% (8/9) of new methylations reversed during 2wks recovery following 2 and 4wks PB exposure, respectively. This illustrates that increases in

Figure 1 Progressive Changes in Methylation: Changes Which Carry Forward From 2 to 4wk in B6C3F1 and C57BL/6 Mice The flow chart illustrates the steps necessary to determine progressive changes in methylation. Separate comparisons between B6C3F1 and C57BL/6, 2 and 4wk regions of altered methylation (RAMs) were performed (a). Each RAMs induced by 2wk, 0.05% PB promotion are represented (Θ) for B6C3F1 and C57BL/6 mice. RAMs induced by 2wk PB were compared to those resulting from 4wk, 0.05% PB in B6C3F1 and C57BL/6 mice. Common RAMs in which the magnitudes of change were equivalent (2-way ANOVA, $p<0.05$) were considered carry forward changes. RAMs unique to 4wk, 0.05% PB (⦿) included common RAMs in which the magnitudes of change were different or the RAMs were only observed with 4wk 0.05% PB (b). Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are segregated. Total unique changes (minus carry forward changes) are tallied and reported for each category of methylation change.

A.


LIVER
C57BL/6
2wk, 0.05% PB
B6C3F1
2wk, 0.05% PB
COMPARE
COMPARE
LIVER
C57BL/6
4wk, 0.05% PB
B6C3F1
4wk, 0.05% PB
Common Regions of Altered Methylation (RAMs)
Unique RAMs at 4 weeks
Methylation Change is Equivalent
Carry Forward Changes
Methylation Change is Statistically Different
Total Unique Methylation Changes at 4wks
1. 2wk B6/C57 methylation change is opposite to 4wk B6/C57 methylation change
2. 4wk B6/C57 changes to a greater extent than 2wk B6/C57

B.

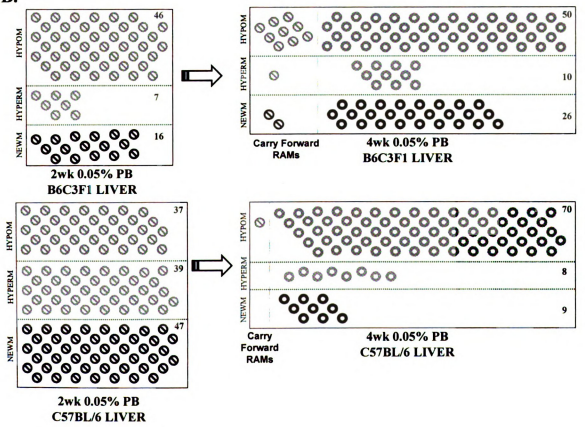
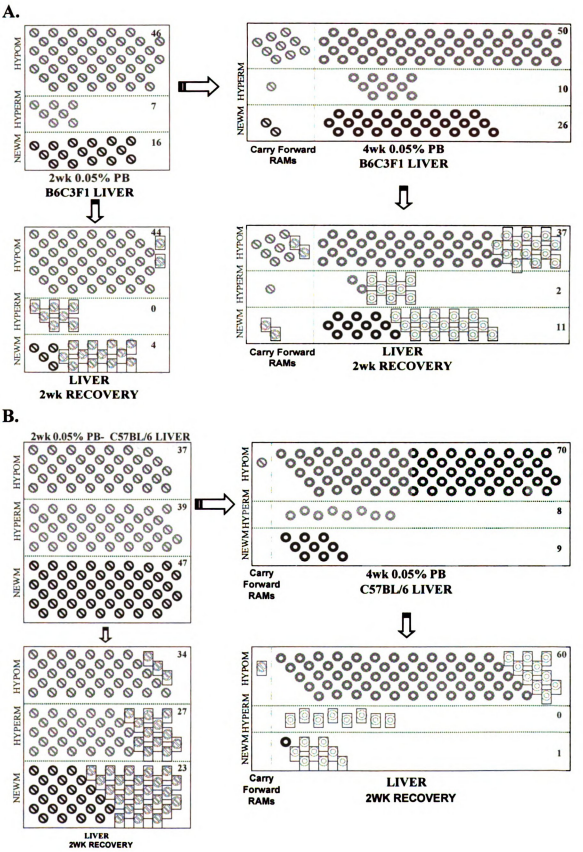

HYPOM
HYPERM
NEWM
46
7
16
2wk 0.05% PB
B6C3F1 LIVER
50
10
26
Carry Forward RAMs
4wk 0.05% PB
B6C3F1 LIVER
37
39
47
2wk 0.05% PB
C57BL/6 LIVER
70
8
9
Carry Forward RAMs
4wk 0.05% PB
C57BL/6 LIVER

Figure 2. Progressive Changes in Methylation: Reversibility of Regions of Altered Methylation Induced by PB Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are represented for 2wk (Θ) and 4wk (⦿) 0.05% PB promotion. RAMs which carried forward from 2 to 4wks are also represented (Θ). RAMs induced by 2 or 4wk PB which reversed during the 2 week recovery periods in B6C3F1 (a) and C57BL/6 (b) are boxed. Total unique changes (minus carry forward RAMs and RAMs which reversed) are tallied and reported for each category of methylation change.


A.
HYPOM
HYPERM
NEWM
46
7
16
2wk 0.05% PB
B6C3F1 LIVER
50
10
26
Carry Forward RAMs
4wk 0.05% PB
B6C3F1 LIVER
44
0
4
LIVER
2wk RECOVERY
37
2
11
Carry Forward RAMs
LIVER
2wk RECOVERY
B.
2wk 0.05% PB- C57BL/6 LIVER
37
39
47
70
8
9
Carry Forward RAMs
4wk 0.05% PB
C57BL/6 LIVER
34
27
23
LIVER
2WK RECOVERY
60
0
1
Carry Forward RAMs
LIVER
2WK RECOVERY

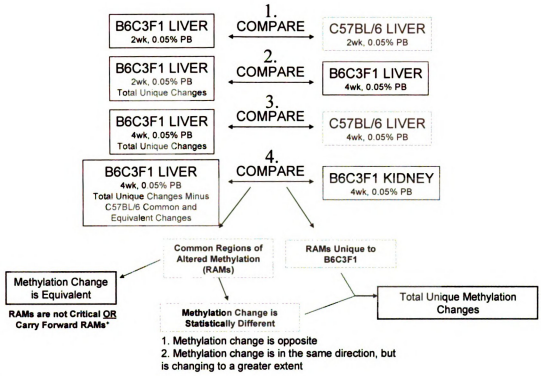
methylation which accumulate in response to the promoting stimuli are largely reversible following a relatively brief period of promoter treatment. However, the reversibility of hypomethylated RAMs in both B6C3F1 and C57BL/6 mice was rather low and might indicate that 2wks are insufficient for "re-methylation" of unmethylated cytosines (Figure 3).

In light of the extreme difference between B6C3F1 and C57BL/6 mice in terms of changes in methylation which carry forward over time, the prime focus of the investigation was on identifying specific differences as well as similarities in RAMs between the liver tumor-prone B6C3F1 and the relatively resistant C57BL/6 mice. We focused on ascertaining which PB-induced increases (i.e. hypermethylations and new methylations) or decreases (i.e. partial and complete hypomethylations) in methylation occurred in the same regions of the genome. For each specific region, changes in methylation identified in B6C3F1 and C57BL/6 hepatic DNA were compared and classified as 1) equivalent, 2) opposite in direction (e.g., hypomethylation in the B6C3F1 and hypermethylation in the C57BL/67), or 3) changing in the same direction, but to a greater extent in the B6C3F1. Additionally, those RAMs which were unique to the B6C3F1 mouse are emphasized. Initially, by using this information, we were able to calculate an overall dissimilarity between PB-induced patterns of altered methylation in B6C3F1 mice as compared to those in the C57BL/6 mice. With 2wk and 4wks of PB exposure, the patterns of altered methylation in the livers of B6C3F1 mice were 81% and 85% dissimilar to those in C57BL/6, respectively (Table 4).

By subtracting out the individual RAMs that were both in common and equivalent between B6C3F1 and C57BL/6 (i.e. the 19% and 15% similarities) at the 2 and 4 wk time

Figure 3. Identification of PB-Induced Unique and Carry Forward RAMs in B6C3F1 Liver. The flow chart illustrates the four steps necessary in defining the unique and carry forward PB-induced RAMs in B6C3F1 liver. Four separate comparisons were performed (a). 1. B6C3F1 unique RAMs induced by 2wk, 0.05% PB promotion are represented (Θ). 2. B6C3F1 unique RAMs induced by 2wk PB were compared to total RAMs resulting from 4wk, 0.05% PB. Common RAMs in which the magnitudes of change were equivalent (2-way ANOVA, $p<0.05$) were considered carry forward changes. RAMs unique to 4wk, 0.05% PB (⦿) included common RAMs in which the magnitudes of change were different or the RAMs were only observed with 4wk 0.05% PB 3. B6C3F1 total RAMs were compared to C57BL/6 total RAMs. RAMs which are in common with C57BL/6 and exhibit equivalent changes in methylation are identified (◎). 4. B6C3F1 total RAMs minus C57BL/6 common and equivalent changes were compared to B6C3F1 RAMs in kidney. RAMs which are in common with C57BL/6 (◎) and kidney (☾) and exhibit equivalent changes in methylation are identified. Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are segregated. Total unique changes (minus carry forward changes and changes in common and equivalent with C57BL/6 or kidney) are tallied and reported for each category of methylation change. (+) RAMs are considered less critical if the RAMs are in common with C57BL/6 and the change in methylation is equivalent. Carry Forward RAMs are only determined with comparison (2.) in which 2wk B6C3F1 total unique RAMs are compared to 4wk B6C3F1 RAMs. (*) RAMs which are in common and equivalent in B6C3F1 and C57BL/6 at 2wks but only observed in B6C3F1 at 4wks.

A.


1. COMPARE
B6C3F1 LIVER 2wk, 0.05% PB
C57BL/6 LIVER 2wk, 0.05% PB
2. COMPARE
B6C3F1 LIVER 2wk, 0.05% PB Total Unique Changes
B6C3F1 LIVER 4wk, 0.05% PB
3. COMPARE
B6C3F1 LIVER 4wk, 0.05% PB Total Unique Changes
C57BL/6 LIVER 4wk, 0.05% PB
4. COMPARE
B6C3F1 LIVER 4wk, 0.05% PB Total Unique Changes Minus C57BL/6 Common and Equivalent Changes
B6C3F1 KIDNEY 4wk, 0.05% PB
Common Regions of Altered Methylation (RAMs)
RAMs Unique to B6C3F1
Methylation Change is Equivalent
RAMs are not Critical OR Carry Forward RAMs*
Methylation Change is Statistically Different
Total Unique Methylation Changes
1. Methylation change is opposite
2. Methylation change is in the same direction, but is changing to a greater extent

B.

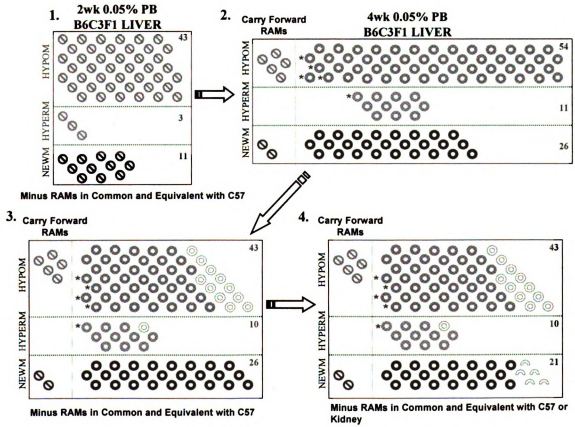

1. 2wk 0.05% PB B6C3F1 LIVER
HYPOM
HYPERM
NEWM
43
3
11
Minus RAMs in Common and Equivalent with C57
2. Carry Forward RAMs
4wk 0.05% PB B6C3F1 LIVER
54
11
26
3. Carry Forward RAMs
43
10
26
Minus RAMs in Common and Equivalent with C57
4. Carry Forward RAMs
43
10
21
Minus RAMs in Common and Equivalent with C57 or Kidney

Table 4. Dissimilarity Between PB-Induced Methylation Changes in B6C3F1 and C57BL/6 Mouse Liver

Dissimilarity Between:		Total RAMs in B6[a]	Total Unique RAMs As Compared to C57BL/6[b]	Percent Dissimilarity To C57BL/6	
2 week 0.05% PB					
B6C3F1	C57BL/6	69	56	56/69	**81%**
4 week 0.05% PB					
B6C3F1	C57BL/6	98	83	83/98	**85%**

[a] Total RAMs in B6C3F1 is reported from Table 1

[b] Total Unique RAMs represents the number of regions exhibiting a statistically significant ($p<0.05$) change in methylation including complete hypomethylations (100%), partial hypomethylations, hypermethylations, or new methylations that were only observed in B6C3F1 mice.

points, we were able to further prioritize which RAMs are most unique to the B6C3F1 mice and, therefore, likely to contribute to liver tumor formation (Figure 3a). Figure 3b, comparison 1, represents the total unique RAMs (i.e. all RAMs that were in common and equivalent to C57BL/6 are not represented) in the B6C3F1 mice at 2 wks. This pool of B6C3F1 total unique RAMs (i.e. total RAMs minus RAMs in common with C57BL/6) was compared to the total B6C3F1 RAMs identified at 4wks and established that 7 of the 57 unique RAMs observed at 2 wk carried forward to 4 wks (Figure 3b, comparison 2.). Additionally, 54 hypomethylations, 11 hypermethylations, and 26 new methylations were all unique to the B6C3F1 mouse at 4 wks, and this adds up to a total of 98 RAMs in B6C3F1 at the 4 wk time point (Figure 3b, comparison 2.). A comparison of 4wk PB-induced RAMs in B6C3F1 and C57BL/6 mice identified 12 common RAMs with equivalent changes in methylation (Figure 3b, comparison 3.) These RAMs were subtracted from the total changes observed in the B6C3F1 mice at 4 wk because they are considered less likely to contribute to tumor formation, and this results in a total of 86 unique RAMs in B6C3F1 at the 4 wk time point (this includes 5 RAMs which were observed in both B6C3F1 and C57BL/6 mice at 2 wk and only in the B6C3F1 at 4 wk, Figure 3b, comparison 3).

Methylation status of the DNA of kidneys of B6C3F1 and C57/BL/6 mice allowed for a comparison between liver and kidney (a non-target for tumorigenesis) tissue. A substantial number of PB-induced RAMs were observed in kidney DNA. Changes in methylation were induced in B6C3F1 kidney at 4wks. However, in comparison to liver, they were fewer in number and, importantly, did not include hypomethylated RAMs, a distinct feature of PB-induced alterations in the liver (Table 5).

Table 5. Methylation Sensitive Digestion with RsaI/MspI or RsaI/HpaII: Summary of GC-Rich Regions of Altered Methylation (RAMs) in Liver and Kidney of B6C3F1 and C57BL/6 Mice in Response to Treatment with 0.05% PB for 4 wk

Treatment	Digest	RAMs HypoM[a]	RAMs HyperM[b]	RAMs "New" Methylation[c]	TOTAL
B6C3F1					
LIVER	**HpaII**	13	4	21	38
	MspI	46	7	7	60
Total[d]		**59**	**11**	**28**	**98**
KIDNEY	**HpaII**	0	8	33	41
	MspI	0	2	23	25
Total[d]		**0**	**10**	**56**	**66**
C57BL/6					
LIVER	**HpaII**	36	8	9	53
	MspI	35	0	0	35
Total[d]		**71**	**8**	**9**	**88**
KIDNEY	**HpaII**	4	0	1	5
	MspI	18	2	2	22
Total[d]		**22**	**2**	**3**	**27**

[a] Hypomethylated (HypoM) RAMs include both statistically significant ($p<0.05$) decreases and 100% decreases.

[b] Hypermethylated (HyperM) RAMs are only those increases which are statistically significant ($p<0.05$).

[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not present under control conditions.

[d] Total RAMs including hypomethylations, hypermethylation, and new methylations for the combined digests are reported for each treatment group

Altered methylation in the kidney of C57BL/6 mice mirrored the pattern of altered methylation in the liver; however, the total number of RAMs were much lower in kidney (Table 5). It was instructive to determine the degree of similarity in altered patterns of methylation between the kidney and the liver of each of the different mice. In B6C3F1 mice RAMs induced by PB in the liver were 94% dissimilar to those in kidney whereas in C57BL/6 mice RAMs in the liver were 85% dissimilar to those in kidney (Table 6).

In much the same way that C57BL/6 was used as a "control" for trying to discern critical RAMs in B6C3F1 liver, patterns of altered methylation in kidney can be compared to liver to further distinguish which changes in methylation are likely to be critical in B6C3F1 liver. This analysis identified 5 common RAMs with equivalent changes in methylation (Figure 3b, comparison 4.) These RAMs were subtracted from the total unique changes because they are considered less likely to contribute to tumor formation. Therefore, of the 98 total RAMs detected in the liver of B6C3F1 mice at 4 wks, 74 unique RAMs plus the 7 RAMs which carried forward from 2 to 4 weeks represent the PB-induced changes in methylation observed solely in B6C3F1 mouse liver.

Analysis of altered methylation was also assessed using the methylation insensitive enzyme BfaI in conjunction with the methylation sensitive enzyme BssHII. BfaI restricts CpG islands less than RsaI (Shiraishi *et al.*, 1995) and BssHII has a 6 base recognition sequence (Shiraishi *et al.*, 1995) as compared to the 4 base recognition sequence of MspI and HpaII. Patterns of methylation detected at 2 and 4 wks in B6C3F1 and C57BL/6 mice using BssHII were similar to those detected with MspI and HpaII. Less total changes were observed at 2wks in both B6C3F1 and C57BL/6 mice (Table 7). At 4wks, B6 exhibited 25 total RAMs as compared to 12 RAMs in C57BL/6 mice (Table

Table 6. Dissimilarity Between Methylation Changes in the LIVER as compared to the KIDNEY following 4 wk of Treatment with PB

Dissimilarity Between:		Total RAMs in LIVER[a]	Total Unique RAMs As Compared to KIDNEY[b]	Percent Dissimilarity To KIDNEY	
B6C3F1					
LIVER	KIDNEY	98	92	92/98	**94%**
C57BL/6					
LIVER	KIDNEY	88	75	75/88	**85%**

[a] Total RAMs in liver is reported from Table 1

[b] Total Unique RAMs represents the number of regions exhibiting a statistically significant ($p<0.05$) change in methylation including complete hypomethylations (100%), partial hypomethylations, hypermethylations, or new methylations that were only observed in the liver.

Table 7. Methylation Sensitive Digestion with BfaI/BssHII: Summary of GC-Rich Regions of Altered Methylation (RAMs) in the Liver of B6C3F1 and C57BL/6 Mice Following Treatment with PB for to 2 or 4wk

Treatment	Digest	RAMs HypoM[a]	RAMs HyperM[b]	RAMs "New" Methylation[c]	TOTAL
2 wk 0.05% PB					
B6C3F1	**BssHII**	2	1	11	14
C57BL/6	**BssHII**	14	1	0	15
4 wk 0.05% PB					
B6C3F1	**BssHII**	18	2	5	25
C57BL/6	**BssHII**	3	3	6	12

[a] Hypomethylated (HypoM) RAMs include both statistically significant ($p<0.05$) decreases and 100% decreases.

[b] Hypermethylated (HyperM) RAMs are only those increases which are statistically significant ($p<0.05$).

[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not present under control conditions.

7). This analysis served to re-enforce the results obtained with RsaI/MspI and RsaI/HpaII digests.

GC-rich regions are associated with the promoter regions of genes and altered methylation within these regions could affect gene expression. Therefore, the methylation status of a 5' promoter region of Ha-*ras* oncogene was analyzed in addition to an upstream region of the LINE-1 element. The methylation status of a 283bp region of the Ha-*ras* promoter, containing 22CpG dinucleotides, which was 950nt upstream of the transcriptional start site was evaluated (Figure 4a). Bisulfite sequencing revealed 3 methylated cytosines within the targeted region under control conditions in both the B6C3F1 and C57BL/6 mice. One additional cytosine was methylated in 50% and 67% of the control animals in B6C3F1 and C57BL/6 mice respectively (Figure 4b). With 2wk 0.05% PB the methylation status of one cytosine at -1174nt decreased only in the B6C3F1 mice. This site of altered methylation (-1174nt) was seen to reverse following 2wks recovery (Figure 4b). Following 4 wk of PB treatment, the B6C3F1, but not C57BL/6 mice, also exhibited hypomethylation of the cytosine at position -1174. In addition, 2/6 animals were hypomethylated at a second cytosine (-1204nt) (Figure 4c). Following the 2wk recovery period the cytosine at -1204 of all 6 animals, and 3/6 at -1174nt reversed to a methylated status (Figure 4c). Bisulfite sequencing was also performed on a 5' region of the LINE-1 element containing 11CpG dinucleotides 975nt upstream of the transcriptional start site. All 11 cytosines were methylated in control animals as well as animals treated with 2 or 4wk 0.05% PB (Figure 5).

With evidence for hypomethylation within a limited region of the Ha-*ras* promoter only in the B6C3F1 mice at 2 and 4wk, the effect on gene expression levels was

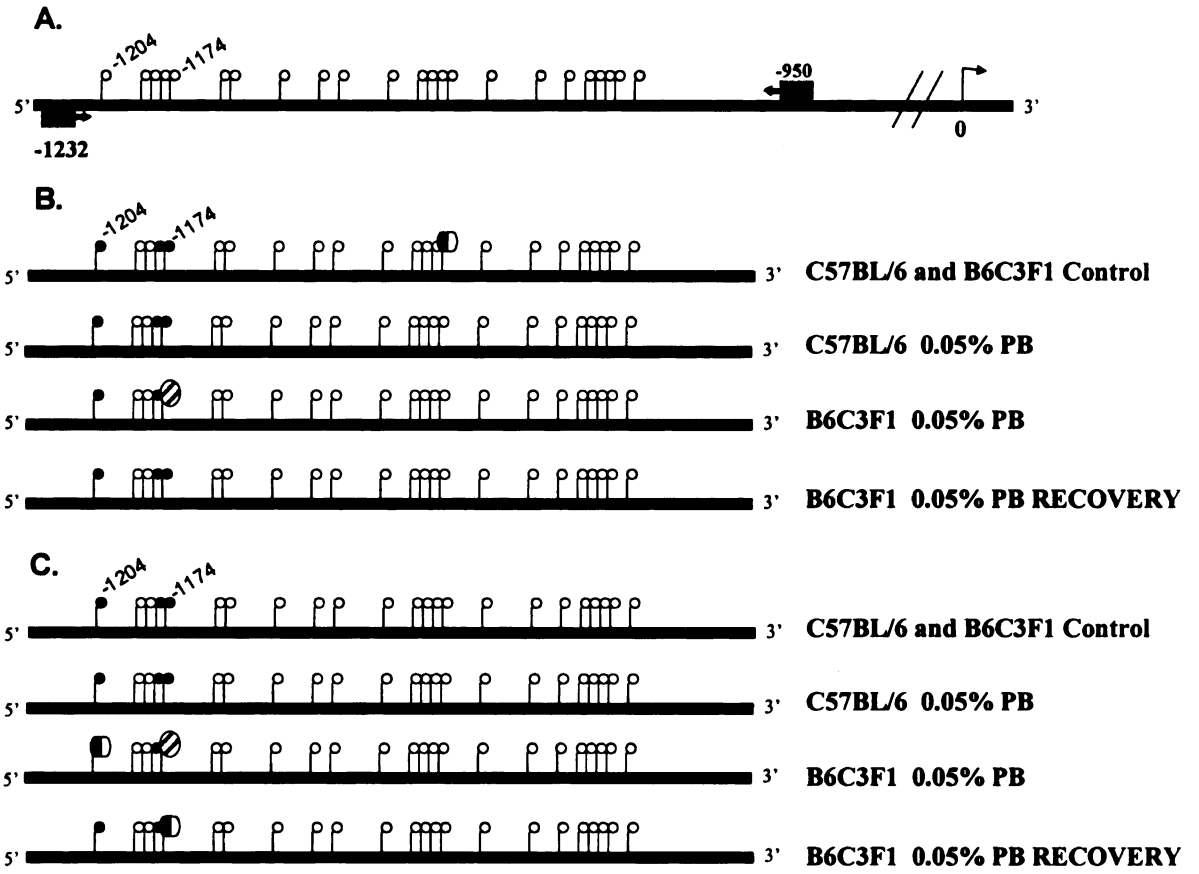


Figure 4. Methylation Status of the Promoter Region of Ha-*ras*. A diagram of the Ha-ras promoter indicating location of PCR primers and CpG sites (gray lollipops) in relation to the transcriptional start site is presented (a). Bisulfite sequencing analysis within the region spanning 283bp revealed that 3 cytosines are methylated (black lollipops) in both B6C3F1 and C57BL/6 control mice. In addition, the cytosine at position -1033 was methylated in 50% and 65% of B6C3F1 and C57BL/6 mice, respectively. This is represented by a half black and half white lollipop. PB-induced hypomethylation (striped lollipop) of the cytosine at -1174nt was only seen the B6C3F1 mice at 2wks. This hypomethylated state reversed following 2wks recovery (b). At 4wks, 3 cytosines were methylated in control animals. PB induced hypomethylation of the cytosine at position -1174nt in B6C3F1 mice and hypomethylation of the cytosine at position -1204nt in 30% of B6C3F1 mice. With 2wks recovery, the methylation status of the cytosine at -1204nt fully reversed while reversal was only seen in 50% of the B6C3F1 mice at position -1174nt (c). Black = methylated, White = unmethylated, Striped = Hypomethylated in 100% of animals.

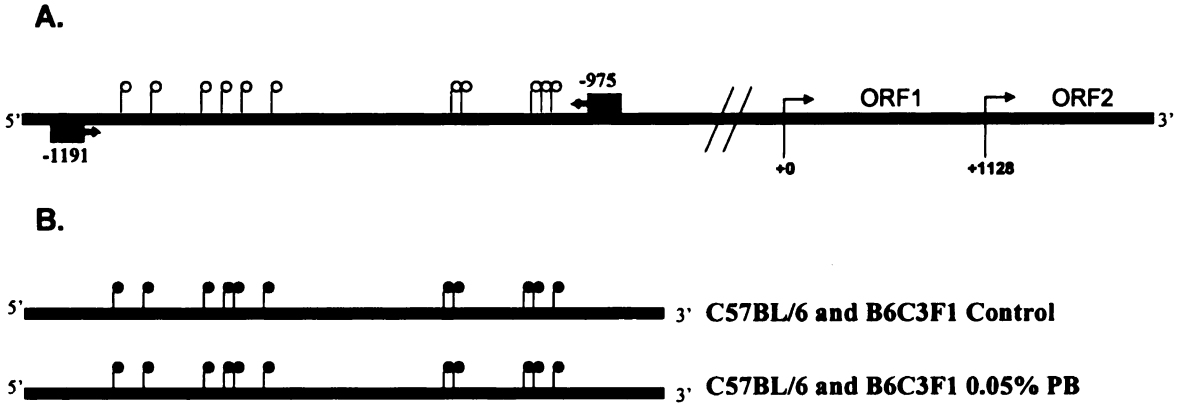


Figure 5. Methylation Status of the Promoter Region of LINE-1 in B6C3F1 and C57BL/6 Mouse Liver. A diagram of the LINE-1 promoter indicating location of PCR primers and CpG sites (gray lollipops) in relation to the transcriptional start site of the 1st open reading frame (ORF) is presented (a). Bisulfite sequencing analysis within the outlined region revealed that all 11 cytosines are methylated (black lollipops) in both B6C3F1 and C57BL/6 control and treated mice. (b).

investigated. Fold change of Ha-*ras* expression over normalized control levels was measured. No change in gene expression was observed between control and PB treated animals at 2wks (Figure 6). At 4wks selective increases in expression were observed in response to PB (Figure 7). Three animals exhibited a level of expression which exceeded the upper 95% confidence limit of the controls (Figure 7). Following two weeks recovery, 4/6 animals expressed basal levels of Ha-*ras* expression (Figure 8). In comparison, Ha-*ras* expression levels in C57BL/6 were unaffected by 2 or 4wks PB treatment (Figure 9). The methylation status of cytosines within the targeted region of the LINE-1 promoter was unchanged by treatment with PB in both B6C3F1 and C57BL/6 mice. Consistent with this, the levels of expression of LINE-1 in control and treated animals at 2 and 4wks were comparable (Figure 10).

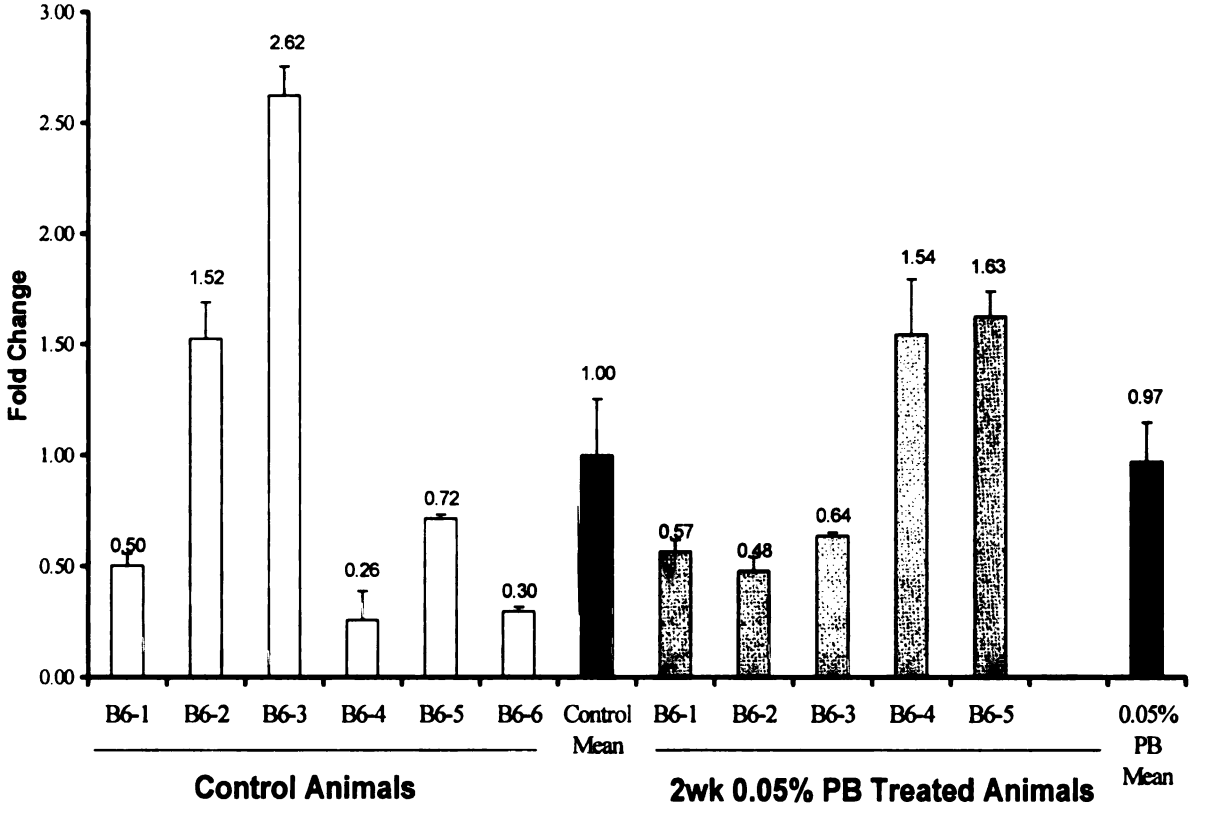


Figure 6. Effect of 2wk 0.05% Phenobarbital Promotion on the Expression of Ha-*ras* in B6C3F1 Mouse Liver. PB-Induced changes in the gene expression of Ha-*ras* at 2wks in B6C3F1 mice as detected by real-time PCR are expressed as fold change over control. Six control animals and 5 PB treated animals were assayed. Treatment did not increase the expression of Ha-*ras* at 2wks.

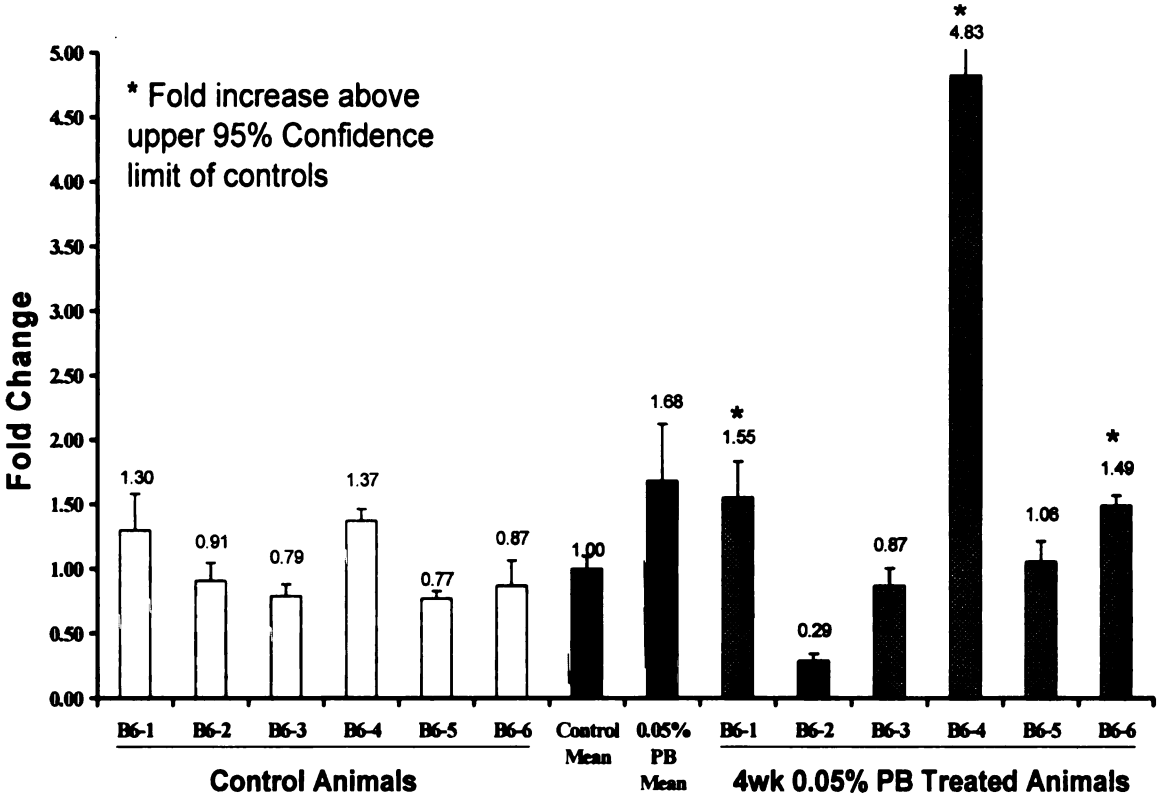


Figure 7. Effect of 4wk 0.05% Phenobarbital Promotion on the Expression of Ha-*ras* in B6C3F1 Mouse Liver. PB-Induced changes in the gene expression of Ha-*ras* at 4wks in B6C3F1 mice as detected by real-time PCR are expressed as fold change over control. Six control animals and 6 PB treated animals were assayed. Increases in Ha-*ras* expression which exceeded the upper limit of the 95% confidence interval of the control animals are noted with an asterisk (*).

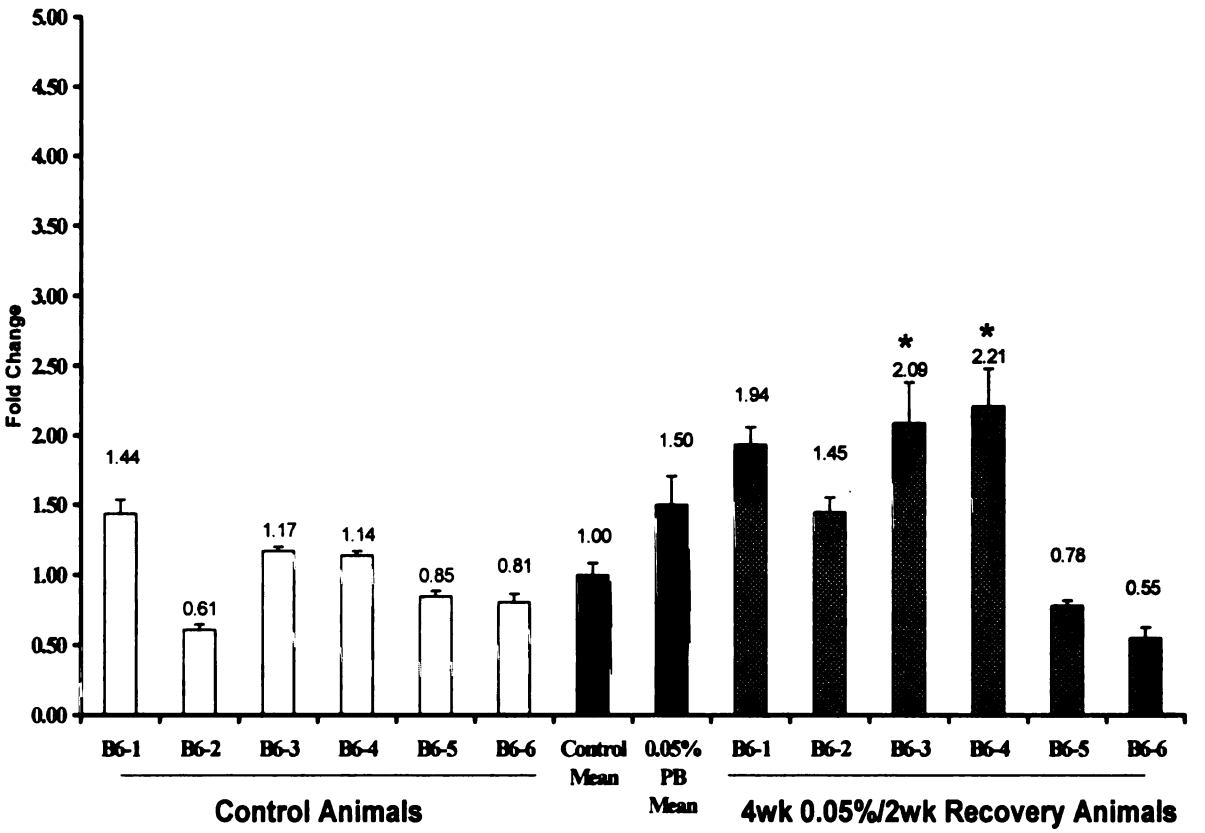
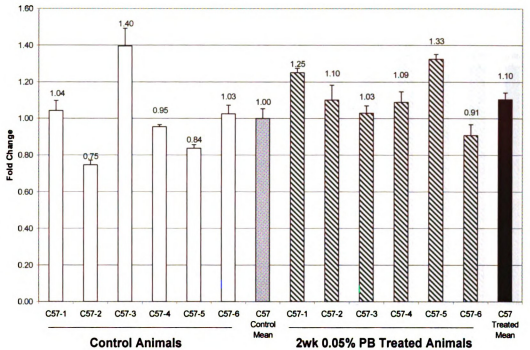


Figure 8. Reversal of Increased expression following 4wk Phenobarbital Exposure in B6C3F1 Mouse Liver. PB-Induced changes in the gene expression of Ha-*ras* following 4wks PB treatment and a 2wk recovery period in B6C3F1 mice was detected by real-time PCR and expressed as fold change over control. Six control animals and 6 treatment/recovery animals were assayed. Increases in Ha-*ras* expression which exceeded the upper limit of the 95% confidence interval of the control animals are noted with an asterisk (*).

A.



B.

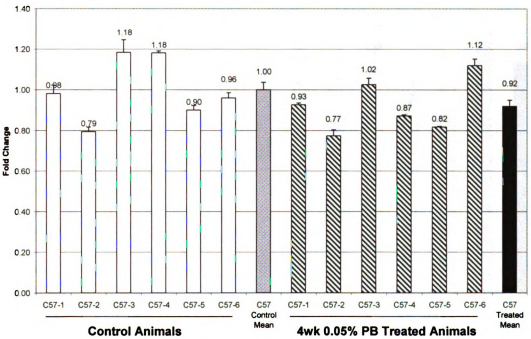


Figure 9. Effect of 2 and 4wk Phenobarbital on the Expression of Ha-ras in C57BL/6 Mouse Liver. PB-Induced changes in the gene expression of Ha-*ras* at 2 wks (a) and 4wks (b) in C57BL/6 mice was detected by real-time PCR and expressed as fold change over control. Six control animals and 6 PB treated animals were assayed at each time point. Treatment did not increase the expression of Ha-*ras* at 2 or 4wks.

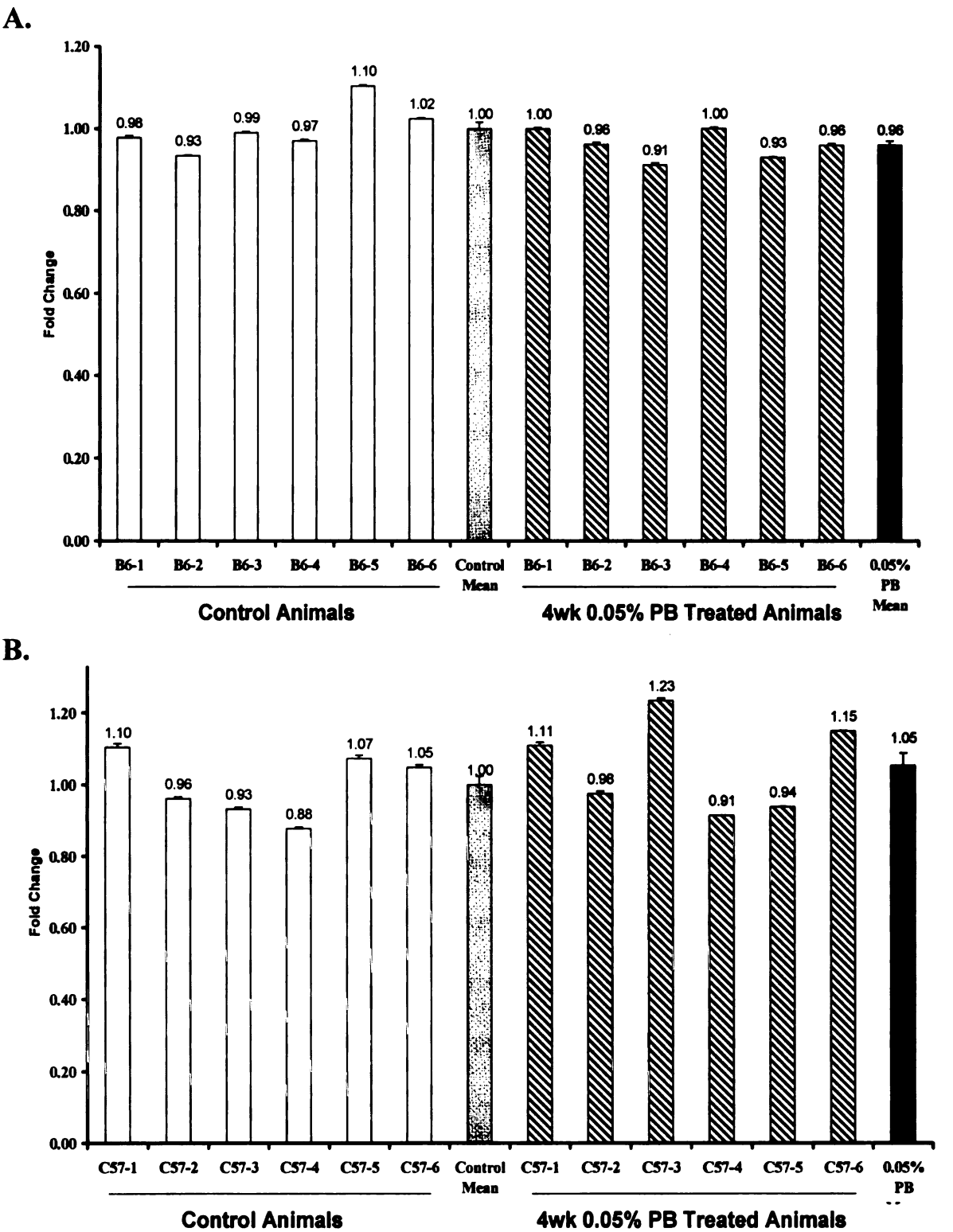


Figure 10. Effect of 4wk Phenobarbital Promotion on the Expression Level of LINE-1 Elements in B6C3F1 and C57BL/6 Mouse Liver. PB-Induced changes in the gene expression of LINE-1 elements at 4 wks in B6C3F1 (a) and C57BL/6 (b) mice was detected by real-time PCR and expressed as fold change over control. Six control animals and 6 PB treated animals were assayed. Treatment did not increase the expression of LINE-1 elements at 4wks in either B6C3F1 or C57BL/6 mouse liver.

DISCUSSION

The initiation stage of tumorigenesis involves an irreversible alteration of the genome via a mutation or possibly epigenetic event (Dragan *et al.*, 1993; Goodman and Watson, 2002). The cells that acquire the first critical initiating event(s) could, theoretically, be derived from stem cells, early stem cell progenitor cells, or differentiated cells (Bjerkvig *et al.*, 2005). These cells which have acquired a growth advantage over neighboring cells within the context that they might be selected for and clonally expand during promotion; thus, tumorigenesis involves the progressive, clonal expansion of cell populations which accumulate heritable changes to their genomes leading to them becoming increasingly abnormal (Nowell, 1976). Cell self-renewal is an important feature of the liver as evidenced by the regenerative response hepatocytes following partial hepatectomy, i.e., the hepatocyte is a differentiated cell that remains capable of replication (Fausto, 1997). Adult mouse liver cells (i.e. mature hepatocytes) transplanted into transgenic mice have the capacity to divide at least 12 times (Tateno and Yoshizato, 1996). This capacity for cell proliferation indicates that hepatocytes themselves can be the functional stem cells of the liver (Forbes *et al.*, 2002). It follows that hepatocytes might also serve as the progenitor cells for liver tumors, and this is not incompatible with a role for stem cells, too. Thus, the production of altered methylation in hepatocytes and the progressive accumulation of these heritable epigenetic changes might contribute to hepatocarcinogenesis

During DNA replication stable patterns of methylation are mainly dependent on the maintenance methyltransferase, DNMT1. However, methylation is also regulated by *de novo* methyltransferases (e.g. Dnmt3a and 3b), demethylases (Pradhan and Esteve,

2003), and the availability of both SAM and methyl groups (Zeisel, 1996). Altered patterns of methylation, specifically hypomethylation, might arise when the levels of Dnmt1 and/or SAM are low in replicating cells. Alternatively, aberrant activity of demethylases and de novo methylases could induce hypo- and hypermethylated states in quiescent cells. In this context, the activity of PB could disrupt multiple factors. One mediator of PB responses is the nuclear receptor CAR (constitutive active/androstane receptor). This relationship is important to consider in that CAR knockout mice do not develop eosinophilic foci or advanced liver tumors when promoted with PB (Yamamoto et al., 2004). Still, of 138 hepatic genes increased or decreased by PB only about 50% are regulated by CAR which implies that PB has numerous CAR-independent actions (Ueda *et al.*, 2002).

The actions of PB result in complex patterns of altered methylation in many if not all of the cells in the liver, with multiple unique changes produced in the hepatic DNA of liver tumor-prone B6C3F1 mice. However, within individual hepatocytes, these changes are not simply random as demonstrated by the fact that PB-induced altered methylation was detected within the promoter region of the Ha-*ras* oncogene whereas the methylation status of LINE-1 elements, a very large portion of the genome (~33%), was unaffected over the course of treatment with PB. Indicating that normal patterns of methylation are maintained in a more stable fashion in some portions of the genome as compared to others. Importantly, the fact that methylation of LINE-1 elements, a portion of the "junk DNA" which makes-up approximately 30% of the genome did not change indicates that the PB-induced altered methylation detected over a four wk course of treatment was concentrated within the pool of "regular" genes. When this occurs within critical genes,

e.g., those controlling portions of genes that regulate cell proliferation, it could facilitate their aberrant expression, providing the cells affected in this fashion with a growth advantage. Thus, the accumulation of aberrant RAMs, particularly those which progress during early times after the start of PB treatment, might be involved in initiation and/or promotion of tumorigenesis. The progressive accumulation of changes during PB promotion was substantiated by the fact that 17% of the RAMs in B6C3F1 mice carried forward from 2 to 4 weeks. Strikingly, less than 1% of the C57BL/6 RAMs carried forward (Figure 1b). These RAMs which carried forward likely represent the aforementioned critical epigenetic changes which contribute to the clonal proliferation of subsets of initiated cells. C57BL/6 PB-induced RAMs were used as the "control" comparison to PB-induced RAMs in B6C3F1 mice at 2 and 4wks. At 2wks 12 RAMs were in common and equivalent between B6C3F1 and C57BL/6. By subtracting out these particular RAMs 57 RAMs (43 hypomethylations, 3 hypermethylations, and 11 new methylations) were identified as unique to the B6C3F1 mice. Comparison at 4wks, identified 86 RAMs (48 hypomethylations, 10 hypermethylations, and 28 new methylations) unique to B6C3F1 mouse liver. A comparison of the 86 unique RAMs in B6C3F1 liver to RAMs in the kidney further refined the number of unique RAMs to 81, in that only 5 new methylations were in common and equivalent to kidney. These 81 RAMs, specific to B6C3F1 mouse liver are likely critical to the development of tumors. In addition, they could be important in defining the inherent susceptibility of the B6C3F1 mice to liver tumorigenesis. The ability to associate each unique RAM with a specific gene will be key to addressing this issue and is presently being pursued.

Tumors arising from promotion with PB have defined characteristics. Specifically, activation of Ha-*ras* via point mutation is a not a common feature of PB-induced tumors (Fox *et al.*, 1990), but is frequently seen in spontaneously arising tumors in B6C3F1 mice (Maronpot *et al.*, 1995). In addition, the down regulation of TGF-β, a cell cycle inhibitor, is dependent on promotion with PB (Reisenbichler *et al.*, 1994). Pre-TGF-β is metabolized to its active form by the mannose 6-phosphate/insulin growth factor II receptor (M6P-IGF2r) and, therefore, the gene encoding M6P-IGF2r can be viewed as a tumor suppressor gene (DeSouza *et al.*, 1995). M6P-IGF2r is an imprinted gene in rodents, but is biallelically expressed in humans (Xu *et al.*, 1993), which is regulated by methylation. Methylation of a CpG site in an intron of the expressed allele is the imprinting single which is required, in addition to hypomethylation of the 5' flanking region, for its normal expression (Stoger *et al.*, 1993). Two key observations of the current study are directly related to these findings: 1) PB induces both increases and decreases of methylation in GC-rich regions, and 2) PB-induced hypomethylation of the Ha-*ras* gene was associated with selective increases in gene-expression. At 4wks, PB induced 48 unique hypomethylated RAMs in addition to 10 unique hypermethylated RAMs and 23 new methylations in the B6C3F1 mice. The simultaneous hypermethylation of tumor suppressor genes and hypomethylation of oncogenes could facilitate the formation of tumors. For instance, either hypermethylation of the promoter region of the M6P-IGF2r gene or hypomethylation leading to removal of the imprinting signal could silence this "tumor-suppressor" and lead to decreased levels TGF-β which would remove the growth inhibition and enhance cell proliferation. In this context, it is instructive to a recent report indicating that hypomethylation leading to disruption of

imprinting can lead to tumorigenesis (Holm, *et al.*, 2005). In addition, hypomethylation might result in up-regulation of oncogenes. This has been suggested earlier for Ha-*ras* and *raf* (Ray *et al.*, 1994; Counts *et al.*, 1996) and with regard to Ha-*ras* more convincing evidence is provided in the current study.

In the working model of carcinogenesis reversibility is characteristic of tumor promotion as continued exposure to the promoting agent is necessary for progressive clonal expansion of cells exhibiting increasingly abnormal phenotypes. In light of this, reversibility of changes in methylation in response to a 2 or 4wk exposure to PB were assessed following 2wk recovery periods. The large majority of hypermethylations and new methylations were reversible however, very few hypomethylations reversed. *De novo* methylation of hypomethylated CCGG sites could restore the normal methylation pattern. However Dnmt3a and 3b prefer AT-rich flanking sequences surrounding the CpG dinucleotide (Handa and Jeltsch, 2005). Biologically, this difficulty in reversing hypomethylation could be necessary for the continued promotion of initiated cells. Global hypomethylation of CCGG sites induced by a methyl-deficient diet in rats was stable during re-exposure to a methyl-adequate diet and correlated to the persistence of altered hepatic foci (Pogribny *et al.*, 2005). In our model, progressive changes in methylation were only seen in the B6C3F1 mice; however, minimal reversibility of hypomethylated RAMs was a common feature of both the sensitive and resistant mice. This indicates that irreversible hyomethylation may be necessary but not sufficient for the continued expansion of clones of initiated cells in response to PB.

Genetic differences likely contribute to the observed divergence in the ability to maintain patterns of DNA methylation during promotion with PB. The hepatocarcinogen

sensitive locus (hcs) has been suggested to account for approximately 85% of the difference in strain susceptibilities (Drinkwater and Ginsler, 1986). Furthermore, this locus appears to affect the growth rate of pre-neoplastic foci during the promotion stage of tumor induction in a strain dependent manner (Hanigan *et al.*, 1988; Goldsworthy and Fransson-Stern, 2002). Therefore, tumor induction facilitated by altered patterns of methylation could be enhanced by the pre-existing genetic predisposition. For instance, strain differences in the activity of one or more *de novo* methyltransferase enzymes could potentially account for the extreme difference between B6C3F1 and C57BL/6 mice at 2 and 4wks. B6C3F1 RAMs with increased methylation, were few in number at 2wks (23 RAMs) but increased by 4wks (39 RAMs). This response was in direct opposition to C57BL/6 where high numbers where seen at 2wks (86 RAMs) and dramatically less at 4wks (17 RAMs). Therefore, the hcs might functionally involve a compromised ability to maintain normal methylation patterns in liver tumor-prone mice, e.g., the B6C3F1 mouse.

Methylation sensitive restriction digestion, arbitrarily primed PCR combined with capillary electrophoretic detection of PCR products is a novel approach to simultaneously measuring increases, decreases, and new methylations in multiple GC-rich regions throughout the genome. GC-rich regions, including both G + C rich and CpG island regions are closely associated with gene promoters. CpG islands are short stretches of DNA, at least 200bp in length, possessing 50% or greater GC content and a higher proportion of CpG dinucleotides than expected (Gardiner-Garden and Frommer, 1987). Basically, three complementary sets of analyses were performed: RsaI/HpaII, RsaI/MspI and BfaI/BssHII. Overall, altered methylation at ~7.45% of all CpG dinucleotides

estimated for the mouse genome is detected by the 4-base cutter isoschizomers, MspI and HpaII which complement each other's sensitivity to inhibition by methylation within their common recognition sequence (Frazarri, and Greally, 2004). Analysis of CpG islands is more directly targeted by using the BfaI/BssHII combination. The 6-base recognition site of BssHII is rare, as compared to the 4-base recognition site of MspI and HpaII, and tightly associated with CpG islands (Shiraishi *et al.*, 1995). Furthermore, concerning the non-methylation sensitive restriction enzymes employed there are fewer restriction sites within CpG islands for BfaI as compared to RsaI (Shiraishi *et al.*, 1995). With each analysis, an increase in the total number of RAMs from 2 to 4 wks in B6C3F1 mouse was identified (Tables 1 and 6). In addition, less RAMs were observed in the C57BL/6 mice at 4wks. Therefore, these restriction enzyme combinations are complementary and reinforce the notion that our method is capable of providing insight regarding the methylation status of the genome.

The differential ability to maintain patterns of DNA methylation is hypothesized to contribute to the variable susceptibilities of B6C3F1 and C57BL/6 mice. As an extension of previous reports (Counts *et al.*, 1996; Watson and Goodman, 2002), we have demonstrated that patterns of methylation are more susceptible to disruption in the sensitive mouse. Importantly a large proportion of the regions of altered methylation in B6C3F1 mouse liver carried forward in comparison to C57BL/6. This is a highly significant observation and shows that B6C3F1 mice accumulate changes much quicker and earlier than C57BL/6 which is in direct agreement with their relative sensitivities to tumor formation. This is strong experimental evidence which indicates that the progressive accumulation of heritable changes are key to the promotion stage and

facilitate tumorigenesis. Gene-specific analysis in B6C3F1 mice revealed a non-random pattern of altered methylation in which Ha-*ras* hypomethylation correlated to selective increases in gene expression and the heavily methylated LINE-1 elements were unaffected by PB. Collectively, these data indicate that: 1) the progressive, non-random changes in methylation play an important role in tumorigenesis; 2) altered DNA methylation is an epigenetic mechanism underlying the ability of PB to cause liver tumorigenesis; and 3) susceptibility to tumorigenesis is related inversely to the capacity to maintain normal patterns of methylation.

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CHAPTER 2 APPENDIX

This appendix contains additional research results that are complementary to the body of data presented in Chapter 2. The materials and methods needed to extend the analysis and detection of altered methylation in response to promotion with PB in the promoter region of the Ha-*ras* gene is provided. Results are presented along with a brief discussion of the findings and relevant references.

MATERIALS AND METHODS

Gene-Specific Methylation Analysis: Methylation Sensitive Restriction Digestion of Ha-ras

Restriction Digests

DNA was restricted with SmaI or ApaI endonuclease (Invitrogen), methylation sensitive enzymes which recognize the CCCGGG and GGGCCC sequence, respectively. SmaI restricts DNA only if the cytosine immediately 5' to guanine is not methylated. ApaI will not restrict the DNA if the cytosine immediately 3' to guanine is methylated. The target region will be amplified with subsequent PCR only if these target sites are methylated. Each reaction was composed of 1ug DNA, 3 units SmaI or ApaI enzyme, 1X React4 Buffer (Invitrogen), and GDW to a final volume of 10ul. Negative control digests (i.e. SmaI/ApaI was omitted) for each animal were also prepared. Reactions included, 1ug DNA, 1X React4 Buffer and GDW to a final volume of 10ul. All samples were incubated at 37^0C for 16hours.

PCR Amplification

PCR was carried out on digested (i.e. incubated with SmaI/ApaI enzyme (+SmaI/ApaI)) or undigested (i.e. incubated without SmaI/ApaI enzyme (-SmaI/ApaI)) DNA. Forward (5' CAG GGT GGA GGC TCT GTA GT 3') and reverse (5' GAG AGG AGC AAG GAA GCA CC 3') primers (Okoji *et al.*, 2002) amplify the –325 to +200 region spanning the transcriptional start site of the Ha-*ras* gene which contains two SmaI restriction sites and 1 ApaI restriction site. Reactions consisted of 1X Failsafe Buffer H (Epicentre Technologies), 2uM each primer, 0.43ug digested or undigested DNA, and GDW to a

final volume of 25ul. Cycling conditions were: 80^0C for 5min, 94^0C for 2min, 24 cycles of 96^0C for 1min, 65^0C for 1min, and 72^0C for 2min, followed by 1 time delay cycle of 72^0C for 5min. PCR products were electrophoresed (3% agarose gel) and visualized by ethidium bromide staining. A Polaroid picture of each gel was taken, scanned and the image was analyzed using the NIH image program. The number of PCR cycles (24) was chosen following a pilot study in which 21, 23, and 25 cycles were tested. The chosen number of cycles was estimated to maximize the difference in PCR product band intensities between the undigested control DNA and the digested DNA. Because a heterogeneous mixture of cells possibly expressing varying degrees of methylation were sampled, increases and decreases in band intensity compared to control is proportional to increases and decreases in methylation of the target restriction sites.

Quantification of Band Intensity

The relative intensity of a PCR product band corresponds to the relative starting concentration of methylated DNA. Each target PCR product band was outlined and measured for pixel number and intensity by using the NIH image program (http:// rsbi.info.nih.gov.). The number of pixels defined the size of the outlined region. The same sized region was used to measure both the PCR product band and the lane background. Total pixel intensity units (TPI) were calculated by multiplying the number of pixels by the mean intensity units within the outlined region. This was done separately for both the background and PCR product band. The TPI units of the background was subtracted from the TPI units of the PCR product band to give a normalized TPI for the outlined region within a lane.

Calculation of Ratios and 95% Confidence Interval (CI)

TPI units were calculated for PCR product bands resulting from amplification of either digested (+SmaI/ApaI) or undigested (-SmaI/ApaI) DNA. For each animal, a ratio was calculated to determine the amount of PCR product formed from digested DNA versus undigested DNA. For this, a ratio of the TPI units from +SmaI/ApaI DNA to TPI units from –SmaI/ApaI DNA was calculated. The mean (n=5) ratio with standard deviation and 95% CI (α=0.05) was calculated for the control animals only. Treated animal ratios were compared individually to the control 95% CI. If a ratio from a treated animal fell above the upper limit of the CI, the internal cytosine within the SmaI/ApaI sites was considered to be hypermethylated. If a ratio from a treated animal fell below the lower limit of the CI, the internal cytosine within the SmaI/ApaI sites was considered to be hypomethylated.

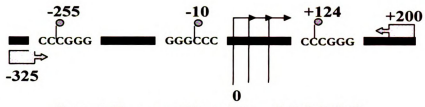
RESULTS

Due to the fact that the upstream 5' promoter region of Ha-*ras* exhibited hypomethylation at both 2 and 4 weeks only in the B6C3F1 mouse, we wanted to investigate the methylation status of CpG sites close to the transcriptional start site. This was achieved via methylation sensitive restriction digestion with SmaI or ApaI endonuclease. Appendix Figure 1a outlines the target 525bp region which spanned the transcriptional start site of the Ha-*ras* gene and contained two SmaI CCCGGG recognition sites and 1 ApaI GGGCCC recognition site. In B6C3F1 mice, the percent of animals exhibiting hypomethylation at the two SmaI sites progressed from 0% to 40%

while hypermethylation was seen to decreased from 33% to 20% with 2 and 4 wks of PB promotion, respectively (Appendix Figure 1b). The incidence of hypomethylation in a group of 6 C57BL/6 mice remained largely the same at 2 and 4wks and hypermethylation increased slightly with 4wks PB (Appendix Figure 1b). The ApaI recognition site is located very near (-10nt) to the transcriptional start site (Appendix Figure 1a). Methylation at this site was generally maintained in the B6C3F1 mice from 2 to 4wks. Low incidences of hypo- and hyper-methylation were observed. Interestingly, the C57BL/6 mice were considerably hypomethylated at 2wks, but by 4wks, the incidence of hypermethylation increased to 100% (Appendix Figure 1c).

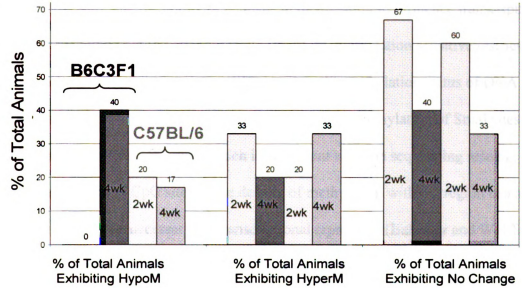
Appendix Figure 1 Analysis of Altered Methylation Near to the Transcriptional Start Site of Ha-ras in B6C3F1 and C57BL/6 Mice at 2 and 4wks. A 325bp region spanning the transcriptional start site of Ha-ras was amplified following digestion with either SmaI or ApaI restriction endonuclease. Recognition sites for each enzyme are diagrammed (a). An increase in the incidence of hypomethylation at the SmaI recognition sites was observed fom 2 to 4wk in B6C3F1 but not C57BL/6 mouse liver (b). By 4wks, 100% of C57BL/6 mice exhibited hypermethylation of the ApaI site while the status of methylation in B6C3F1 mouse liver DNA was unchanged (c). For each set of 4 bars in parts (a) and (b), the first two bars represent the 2 and 4wk time points respectively for B6C3F1 mice and the second two bars represent the 2 and 4 wk time points for C57BL/6 mice. Analysis was performed on DNA from 6 control and 6 treated animals at each time point

A.

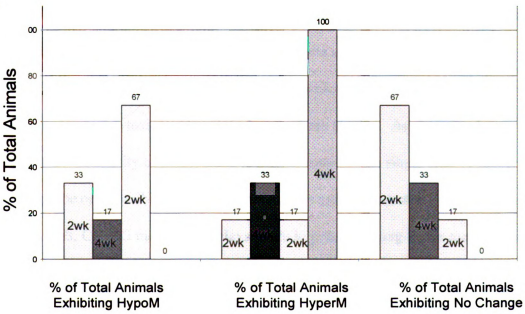


Sma I Recognition Site CCCGGG
Apal Recognition Site GGGCCC

B.



C.



DISCUSSION

The results presented here are an extension of data obtained via bisulfite sequencing of a 283bp region containing 22 CpG sites 950nt upstream of the Ha-*ras* transcriptional start site. Hypomethylation exhibited by only the B6C3F1 mouse at one possibly critical site (-1174nt) was observed. This site was consistently hypomethylated at 2 and 4wks in response to PB. Because this site was relatively far from the transcriptional start site, the ability of changes in methylation to influence transcription of the gene needed to be put in better context. Therefore, methylation sensitive restriction digestion afforded the ability to detect differences in the methylation status of DNA at three additional sites in control and treated animals. Hypomethylation of SmaI sites were observed only in the B6C3F1 mice which is consistent with the sequencing results. Decreases in particular CpG sites or the density of methylation within a region can affect the binding of proteins necessary for transcriptional repression (Ballestar and Wolffe, 2001). Digestion with ApaI revealed hypermethylation of the internal cytosine of the GGGCCC recognition sequence. Methylation of CpC sites is not a common occurrence (Dodge *et al.*, 2002). Therefore, the sequence context of this recognition site must be considered. The 5 base flanking sequences of this site are as follows: 5' AGCGC GGGCCC GGCCA 3'. Methylation of cytosines which are not directly 5' to guanine but appear in a string of cytosines, $Cp_{(x)}CpG$, has not been studied. Again, it has been proposed that the density of methylation is just as important as single sites of methylation which could be one possibility for an increase in the methylation status of this cytosine. In *Arabidopsis*, CpNpG methylation plays a role in gene silencing and is mediated by histone H3 lysine 9 methylation through interaction of the DNA methyltransferase gene

with methylated chromatin (Jackson *et al.*, 2003). Therefore, both the increased incidence of hypomethylation observed at the SmaI only in the sensitive mice coupled to the complete hypermethylation of the non-CpG site in the C57BL/6 mice supports a role for activated Ha-*ras* in the facilitation of tumorigenesis and the underlying susceptibility of the B6C3F1 mice.

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CHAPTER 3

ALTERED METHYLATION IN GENE-SPECIFIC AND GC-RICH REGIONS OF DNA IS PROGRESSIVE AND NON-RANDOM DURING PROMOTION OF SKIN TUMORIGENESIS

This chapter represents a manuscript that will be submitted to Toxicological Sciences in January, 2006. Authors include: Bachman, Ammie N., Curtin, Geoffrey M., Doolittle, David J. and Goodman, Jay I.

ABSTRACT

Altered DNA methylation, an epigenetic mechanism, likely contributes to tumorigenesis, with an inverse relationship existing between methylation in a promoter region and transcription. Using the SENCAR 2-stage mouse skin tumorigenesis model, altered methylation was characterized in precancerous tissue and in tumor tissue. Mouse skin was initiated with 7, 12-dimethylbenz[*a*] anthracene (DMBA) and promoted 3X/wk with 3, 9, 18, or 27mg cigarette smoke condensate (CSC) for 4, 8, or 29 wks; tumors were collected at 29wks. In addition, reversibility of changes in methylation were assessed following cessation of the promoting stimulus. DNA was isolated, and GC-rich methylation was assessed quantitatively via methylation sensitive restriction digestion, arbitrarily primed PCR, and electrophoretic separation of PCR products. Analysis focused on regions of altered methylation (RAMs) which persisted from 4 to 8wks and from 8wks to tumor tissue. Persistent RAMs (i.e. seen in precancerous tissue and carried forward to tumors) are likely to play a key role in tumorigenesis. Twenty-two CpG sites in an upstream region of the Ha-ras promoter were unmethylated in control skin, 27 mg CSC and tumor tissue. At 2 CpG sites closer to the transcriptional start site the incidence of hypomethylation increased with dose of CSC. Hypomethylation was detected in all tumor samples. Expression of Ha-*ras* increased with 18 and 27mg CSC promotion, and more so in tumor tissue. These data support our hypothesis that tumor promotion involves an instability of the epigenome, providing an environment where changes in the methylation status of specific regions of the genome accumulate progressively and contribute to the clonal expansion of initiated cells that leads to tumor formation.

INTRODUCTION

Cancer is characterized by six fundamental changes in cell physiology including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and the ability to invade and metastasize (Hanahan and Weinberg, 2000). These characteristics are the result of a step-wise clonal expansion of cell populations bearing accumulated heritable genetic changes which can involve the mis-regulation of critical cell cycle proteins, transcription factors and signal transduction proteins.

A central paradigm in explaining events leading to tumorigenesis involves a multistage and multi-step (e.g., multi-mechanism) model for the development of precancerous lesions and their evolution into frank carcinomas. The stages defined by this model are initiation, promotion and progression (Dragan *et al.*, 1993). During initiation, a heritable change occurs in the genome. The nature of this change can be rooted in direct mutation of the DNA sequence or it could occur by an epigenetic modification (Goodman and Watson, 2002). Exceedingly aberrant subclone populations arise from the progressive clonal expansion of initiated cells during promotion. Promoting agents likely foster the growth of initiated cells by enhancing cell proliferation or by inhibiting apoptosis (Schulte-Hermann *et al.*, 1990). Reversibility is a key characteristic of promotion (Dragan *et al.*, 1993). If the promoting stimulus is withdrawn, the altered cells possessing advantageous growth characteristics stop proliferating and altered foci may "remodel". Subsequent to the iterative nature of this process is progression in which cells clonally expand, even in the absence of the promoting stimulus, and typically exhibit marked changes in ploidy (Dragan *et al.*, 1993).

The progressive accumulation of heritable changes is fundamental to the process of carcinogenesis. A highly effective model to study this is the SENCAR 2-stage mouse skin tumorigenesis model which demarcates the stages of initiation and promotion. SENCAR mice are particularly sensitive to carcinogenesis induced by the combination of 7, 12-dimethylbenz[*a*]anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA), when utilized as initiator and promoter; moreover, this stock of mice consistently exhibits increased sensitivity for the induction of skin tumors when compared to other available strains (Hennings *et al.*, 1997; Coghlan *et al*, 2000). With this model the contribution of both mutations and, importantly, epigenetic mechanisms can be evaluated. Epigenetics is defined broadly as processes that establish heritable states of gene expression without altering the DNA sequence. Specifically, altered patterns of DNA methylation, (i.e. 5-methylcytosine content of DNA), have been shown to occur during the promotion stage of skin tumorigenesis. Previous studies in our lab showed that cigarette smoke condensate, an effective promoting agent in the 2-stage model, induced reversible dose and time-dependent changes in methylation in GC-rich regions of DNA as well as global decreases in methylation in tumor tissue (Watson *et al.*, 2003).

GC-rich regions of DNA are frequently associated with the promoter regions of genes, and the methylation status of promoter regions can be linked to the regulation of gene expression. Specifically, increased methylation in a promoter region might decrease gene expression, while a decrease in promoter methylation possesses the potential for up regulating gene expression (Jones and Laird, 1999). The silencing of tumor suppressor genes via an increase in promoter methylation has been demonstrated for genes such as O^6-methylguaunine-DNA methyltransferase, cyclin-dependent kinase inhibitor 2B, and

RASSF1A (Watson *et al.*, 2004; Jones and Baylin, 2002). In addition HoxA5 which upregulates p53 expression was shown to be hypermethylated following promotion with 27 and 36mg CSC for 9wks which was associated with a decrease in expression (Watson *et al.*, 2004). Aberrant patterns of methylation, specifically hypomethylation, may also facilitate the activation of oncogenes. Ha-*ras*, a classic oncogene, is reproducibly activated in mouse epidermal tumors (Balmain and Pragnell, 1983; Balmain, *et al.*, 1984). This has mainly been attributed to early mutation induced by initiating agents such as DMBA and dibenzo[α,l]pyrene (DB[α,l]P). However, one study suggests that mutations in codon 61 of Ha-*ras* induced by DB[α,l]P simply results in a transient proliferation of cells. Over time only a small subpopulation of these cells persist whereas the majority are lost (Khan, *et al.*, 2005). It is possible that continued upregulation of Ha-*ras* may be governed by changes in DNA methylation. In support of this assertion, decreased methylation at one XhoI site within the vicinity of the Ha-*ras* gene exhibited hypomethylation as compared to normal epidermis in some papillomas and carcinomas (Ramsden *et al.*, 1985). Based on these intriguing but limited data, further investigation of Ha-*ras* promoter methylation is necessary.

We hypothesize that progressive, non-random changes in DNA methylation contribute to tumorigenesis. SENCAR mice were initiated with DMBA and promoted with increasing doses of CSC for 4 or 8wks. The GC-rich methylation patterns were analyzed for dose- and time-relationships as well as reversibility in precancerous skin tissue via a sensitive and quantitative arbitrarily primed PCR and capillary electrophoretic approach. Regions of altered DNA methylation which persist from

precancerous to tumor tissue are identified, and changes in the methylation status of the promoter region of Ha-*ras* are associated with changes in gene expression.

MATERIALS AND METHODS

Animals

Female SENCAR mice (ages 7-10 weeks) were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, Maryland). Animals were quarantined and allowed to acclimate for a minimum of 10 days prior to being randomly assigned to treatment groups based on 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 risk level to ensure groups of similar mean body weight. Animals were housed and cared for at RJ Reynold's facilities and in accordance with the Institute of Laboratory Animals Resources (ILAR), Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Mice, 6-7 animals per group, were initiated with a single, topical application of 75 ug DMBA or acetone (vehicle control), followed by thrice-weekly applications of 27, 18, 9, or 3 mg CSC or acetone (vehicle control) promotion for 4, 8 or 29 wks, and sacrificed immediately afterwards. The recovery groups were treated with 27, 18, 9, or 3mg CSC for 8wks and allowed an 8 wk recovery period prior to sacrifice. Two additional recovery groups for mice treated with 27mg for 4 wks were allowed a 4 or 8 wk recovery period prior to sacrifice. Mice were euthanized with 70% CO_2, and skin tissue was collected from the chemical application site. Skin masses that arose during the 29wk post-initiation period were excised and identified histologically for tumor type,

number, and factors associated with whether the tumor was benign or malignant. All collected samples were snap-frozen at -80^0C and kept until analyzed.

DNA and RNA Isolation

In order to isolate DNA, frozen skin tissue or tumor tissue was pulverized using a mortar and pestle and then allowed to thaw following the addition of TRI Reagent (Sigma). A dounce homogenizer was used to thoroughly homogenize the sample. RNA was isolated according to the manufacturer's protocol. DNA isolation was carried out according to an alternative protocol obtained from the manufacturer. DNA is extracted with Back Extraction Buffer in lieu of ethanol precipitation.

Preparation of CSC

Cigarette smoke condensate (CSC) was prepared as described previously (Watson *et al.*, 2003), and doses of 27, 18, 9, and 3mg were applied to the animals 3 times/wk.

A detailed description of the methodology associated with the following can be found in the Materials and Methods Section of Chapter 2.

Reversibility of altered methylation: Calculations

Assumptions of the AP-PCR Data Analysis

Common and Unique Regions of Altered Methylation (RAMs)

Evaluating Total Regions of Altered Methylation (RAMs)

Percent Dissimilarity Calculations

Gene-Specific Methylation Analysis:

Sequencing of 5' Promoter Region of Ha-ras

SmaI Methylation Sensitive Restriction Digestion of Ha-ras

Expression of Ha-ras

Reverse Transcription of RNA

RNA samples were treated with DNaseI (Invitrogen) to purify the RNA from contaminating DNA remaining after isolation. Each reaction contained 2ug RNA, 1X DNaseI reaction buffer, 2 units DNaseI, and DEPC-treated GDW to a final volume of 20ul. Samples were incubated at room temp. for 15min followed by addition of $MgCl_2$ to a final concentration of 2.27mM. RNA was heated to 65^0C for 10min to inactivate the DNaseI enzyme. The TaqMan Reverse Transcription Kit (Applied Biosystems; Foster City, CA) was used to reverse transcribed the DNaseI treated RNA. Each reverse transcription reaction contained, 1X Reverse Transcription Reaction Buffer, 5.5mM MgCl2, 200uM of each dNTP, 2.5uM random hexamer, 20 units RNase Inhibitor, 62.5 units Multiscribe Reverse Transcriptase and DEPC-treated GDW to a final volume of 50ul. The reactions are incubated at 25^0C for 10min, 42^0C for 1hr, and 95^0C for 5 min. All samples were stored at 4^0C until needed.

Real Time PCR

A custom TaqMan assay including primers (For 5' TGG TGG GCA ACA AGT GTG A3' and Rev 5' GGC CTG CCG AGA CTC A 3') and probe (5' FAM CTG GCT GCT CGC ACT GT 3') specific for Exon 3 of the Ha-*ras* gene was purchased from Applied Biosystems. The assay was designed based on sequence information obtained from Brown *et al.*, 1988 and Neades *et al.*, 1991. In addition an Applied Biosystems custom TaqMan assay including primers (For 5' CTA CTA CCG ATT GGA TGG TTT AGT GA 3' and Rev 5' GTC AAG TTC GAC CGT CTT CTC A 3' and probe (FAM 5' CCG TGG GCC GAC CC3') was used for the control gene, 18S rRNA (Accession X00686).

Triplicate reactions for both the gene of interest (Ha-*ras*) and the control gene (18S) were prepared per sample. Standards were also prepared for 18S and Ha-*ras* and ranged from 5×10^1 copies/ul to 5×10^7 copies/ul. Each Ha-*ras* reaction contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 11ul cDNA and GDW to a final volume of 25ul. Each 18S reaction contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 11ul cDNA and GDW to a final volume of 25ul. Reactions for each standard contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 1X TaqMan Universal PCR MasterMix, 2ul standard and GDW to a final volume of 25ul. Cycling conditions were as follows: 50^0C for 2 min, 95^0C for 10min, and 40 cycles of 95^0C for 15sec. and 60^0C for 1 min. The absolute standard curve method for quantifying fold change over control was employed.

RESULTS

Analysis of GC-rich regions of DNA allowed for a genome-wide snapshot of altered methylation including hypomethylations, hypermethylations and new methylations in response to treatment. Dose-dependent changes in methylation were discerned following promotion of mouse skin with 3, 9, 18, or 27mg CSC for 8wks. In addition, tumor tissue was evaluated for aberrant patterns of methylations. During promotion, a treatment-related increase in the total number of RAMs was observed whereby the RAMs induced by 3mg and 9mg CSC were approximately half of the total RAMs induced by 18 and 27mg CSC (Table 1). Regions of increased methylation, including hypermethylations and new methylations, were predominant and clearly increased with dose (Figure 1). In contrast, regions of hypomethylation did not exhibit a dose-response relationship. Tumor tissue was somewhat distinct from precancerous tissue in that the incidence of hypermethylations and new methylations decreased while total regions of hypomethylation increased and comprised over half of the total alterations detected (Figure 1).

A comparative evaluation of the total changes in altered methylation between treatment groups allowed for the identification of developing patterns and trends which developed with increasing doses of CSC. However, in order to clearly and simply demonstrate the extent to which RAMs that developed during promotion and in tumor tissue differed from controls, a percent dissimilarity was calculated for each separate dose and for tumor tissue. Methylation patterns arising from 3 and 9mg CSC promotion were 49% and 48% dissimilar to methylation patterns of controls (Table 2). With 18mg and 27mg CSC promotion, dissimilarity increased to 70% and 66% (Table 2). This illustrates

Table 1. Summary of GC-Rich Regions of Altered Methylation: Comparison of DMBA Initiated, CSC 8wk Promotion and Tumor Tissue to Control

Treatment	Digest	Regions of Hypomethylation[a]	Regions of Hypermethylation[b]	Regions of New Methylation[c]	TOTAL
3mg CSC	HpaII	5	0	3	8
	MspI	9	0	3	12
Total[d]		**14**	**0**	**6**	**20**
9mg CSC	HpaII	2	6	6	14
	MspI	0	2	5	7
Total[d]		**2**	**8**	**11**	**21**
18mg CSC	HpaII	6	10	19	35
	MspI	1	0	2	3
Total[d]		**7**	**10**	**21**	**38**
27mg CSC	HpaII	2	0	1	3
	MspI	0	10	27	37
Total[d]		**2**	**10**	**28**	**40**
Tumor Tissue	HpaII	19	0	3	22
	MspI	3	1	11	15
Total[d]		**22**	**1**	**14**	**37**

[a] Hypomethylated RAMs include both statistically significant (Student's *t*-test, $p<0.05$) decreases and 100% decreases.

[b] Hypermethylated RAMs are only those increases which are statistically significant (Student's *t*-test, $p<0.05$).

[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

[d] Total RAMs including hypomethylations, hypermethylations, and new methylations for the RsaI/MspI and RsaI/HpaII digests combined are reported for each treatment.

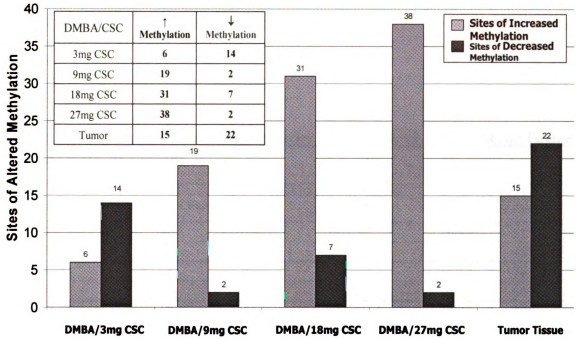


Figure 1 Increases (Hypermethylations and New Methylations) in GC-Rich Methylation are Dose-Dependent Regions of altered methylation are categorized as increased methylation and decreased methylation. Increases in methylation exhibit a clear dose-response relationship. Tumor tissue is shown for comparison. A table tallying the regions of altered methylation for each dose and tumor tissue is shown as an inset in the chart. Regions of altered methylation exhibiting increased methylation include all hypermethylations and new methylations. Hypermethylations are increases that are significantly different from control values (Student's t-test, $p<0.05$). Regions of altered methylation exhibiting decreased methylation include all partial hypomethylations and complete hypomethylations (100% decreases from control). Partial hypomethylations are decreases that are significantly different from control values (Student's t-test, $p<0.05$).

Table 2. Measure of the Percent Dissimilarity of 8wk, 3, 9, 18, 27mg CSC or Tumor to DMBA/Acetone Control

	Total Number of Regions[a]	Total Regions of Altered Methylation[b]	Percent Dissimilarity to DMBA/Acetone Control	
3mg CSC	39	20	20/39	**51%**
9mg CSC	44	21	21/44	**48%**
18mg CSC	54	38	38/54	**70%**
27mg CSC	61	40	40/61	**66%**
Tumor	47	37	37/47	**79%**

[a] The total number of regions includes every PCR product size reporting between control and treated groups. Each PCR product represents a region of the genome.
[b] Total Regions of Altered Methylation represents the number of regions exhibiting complete hypomethylation (100%), partial hypomethylation, hypermethylation or new methylation.

that patterns of methylation become more abnormal as the dose of CSC increases from 9 to 18mg CSC. As expected, the status of methylation in tumor tissue was the most dissimilar (79%) to control (Table 2).

Dose-dependent changes in methylation were consistent with tumor incidence reported for each dose of CSC. At 29wk, 297 total tumors arose due to 27mg CSC promotion (Figure 2). Just under 200 total tumors were induced by 18mg CSC promotion and 79 total tumors were seen with 9mg CSC promotion; 3mg tumor promotion did not increase tumor incidence above that of control (Figure 2). Therefore, tumor number could reflect, in part, the extent of altered patterns of methylation and the increasing dissimilarity of patterns as the dose of CSC is increased.

With promoter stimulation, an accumulation of changes in methylation could occur over time. Time-dependent changes in methylation were tracked following promotion of mouse skin with 27mg CSC for 4wks and 8wks. Total RAMs increased from 21 at 4 weeks to 40 at 8 weeks where most changes were attributable to hypermethylations and new methylations (Table 3). This supports the notion that the number of changes in methylation increase and accumulate with time.

A unique feature of the arbitrarily primed PCR, capillary electrophoresis approach described is that it allows methylation changes within particular regions of the genome to be directly compared over time. Changes in methylation induced by 4wk 27mg CSC promotion were compared to those induced by 8wk, 27mg CSC promotion. Common RAMs and unique RAMs were identified (Figure 3a). Common RAMs exhibiting equivalent changes in methylation persisted from 4wk to 8wk. The persistent, "carry forward," RAMs included 1 hypomethylation, 3 hypermethylations, and 10 new

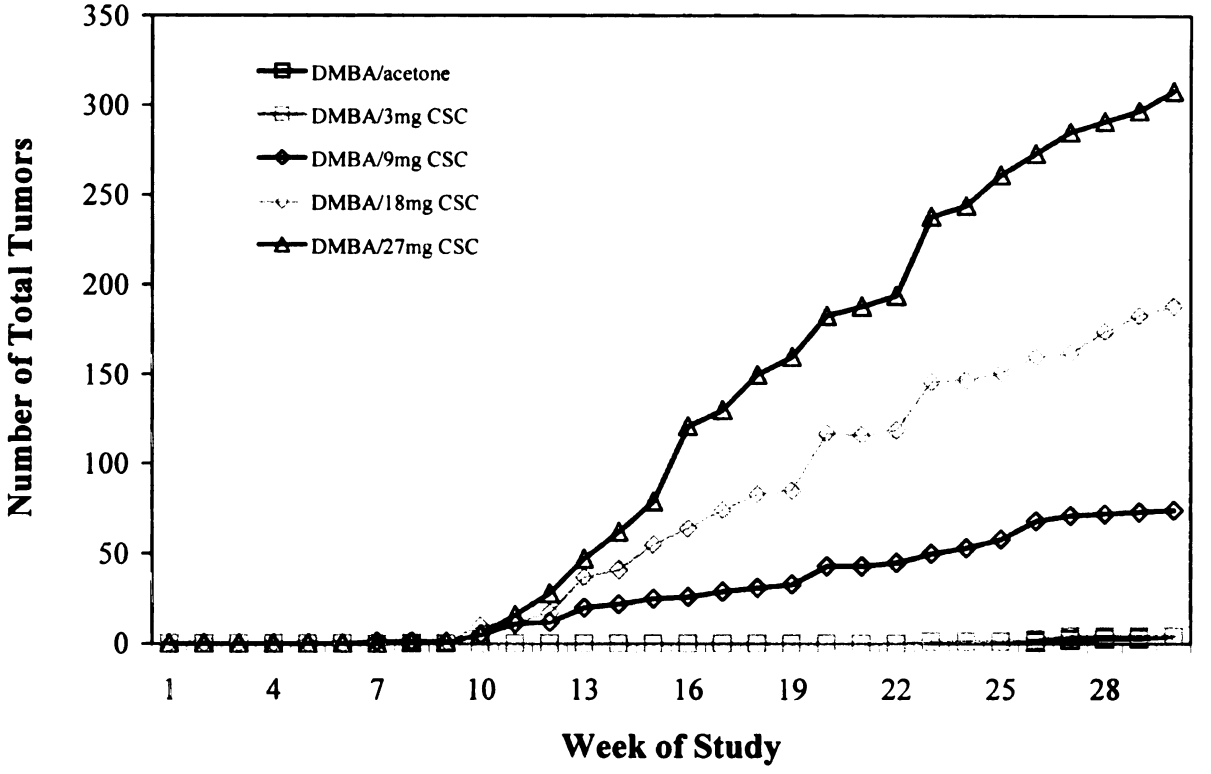


Figure 2 Tumor Incidence Increases with Dose of CSC Tumor incidence for animals initiated with DMBA and promoted thrice weekly with 3, 9, 18, or 27mg CSC for 29wks is shown. Total number of tumors from 40 animals in each dosing group are expressed over time.

Table 3. Summary of GC-Rich Regions of Altered Methylation: Comparison of DMBA Initiated, CSC 4 and 8wk Promotion to Control

Treatment	Digest	Regions of Hypomethylation[a]	Regions of Hypermethylation[b]	Regions of New Methylation[c]	TOTAL
4wk Promotion					
27mg CSC	HpaII	1	2	2	5
	MspI	0	3	13	16
Total[d]		**1**	**5**	**15**	**21**
8wk Promotion					
27mg CSC	HpaII	2	0	1	3
	MspI	0	10	27	37
Total[d]		**2**	**10**	**28**	**40**

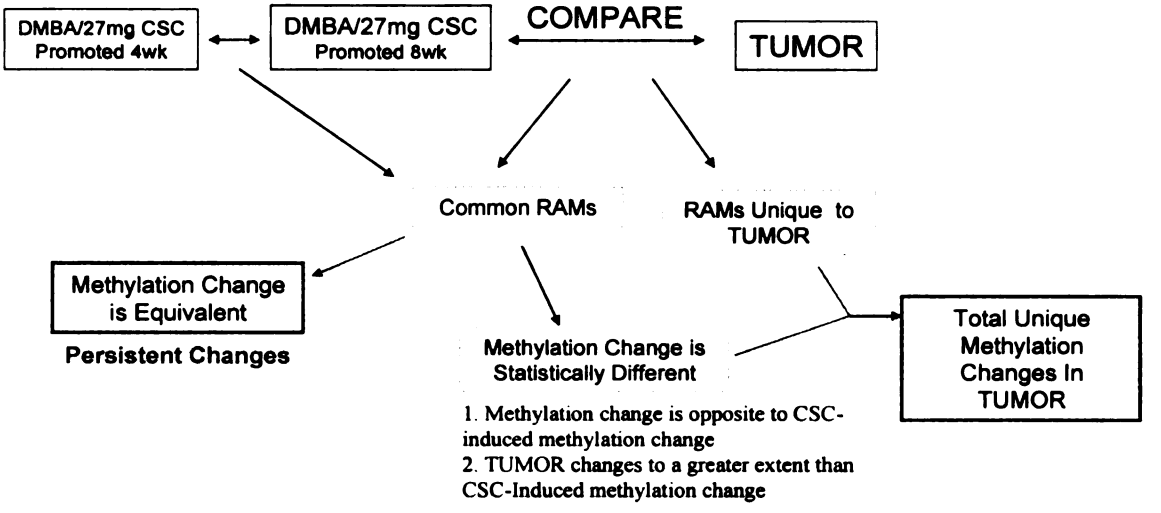
[a] Hypomethylated RAMs include both statistically significant (Student's *t*-test, $p<0.05$) decreases and 100% decreases.

[b] Hypermethylated RAMs are only those increases which are statistically significant (Student's *t*-test, $p<0.05$).

[c] New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

[d] Total RAMs including hypomethylations, hypermethylations, and new methylations for the RsaI/MspI and RsaI/HpaII digests combined are reported for each treatment

A.



B.

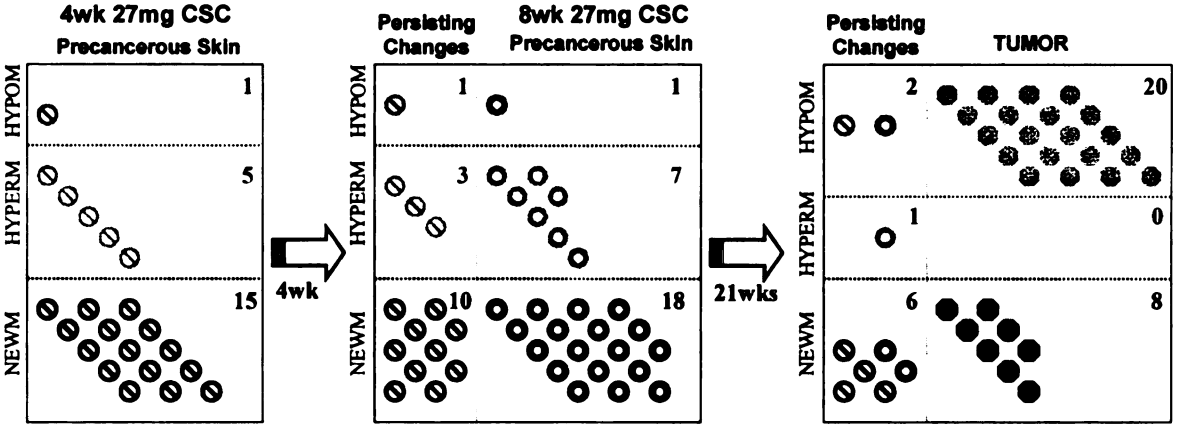


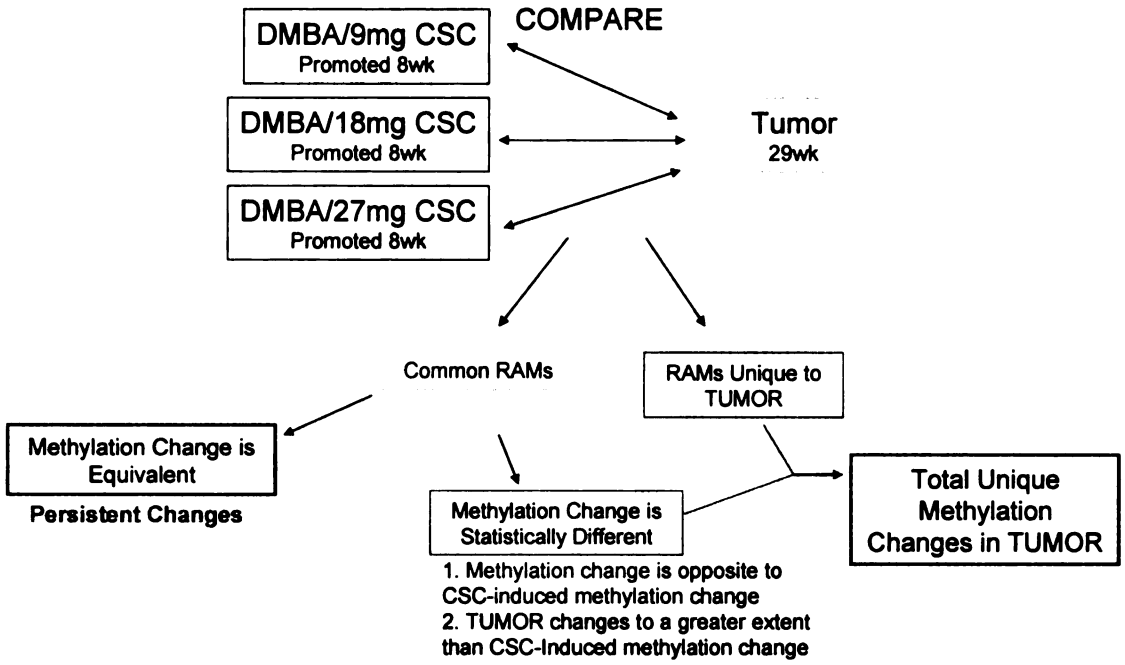
Figure 3 Progressive Changes in Methylation: Changes Which Persist From 4 to 8wk and from 8wks to Tumor A flow chart illustrates the steps necessary to determine progressive changes in methylation (a). Regions of altered methylation (RAMs) induced by 4wk, 27mg CSC promotion were compared to those resulting from 8wk, 27mg CSC promotion. Common regions of altered methylation in which the magnitudes of change were equivalent (2-way ANOVA, $p<0.05$) were considered persistent changes. RAMs unique to 8wk, 27mg CSC included common RAMs in which the magnitude of change was different or the RAMs were only observed with 8wk, 27mg CSC. Similarly, RAMs induced by 8wk, 27mg CSC promotion were compared to those in tumor. Common regions of altered methylation in which the magnitudes of change were equivalent (2-way ANOVA, $p<0.05$) were considered persistent changes. RAMs unique to tumor included common RAMs in which the magnitude of change was different or the RAMs were only observed in tumor tissue. Regions of altered methylation in which the magnitude of change was different included common regions in which the changes in methylation were opposite in direction and common regions in which the change was in the same direction, but the magnitudes of change were statistically different (2-way ANOVA, $p<0.05$). Persistent and unique RAMs induced by 4wk, 27mg CSC (Θ), 8wk, 27mg CSC (○), and tumor (hexagons) are represented (b). Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are segregated. Total unique changes (minus any persistent changes) are tallied and reported for each category of methylation change.

methylations (Figure 3b). A further comparison was made between 8wk, 27mg CSC-induced methylation changes and those identified in tumor tissue (29wk). We were able to categorize the 9 persisting changes into two groups 1) those that persisted from 8 to 29wks and 2) those that originated with 4wk promotion and persisted through the 8wk time point to the tumor tissue (Figure 3b). Of the 9 persisting changes 1 of the 2 hypomethylations, and 4 of the 6 new methylations originated from the 4wk, 27mg CSC promotion (Figure 3b). This step-wise accumulation of RAMs over time as precancerous skin is promoted with 27mg CSC and progresses to tumor tissue likely represents critical changes in methylation.

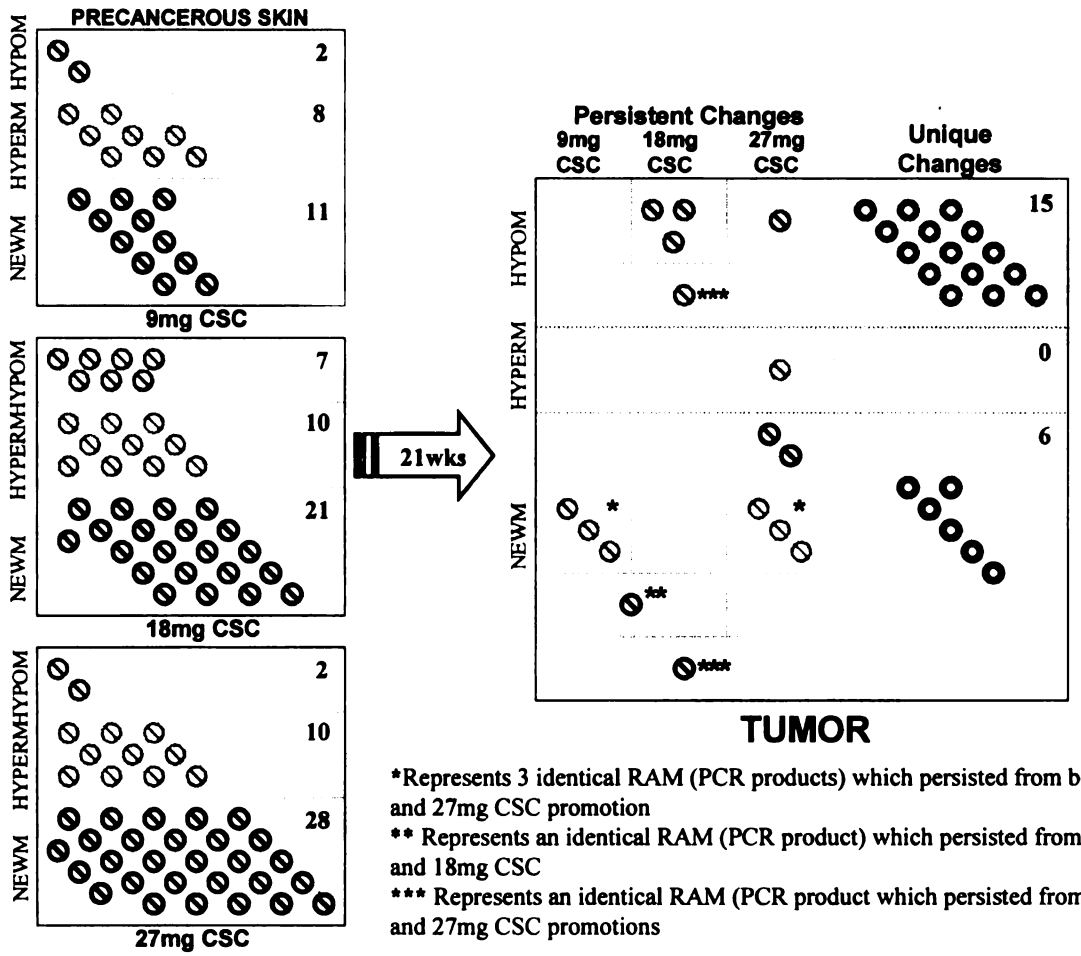
RAMs exhibited by the tumor tissue were a compilation analysis of 9 tumors; 3 tumors each arose from the 9, 18, and 27mg CSC promotion. Common and equivalent RAMs (persisting changes) were identified by separately comparing 8wk, 9, 18, and 27mg CSC-induced changes to tumor tissue. Persisting changes in methylation originating from 9, 18, and/or 27mg CSC are depicted in Figure 4b along with the unique changes exhibited by the tumor tissue. As expected, the highest number of critical changes in methylation which carried through to tumor was seen with 27mg CSC. Of the 13 total persistent changes in tumor, 4 (1 hypomethylation, 1 hypermethylation, and 2 new methylations) were solely attributable to the 27mg CSC promotion and 3 hypomethylations were solely attributable to the 18mg CSC promotion. Notably, 1 hypomethylation and 1 new methylation induced by all three promoting doses persisted to the tumors. Each dose of CSC elicited changes in methylation that persisted and could contribute to the altered pattern of methylation observed in tumor tissue (Figure 4a and b).

Figure 4 Progressive Changes in Methylation: Changes Induced by 8wk, 9, 18, 27mg CSC and Persist to Tumor A flow chart illustrates the steps necessary to determine progressive changes in methylation (a). Regions of altered methylation (RAMs) induced by 8wk, 9, 18 and 27mg CSC promotion were compared to those identified in tumor tissue. Common regions of altered methylation in which the magnitudes of change were equivalent (2-way ANOVA, $p<0.05$) were considered persistent changes. RAMs unique to tumor included common RAMs in which the magnitude of change was different or the RAMs were only observed in tumor tissue. Regions of altered methylation in which the magnitude of change was different included common regions in which the changes in methylation were opposite in direction and common regions in which the change was in the same direction, but the magnitudes of change were statistically different (2-way ANOVA, $p<0.05$). Persistent and unique RAMs induced by 8wk, 9,18, or 27mg CSC (Θ) and tumor (hexagons) are represented (b). A total of 6 RAMs each persisted from 9 and 18mg CSC to tumor while 9 RAMs persisted from 27mg CSC to tumor. Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are segregated for each treatment. Total unique changes (minus any persistent changes) are tallied and reported for each category of methylation change. (*) Represents 3 identical RAMs which persisted from both 9 and 27mg CSC promotion

A.



B.



*Represents 3 identical RAM (PCR products) which persisted from both 9 and 27mg CSC promotion

** Represents an identical RAM (PCR product) which persisted from from 9 and 18mg CSC

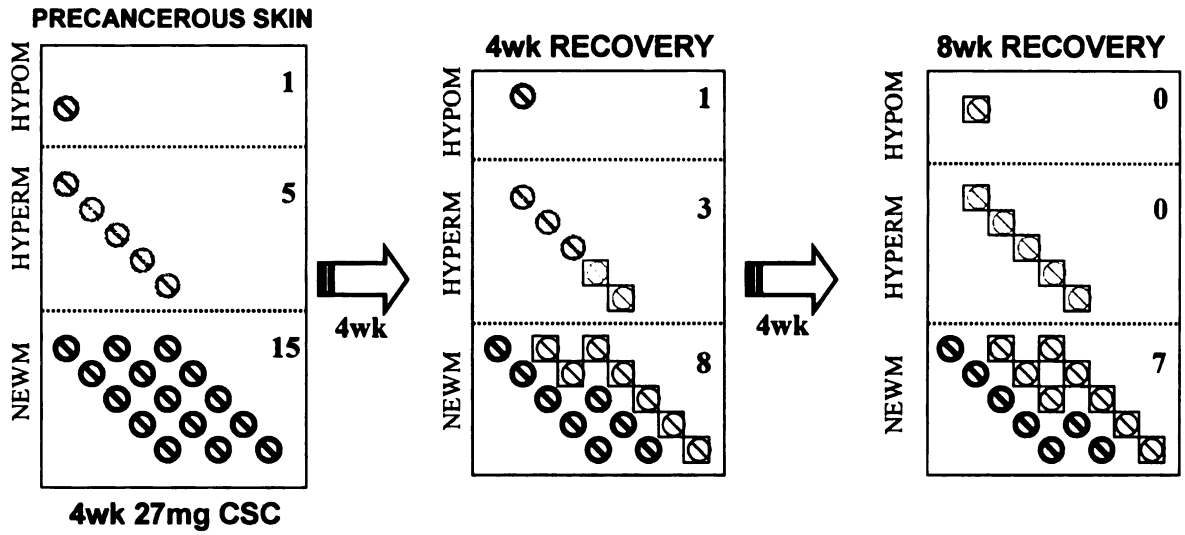
*** Represents an identical RAM (PCR product which persisted from 9, 18, and 27mg CSC promotions

Reversibility, as an operational definition, is characteristic of tumor promotion as continued exposure to the promoting agent is necessary for progressive clonal expansion of initiated cells. In light of this, changes in methylation were assessed following a 4 or 8wk recovery period. Specifically, RAMs previously induced by 4 or 8wk 27mg CSC promotion were re-analyzed following the recovery period. Reversal of the methylation changes induced by 4wk, 27mg CSC was more complete as the duration of recovery was lengthened. Following 4wk recovery, 2 hypermethylations and 7 new methylations had reversed (Figure 5a). With 8wk recovery, an additional 5 RAMs had reversed (Figure 5a and b). In comparison, 8 wk promotion with 27mg CSC followed by 8wk recovery resulted in the reversal of 1 of 2 hypomethylations, 10 of 10 hypermethylations, and 24 of 28 new methylations (Figure 5b). Importantly, all changes, with the exception of 1 new methylation, which persisted from the 4wk promotion, were recoverable which clearly demonstrates that changes in methylation which accumulate in response to the promoting stimuli are largely reversible.

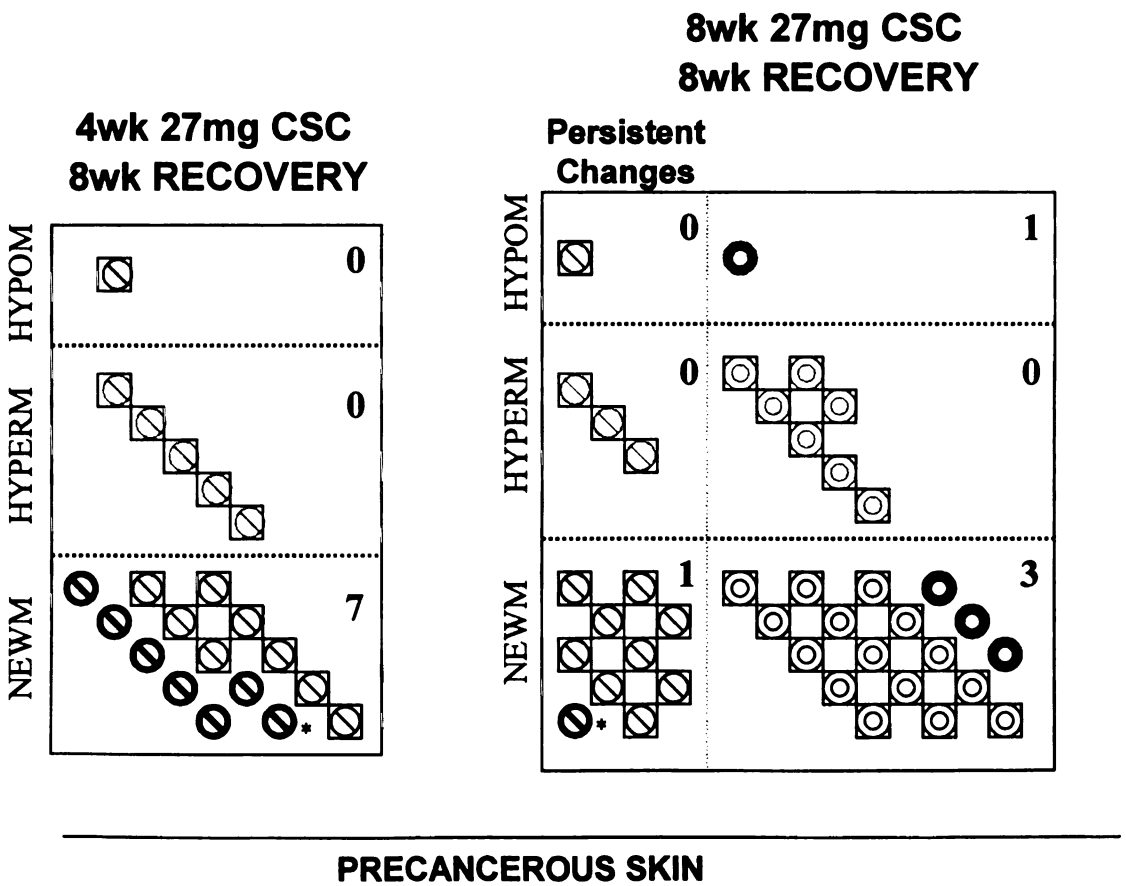
GC-rich regions of the genome are frequently found in the promoter regions of genes, and changes in methylation can be associated with changes in gene expression. Therefore, in light of the number of CSC and tumor-induced RAMs within GC-rich regions, a gene-specific approach was used to assess CSC-induced changes in methylation within the promoter region of Ha-*ras*. Upregulation of Ha-*ras*, an oncogene, via hypomethylation of its promoter region could lead to increased expression which might contribute to tumorigenesis. We first analyzed a 283bp region of the Ha-*ras* promoter containing 22CpG dinucleotides which was 950nt upstream of the transcriptional start site (Figure 6a). All 22 CpG sites were unmethylated in control skin

Figure 5 Progressive Changes in Methylation: Reversible Methylation Changes Following 4 and 8wk Recovery Hypomethylations (HYPOM), hypermethylations (HYPERM), and new methylations (NEWM) are represented for 4wk, 27mg CSC and 8wk, 27mg CSC promotion. Following 4 and 8wk recovery periods, increasing numbers of changes in methylation reversed (a). RAMs induced by 4wk, 27mg CSC (Θ) which reversed during each recovery period are boxed. Promotion with 4 and 8wk, 27mg CSC followed by 8wk recovery identifies numerous reversible changes in methylation (b). RAMs induced by 4wk, 27mg CSC (Θ) or 8wk, 27mg CSC (○) which reversed during the recovery period are boxed. Total unique changes (minus any persistent changes) are tallied and reported for each category of methylation change. (*) One new methylation induced by 4wk, 27mg CSC which persisted through to tumor was not reversible following either 4 or 8wk recovery periods.

A.


PRECANCEROUS SKIN
HYPOM
HYPERM
NEWM
1
5
15
4wk
4wk RECOVERY
1
3
8
4wk
8wk RECOVERY
0
0
7
4wk 27mg CSC

B.


4wk 27mg CSC
8wk RECOVERY
HYPOM
HYPERM
NEWM
0
0
7
*
8wk 27mg CSC
8wk RECOVERY
Persistent
Changes
0
1
0
0
1
3
*
PRECANCEROUS SKIN

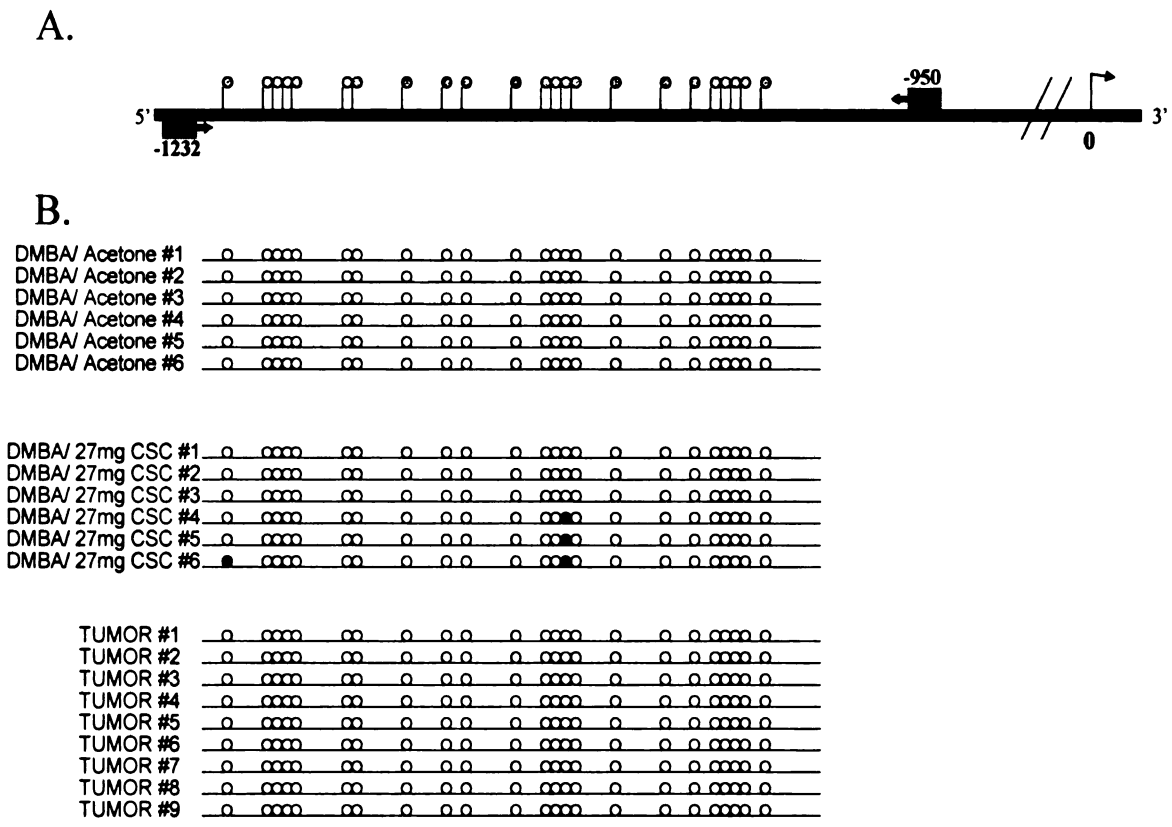


Figure 6 Methylation Status of the Promoter Region of Ha-*ras*. A diagram of the Ha-*ras* promoter indicating location of PCR primers and CpG sites (gray lollipops) in relation to the transcriptional start site is presented (a). Bisulfite sequencing analysis within the region spanning 283bp revealed that all 22 CpG sites were unmethylated (open circles) in control, 27mg CSC promoted skin and tumor tissue except for 3 samples which exhibited methylation (closed circles) at 1 or 2 CpG sites (b).

tissue, 27mg CSC promoted precancerous skin tissue and tumor tissue (Figure 6b). Analysis of changes in methylation occurring closer to the transcriptional start site was similarly conducted. This was achieved via methylation sensitive restriction digestion with SmaI endonuclease. Figure 7a outlines the target 525bp region which spanned the transcriptional start site of the Ha-*ras* gene and contained two SmaI CCCGGG recognition sites. The percent of animals exhibiting hypomethylation at these two sites progressed from 43% to 57% to 85% as the promoting dose of CSC was increased from 3 to 9 to 18mg CSC (Figure 7b). Promotion with 27mg CSC resulted in hypomethylation being observed in 67% of the total animals (Figure 7b). Hypomethylation of the region of interest in the Ha-*ras* promoter was discerned in all 9 of the tumor tissues (Figure 7b).

Due to the fact that the 5' promoter region of Ha-*ras* was determined to be unmethylated and two CpG sites within SmaI recognition sequences were hypomethylated in a generalized dose-dependent manner, changes in gene expression were assessed. Promoting doses of 18 and 27mg CSC resulted in statistically significant (10 and 6 fold) increases in gene expression over control (Figure 8) which can be associated with the higher incidence of hypomethylation at the CpG sites analyzed. Although 3 and 9mg CSC also seemed to increase gene expression, the fold changes did not reach statistical significance and this might indicate that a threshold incidence of hypomethylation required for upregulation of Ha-*ras*. Strikingly, in tumor tissue the expression of Ha-*ras* increased by 104 fold (Figure 8). Hypomethylation of CpG sites close to the transcriptional start site coupled with the absence of methylation in a distant 5'promoter region could contribute to the dramatic upregulation of Ha-*ras* in tumor tissue.

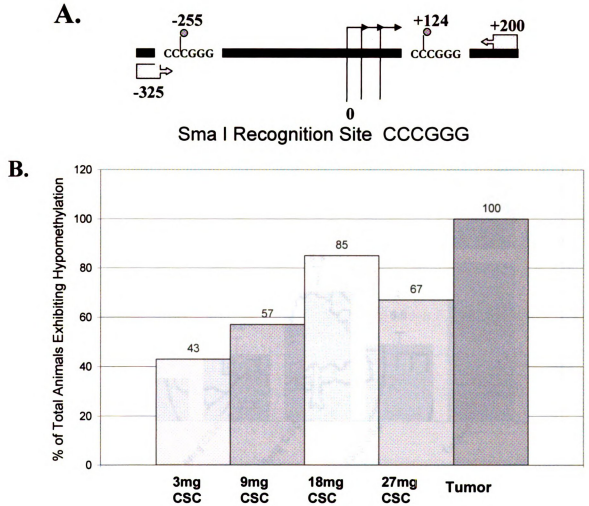


Figure 7 SmaI Restriction Digest Analysis of Ha-*ras* A schematic illustrates the targeted region spanning 325bp around the transcriptional start site of Ha-*ras* which contains 2 SmaI recognition sequences CCCGGG (a). SmaI will not cut its recognition sequence if the internal cytosine is methylated. The amount of PCR product generated is representative of the level of methylation at the internal cytosine. In general, the incidence of hypomethylation of the two CpG sites increased with dose of CSC (b). Hypomethylation was detected in 100% of the tumor tissues analyzed.

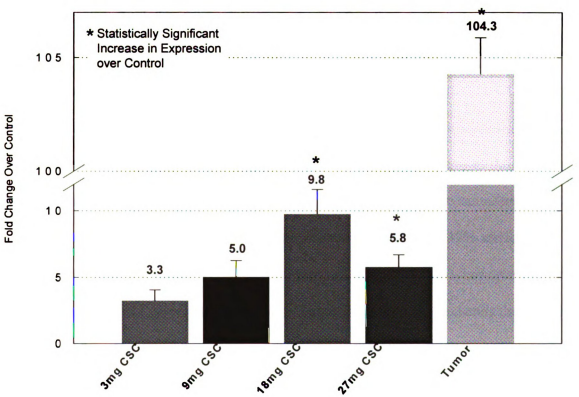


Figure 8 Expression of Ha-*ras* Changes in the gene expression of Ha-*ras* as detected by real-time PCR are expressed as fold change over control. A statistically significant increase (Student's *t*-test, $p<0.05$) over control was observed in response to 18 and 27mg CSC promotion. Tumor tissue exhibited a very large and highly significant ($p<0.001$) induction of Ha-*ras* expression.

DISCUSSION

The arbitrarily primed PCR and capillary electrophoresis method described here is a novel approach to evaluating the methylation status of GC-rich regions of DNA, and it allows for the simultaneous identification of three possible types of methylation changes: hypomethylations, hypermethylations, and new methylations. As demonstrated, dose and time dependent relationships, as well as aspects of methylation reversibility are clearly defined. The scope of this method, when employing the methylation-sensitive isoschizomers HpaII and MspI, relies on changes in methylation within their CCGG recognition site. Approximately 35.25% of these CCGG sequences lie in transposable elements, while 64.19% are located in gene coding and promoter regions of the mouse genome (Fazarri and Greally, 2004). By targeting CCGG sites, we are sampling ~7.45% of all CpG dinucleotides estimated for the mouse genome of which 4.37% are found within gene coding and promoter regions (Frazarri, and Greally, 2004). Importantly, the methylation status of CpCpG sites are concurrently evaluated which expands the capacity and power of our technique to detect overall altered methylation.

Comparatively, common alternative genome-wide methylation status assays (i.e. restriction landmark genomic scanning (RLGS) and amplification of intermethylated sites (AIMS)) which rely on methylation sensitive restriction with enzymes such as NotI and SmaI, further limit the proportion of CpG dinucleotides that can be sampled (Costello *et al.*, 2002; Frigola *et al*, 2002). RLGS targets GCGGCCGC sites via NotI and drastically reduces the proportion of CpG dinucleotides evaluated in CpG islands in the mouse genome to 0.03% (Frazzari and Greally, 2004). Approximately 14% of all HpaII and MspI recognition sequences are located within CpG islands. Therefore, our method is

less restricted allowing for a more comprehensive approach to addressing treatment (i.e. CSC) related disruption of DNA methylation during the promotion stage of skin tumorigenesis.

Promoting agents have been shown to act via a threshold exhibiting dose-response with regard to changes in methylation (Watson *et al.*, 2003). Consistent with this dose-response characteristic, CSC was shown to induce a clear dose-dependent increase in hypermethylations and new methylations. However, a fundamental shift in the nature of the observed changes in methylation over time was evident. Hypermethylations and new methylations were the predominant alteration detected in precancerous tissue at early and intermediate time points while tumor tissue (29wk) was predominantly hypomethylated. This key feature was reported previously in that global hypomethylation was seen to be specific to tumor tissue and GC-rich regions of hypomethylation were infrequently detected in precancerous skin (Watson *et al.*, 2003). In a separate study, global loss of 5-methylcytosine content was reported to progress in two steps. The first occurred during the early stages of benign tumor growth and further loss of 5-methylcytosine content was seen during the transition from an epithelial phenotype to a highly metastatic dedifferentiated/spindle morphology (Fraga *et al.*, 2004). Given that distinct methylation profiles are consistently identified for precancerous and tumor tissues, defining the intermediary steps is critical. In light of the fact that carcinogenesis involves a progressive clonal expansion of cells bearing heritable alterations of their genomes, the key question, addressed in this report, is "Do the changes in methylation at early stages of promotion persist and accumulate over time to facilitate tumorigenesis?"

The promotion stage of tumorigenesis involves the step-wise accumulation of heritable changes which are critical for the selection and clonal expansion of initiated cells (Dragan *et al.*, 1993). A number of changes in DNA methylation persisted from 4 to 8wks with the highest promoting dose of CSC and from 8wks to the tumors. Importantly, a few changes originating with the 4wk promotion were identified at 8wks and, also, persisted to the tumors. Those altered regions of methylation which persisted during promotion are likely to be critical epigenetic changes that contributed to the clonal expansion of subsets of initiated cells. As this effect was observed with the doses that caused tumors (i.e., 9, 18 and 27 mg/CSC), a relationship appears to exist between the tumor incidence elicited with each dose of CSC and the number and type of persisting changes in methylation. This supports the view that altered DNA methylation is a causative factor in tumor formation. The notion that altered DNA methylation is a cause and not an effect was previously tested in mice with decreased Dnmt1 expression and substantial genome-wide DNA hypomethylation in all tissues. These mice developed aggressive T cell lymphomas which were linked to the development of chromosomal instability and the inappropriate activation of oncogenes (Gaudet *et al.*, 2003). Although, at this time, cause and effect can not be established unequivocally, these are fundamental observations illustrating that changes in DNA methylation occur during the promotion stage of tumorigenesis and precede the formation of tumors.

Reversibility is a key aspect of the promotion stage of tumorigenesis (Dragan *et al.*, 1993) and the potential exists for reversing altered methylation of DNA; therefore, altered methylation may be a mechanism underlying promotion (Goodman and Watson, 2002). The balance of methylation-demethylation reactions is regulated by numerous

enzymes including maintenance methylases, de novo methylases and demethylases. Therefore, changes in DNA methylation can "reverse" by a number of mechanisms (Goodman and Watson, 2002). Active demethylation can involve removing the methyl group from cytosine which could effectively restore a hypermethylated state to control levels (Ramchandani *et al.*, 1999). Erroneous maintenance methylation following DNA replication could lead to both loss of methylation in hypermethylated regions and gain of methylation in hypomethylated regions. Finally, proliferation of "normal" cells and apoptosis of cells exhibiting abnormal methylation profiles could both contribute to a "reversal" of altered methylation. Within 8wk following cessation of the 4 and 8wk promoting stimuli, regions exhibiting altered methylation were seen to "reverse". With the exception of one new methylation, all persistent changes were recoverable. This is a clear demonstration that progressive, critical changes in methylation are reversible, a hallmark of tumor promotion.

The altered methylation observed in GC-rich regions could lead to aberrant gene expression. Increased methylation in the promoter regions of p16, and MGMT and E-cadherin, among others were observed during mouse skin promotion (Watson *et al.*, 2004; Fraga *et al.*, 2004). As demonstrated by Estellar *et al.*, 2004, unique profiles of promoter hypermethylation can be created and used to characterize the disruption of critical pathways in tumorigenesis. Hypermethylation associated gene silencing is extremely important when considering the mechanisms involved in tumor formation. However, both hypermethylation and hypomethylation are occurring simultaneously in GC-rich regions, illustrating the point that a variety of alterations in methylation may play a role in carcinogenesis (Counts and Goodman, 1995).

Activation of *Ha-ras* is a common feature of papillomas (Balmain and Pragnell, 1983; Balmain, *et al.*, 1984). A novel aspect of the current research is the finding that tumors arising from CSC-promoted skin exhibited an increased incidence of hypomethylation of the promoter region of Ha-*ras*. Decreased methylation, in addition to significant increased expression of the oncogene, suggests altered methylation during promotion, in addition to the possibility of mutation in response to DMBA-initiation, might contribute directly to the activation of Ha-*ras* which likely plays role in tumorigenesis. At first glance, the 100 fold increase in expression of Ha-*ras* that was observed in the papillomas might appear extreme. However, in a related study, expression of Ha-*ras* in normal skin was virtually undetectable by western blot analysis (Rodriguez-Puebla *et al.*, 1999). Therefore, given that basal levels of Ha-*ras* expression in normal skin are extremely low, the 100 fold increase observed in tumor tissue is not unrealistic, and it may play a fundamental role in facilitating tumorigenesis.

The aberrant activation of Ha-*ras* has been implicated in facilitating numerous aspects of a malignant phenotype (i.e. proliferation, invasion and metastasis) (Giehl, 2005). Over-expression of Ha-*ras* might lead to a cascade of protein kinases resulting in the phosphorylation of *Jun*, which can then upregulate the activity of DNA methyltransferase thereby increasing the methylation capacity of the cell and resulting in aberrant methylation patterns (MacLoed *et al.*, 1995). A consequence of this upregulated methylation capacity includes the possibility of silencing tumor suppressor genes through promoter hypermethylation. It was demonstrated that an oncogene, *v-src*, induced overexpression of Dnmt1 which led to down regulation of a candidate tumor suppressor gene, *tsg*, through promoter hypermethylation (Sung *et al.*, 2004). Therefore,

upregulation of *ras* in response to promoter hypomethylation could facilitate further aberrant patterns of methylation, which can facilitate tumorigenesis including hypermethylation of tumor suppressor genes, through indirect activation of DNA methyltransferases.

Genetic instability is a basic feature of carcinogenesis. Mutations in genes that normally function to maintain genetic stability might cause the formation of a mutator phenotype (Loeb, 2001), DNA hypomethylation may lead to both elevated mutation rates (Chen *et al.*, 1998) and chromosomal instability (Eden *et al.*, 2003) in addition to a CpG island methylator phenotype (Abe *et al.*, 2005). We have discerned widespread altered DNA methylation on all three levels, i.e., hypomethylation, hypermethylations plus new regions of methylation which occur in a progressive fashion during tumorigenesis. Thus, it is now appropriate to talk about instability of the epigenome as a fundamental component of the genetic instability that provides an environment which fosters the aberrant gene expression involved in the transformation of a normal cell into a frank carcinoma.

ACKNOWLEDGEMENT

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CHAPTER 3 APPENDIX

This appendix contains additional research results that are complementary to the body of data presented in Chapter 3. The materials and methods needed to extend the analysis of reversibility of altered methylation in GC-rich regions and to test the status of methylation of LINE-1 sequences in precancerous and tumor tissue is described. In addition experimental methods for the evaluation of expression of LINE-1 elements is described. Results are presented along with a brief discussion of the findings and relevant references.

MATERIALS AND METHODS

Reversibility of altered methylation: Calculations

Determining the reversibility of changes in methylation is discussed in Chapter 2, Pgs. 102-104. The following is an extension of the calculations.

PCR product sizes corresponding to statistically significant changes in methylation that are exclusive to the recovery groups are considered unique because they arose during the recovery period. These hypomethylations, hypermethylations, and new methylations are accounted for separately and designated as new RAM following recovery.

Gene-Specific Methylation Analysis: Combined Bisulfite Restriction Analysis of LINE-1

Bisulfite Conversion and PCR Amplification

Bisulfite conversion of DNA effectively deaminates all un-methylated cytosines to uracil leaving methylated cytosines unaffected. 2μg DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). PCR is performed with bisulfite converted DNA which allows for the replacement of uracil with thymine and 5-methylcytosine with cytosine. Consequently, only cytosines that were originally methylated remain in the DNA sequence. PCR is carried out using primers specific for bisulfite converted DNA and containing no CpG sites. The two LINE primers (Accession M13002), 5' AAT TTT TGT TAG GAG TTT GGT T 3' and 5' ATT TTT AAA TCT AAA TCT AAA TTT TC 3' were used to amplify the -205nt to +132nt (337bp) region of the LINE-1 element relative to the transcriptional start site. Each PCR reaction contained 2.0ul bisulfite converted DNA, 1X Failsafe™ Buffer E (Epicentre®; Madison, WI) 2.5μM each primer, 1.5 units Taq Polymerase (Invitrogen™) and GDW to a final volume of 25ul. Taq Polymerase was added following incubation at 94^0C for 3

min Cycling conditions were: 35 cycles of 94^{0}C for 45 s, 54^{0}C for 45 s, and 72^{0}C for 1 min, followed by 1 time delay cycle of 72^{0}C for 3min and a 4^{0}C soak. Amplification of the target region was verified by gel electrophoresis on a 3% agarose gel.

Restriction Digestion with TaqI or HphI restriction endonucleases

Restriction digestion with TaqI or HphI was carried out on the PCR products. TaqI digests contain 1x NEB Buffer 3 (NewEngland Biolabs), 1x BSA (NewEngland Biolabs), 3.0ul PCR product 20units TaqI and GDW to a final volume of 10.0ul. Digests were incubated overnight at 65^{0}C. HphI digests contain 1x NEB Buffer 4, 3.0ul PCR product, 5units HphI and GDW to a final volume of 10ul. Digests were incubated over night at 37^{0}C. 2.5ul of each digest was electrophoresed via a 3% agarose gel.

Quantification of Band Intensity with the HphI Digest

The relative intensity of a PCR product band corresponds to the relative starting concentration of methylated DNA. Four regions of each lane were outlined and measured for pixel number and intensity with NIH image analysis. 1. the 337bp PCR product band 2. Lane background just below the 337bp PCR product 3. Restriction Digestion Fragment (218bp) 4. Lane background just below the restriction digestion fragment. The number of pixels defined the size of the outlined region. The same sized region was used to measure the PCR product band, restriction digestion fragment and the lane backgrounds. Total pixel intensity units (TPI) were calculated by multiplying the number of pixels by the mean intensity units within the outlined region. This was done separately for the background measurements, the PCR product band, and the 218bp

fragment. The TPI units of the respective background were subtracted from the TPI units of either the PCR product band or the 218bp fragment band to give a normalized TPI for the outlined region within a lane. The ratio of the normalized 218bp band over the normalized 337bp band was calculated and multiplied by 100%. This percentage represents the fraction of intensity the 218bp band is of the 337bp band. Statistical significance was determined by Student's *t*-test, $p<0.05$.

Expression of LINE-1

Reverse Transcription of RNA

RNA samples were treated with DNaseI (Invitrogen) to purify the RNA from contaminating DNA remaining after isolation. Each reaction contained 2ug RNA, 1X DNaseI reaction buffer, 2 units DNaseI, and DEPC-treated GDW to a final volume of 20ul. Samples were incubated at room temp. for 15min followed by addition of $MgCl_2$ to a final concentration of 2.27mM. RNA was heated to 65^0C for 10min to inactivate the DNaseI enzyme. The TaqMan Reverse Transcription Kit (Applied Biosystems; Foster City, CA) was used to reverse transcribed the DNaseI treated RNA. Each reverse transcription reaction contained, 1X Reverse Transcription Reaction Buffer, 5.5mM MgCl2, 200uM of each dNTP, 2.5uM random hexamer, 20 units RNase Inhibitor, 62.5 units Multiscribe Reverse Transcriptase and DEPC-treated GDW to a final volume of 50ul. The reactions are incubated at 25^0C for 10min, 42^0C for 1hr, and 95^0C for 5 min. All samples were stored at 4^0C until needed.

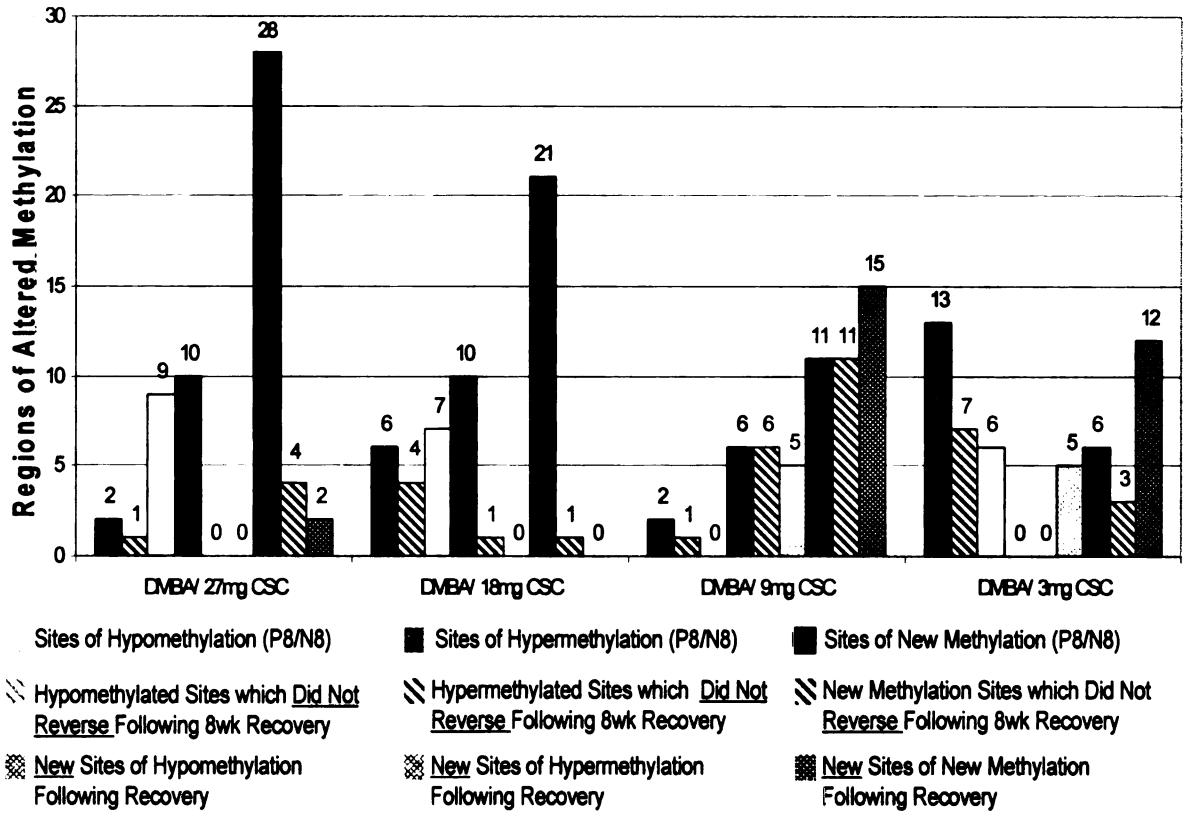
Real Time PCR

A custom TaqMan assay including primers (For 5' GGT CAA ATC TAA GTG GAT CAA GGA ACT 3' and Rev 5' GCT TTT CCC CAC TTT CTC CTC TAT 3') and probe (5' FAM CAG AGA CAC TGA AAC TT 3') specific for ORF2 of the LINE-1 element (Accession M13002) was purchased from Applied Biosystems. In addition an Applied Biosystems custom TaqMan assay including primers (For 5' CTA CTA CCG ATT GGA TGG TTT AGT GA 3' and Rev 5' GTC AAG TTC GAC CGT CTT CTC A 3' and probe (FAM 5' CCG TGG GCC GAC CC3') was used for the control gene, 18S rRNA (Accession X00686). Triplicate reactions for both the gene of interest (LINE-1) and the control gene (18S) were prepared per sample. Standards were also prepared for 18S and Ha-*ras* and ranged from 1 x 10^2 copies to 1 x 10^8 copies. Each reaction contained 1 x Custom Assay Mix (Applied Biosystems; Foster City, CA), 11ul cDNA or 2ul standard, and GDW to a final volume of 25ul. Cycling conditions were as follows: 50^0C for 2 min, 95^0C for 10min, and 40 cycles of 95^0C for 15sec. and 60^0C for 1 min. The absolute standard curve method for quantifying fold change over control was employed.

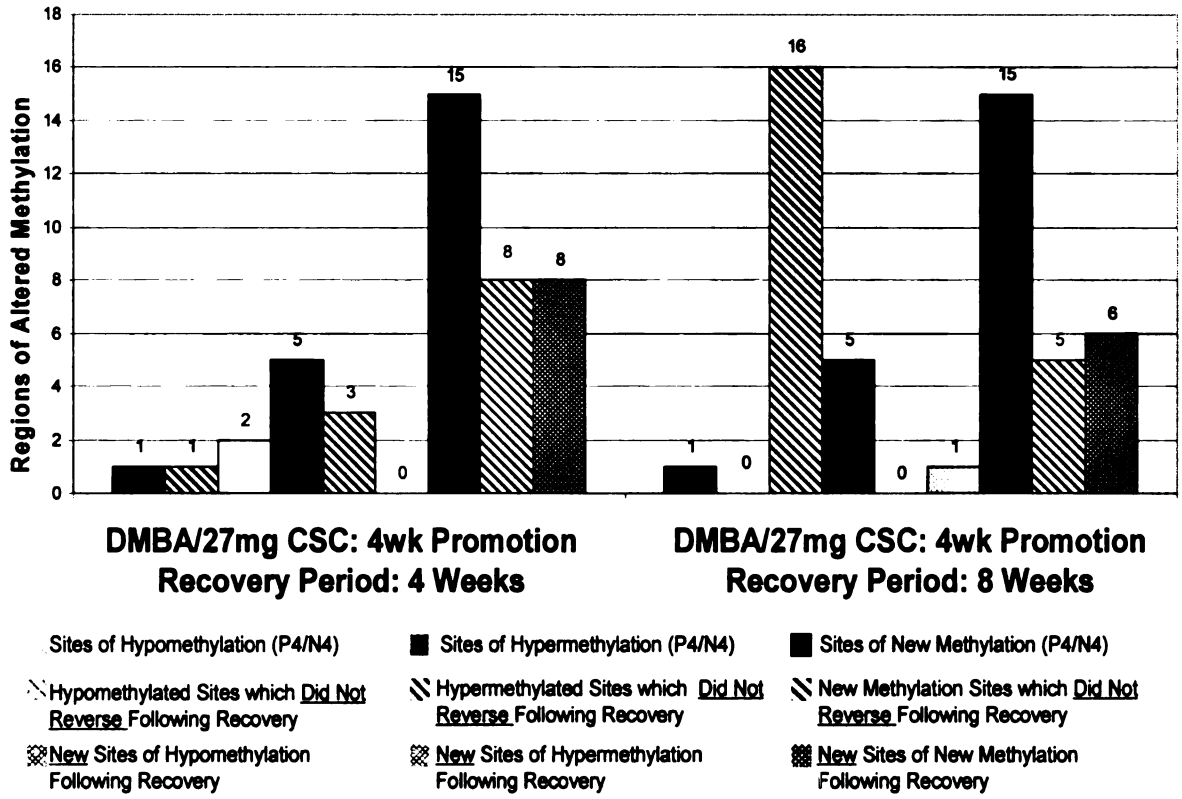
RESULTS

Tumor promotion involves a basic step-wise accumulation of heritable changes. If the promoting stimulus is withdrawn, accumulated changes in methylation could reverse. Reversal could also result from the apoptosis of the initiated cells. Therefore, the reversibility of regions exhibiting altered methylation in response to 4 and 8wk promotion was measured. Specifically, RAMs previously induced by 4 or 8wk 27mg CSC promotion were re-analyzed following the recovery period. Reversal of the methylation changes induced by 4wk, 27mg CSC was more complete as the duration of recovery was lengthened. Following 4wk recovery, 2 hypermethylations and 7 new methylations had reversed (Figure 1). With 8wk recovery, an additional 4 RAMs had reversed (Figure 1). In comparison, 8 wk promotion with 27mg CSC followed by 8wk recovery resulted in the reversal of 1 of 2 hypomethylations, 10 of 10 hypermethylations, and 24 of 28 new methylations (Figure 2). Importantly, all changes, with the exception of 1 new methylation, which persisted from the 4wk promotion, were recoverable which clearly demonstrates that changes in methylation which accumulate in response to the promoting stimuli are largely reversible.

In addition to assessing which RAMs which did or did not reverse, unique methylation changes occurring during recovery were analyzed. The number of hypomethylations after 8wks induced by 3, 9, 18, and 27mg CSC were relatively few in number; however, a notable increase in hypomethylations was evident during the 8wk recovery period for all dosing groups except 9mg CSC (Figure 2). Unique hypermethylations only increased with recovery following 3 and 9mg CSC but not 18 and 27mg CSC (Figure 2). This same pattern was seen with the new methylations.



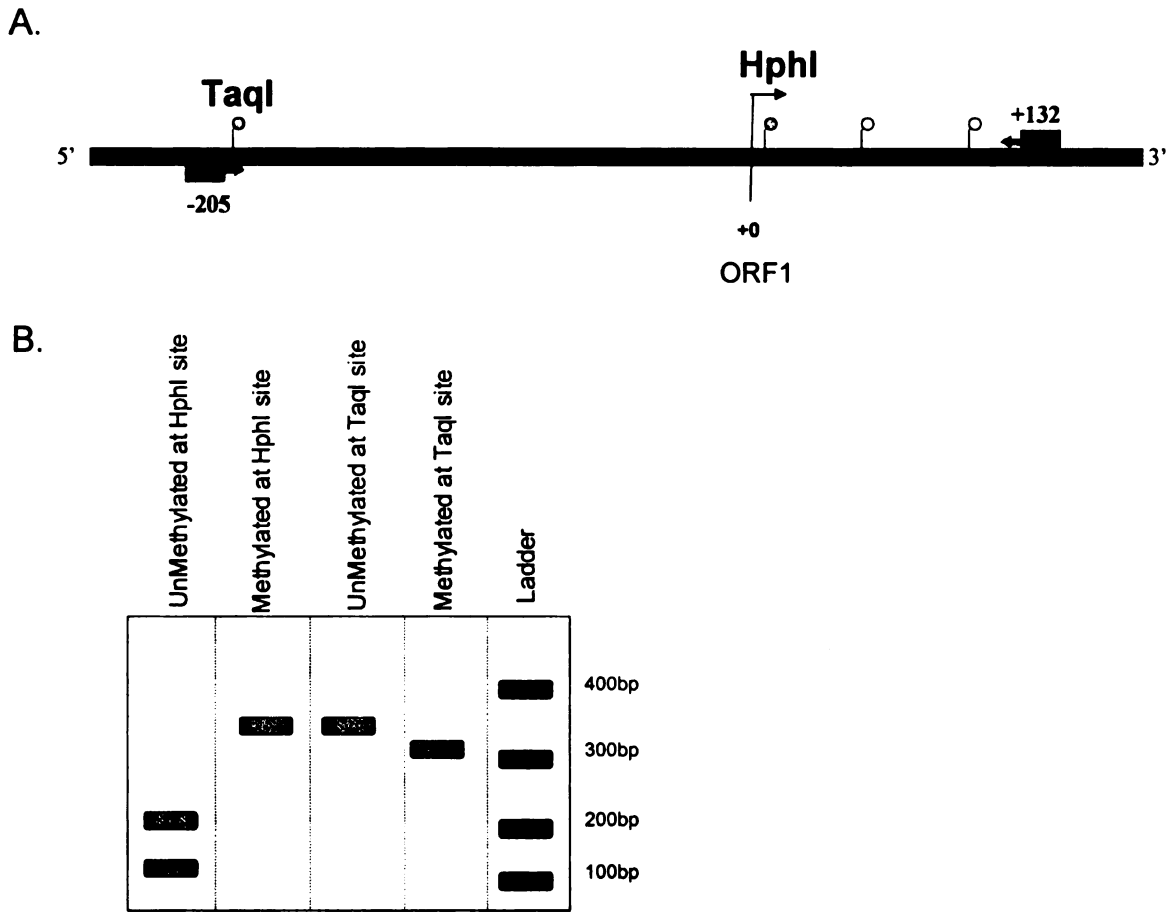
Appendix Figure 1 Reversible Methylation Changes Following 8wk Promotion
Hypomethylations, hypermethylations, and new methylations are represented for 8wk, 3, 9, 18, and 27mg CSC promotion. Of those changes induced by 8wk promotion, the number of regions of altered methylation which did not reverse following an 8wk recovery period are presented in addition to unique changes in methylation identified following recovery. Hypomethylated RAM include both partial hypomethylations (statistically significant ($p<0.05$) decrease as compared to control) and complete (100%) hypomethylations. Hypermethylated RAMs are only those increases which are statistically significant (Student's *t*-test, $p<0.05$). New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing.



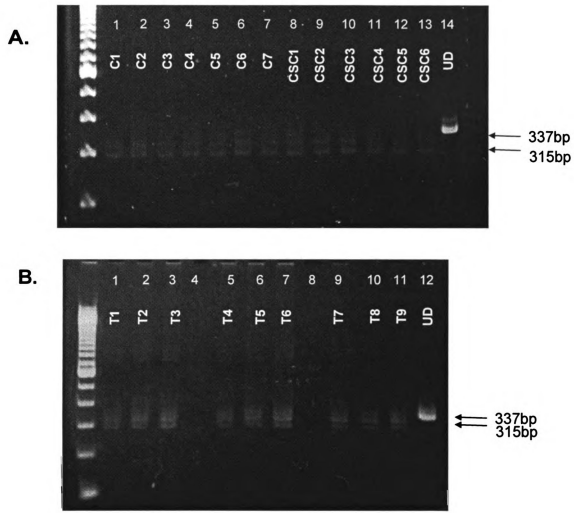
Appendix Figure 2 Reversible Methylation Changes Following 4 and 8wk Promotion with 27mg CSC Hypomethylations, hypermethylations, and new methylations are represented for 4wk, 27mg CSC promotion. Of those changes induced by 4wk promotion, the number of regions of altered methylation which did not reverse following a 4 or 8wk recovery period are presented in addition to unique changes in methylation identified following recovery. Hypomethylated RAM include both partial hypomethylations (statistically significant ($p<0.05$) decrease as compared to control and complete (100%) hypomethylations. Hypermethylated RAMs are only those increases which are statistically significant (Student's *t*-test, $p<0.05$). New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing.

Twelve and 15 unique new methylations were observed during recovery following 3 and 9mg CSC respectively whereas only 2 were identified post 27mg CSC (Figure 2). Similar to this, an increase in unique hypomethylations from 2 to 16 was observed as the duration of recovery was extended following 4wk promotion with 27mg CSC (Figure 1). Unique hypermethylations increased by 1 and unique new methylations decreased from 8 following 4wk to 6 after 8wks recovery (Figure 1).

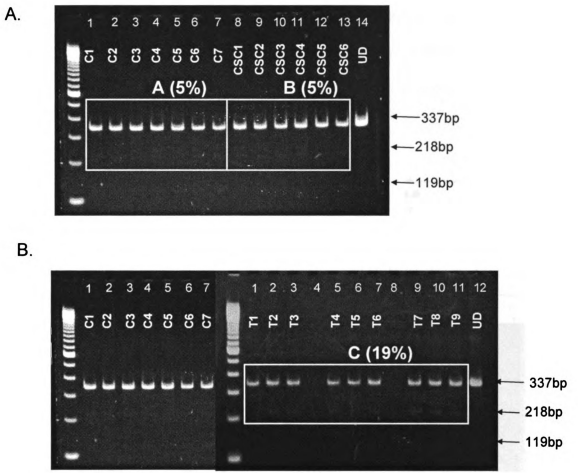
GC-rich regions of the genome are frequently found in the promoter regions of genes, and changes in methylation can be associated with changes in gene expression. In that regard, the disregulation of LINE-elements can be associated with genomic instability. The methylation status of two CpG sites close to the transcriptional start site for the first ORF were measured via COBRA analysis with TaqI and HphI enzymes (Figure 3a and b). The methylation status of the CpG dinucleotide contained within the TaqI recognition site was similar in control, 27mg CSC promoted skin (Figure 4a) and tumor samples (Figure 4b). This CpG dinucleotide was partially (~50%) unmethylated as demonstrated by the visualization of both the 337bp PCR product and the 315bp digestion fragment which occurred with approximately equal intensities (Figure 4a and b). The HphI CpG dinucleotide located within 3bp of the transcriptional start site was methylated in both control and 27mg CSC promoted skin samples (Figure 5a). However, the intensity of the 218bp digestion fragment significantly increased in tumor samples; this indicates that the site was partially hypomethylated (Figure 5b). Expression of the LINE-1 element showed a slight, but statistically significant, increase over control with 27mg CSC promotion while this was not seen in tumor (Figure 6).



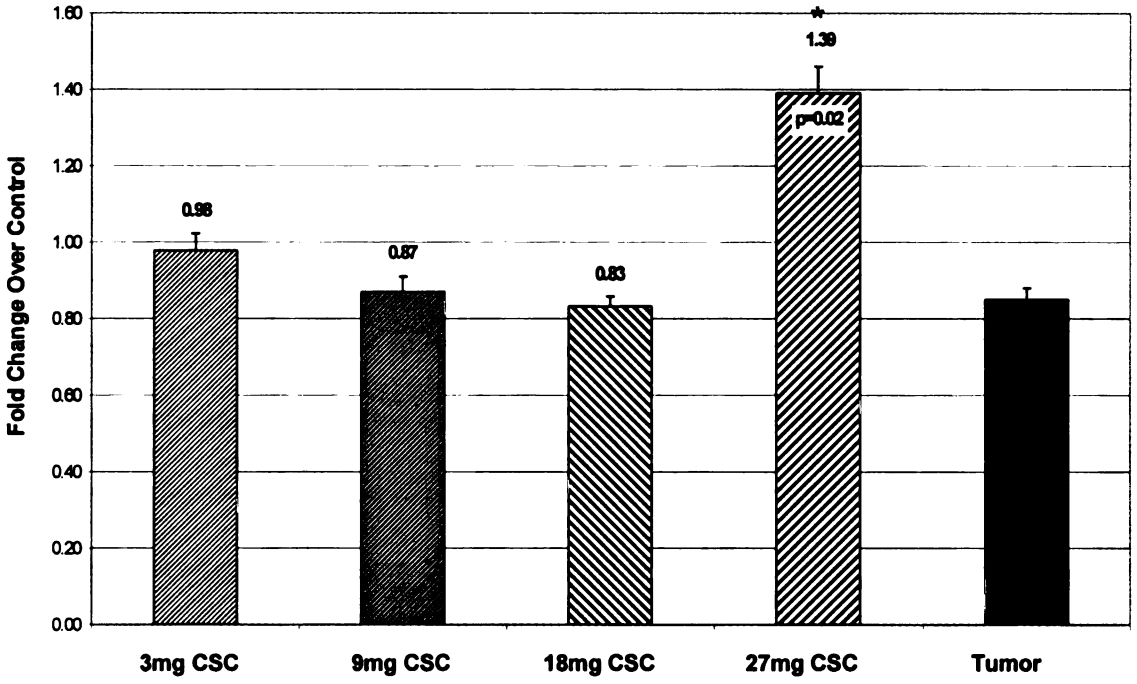
Appendix Figure 3 Targeted Region of LINE-1. The LINE-1 promoter and part of the first ORF (open reading frame) indicating location of PCR primers and CpG sites (gray lollipops) in relation to the transcriptional start site is presented (a). Open lollipops represent CpG sites which are not analyzed using the COBRA technique. Expected fragment sizes based on the methylation status of either the TaqI or HphI sites is diagrammed (b). DNA is first bisulfite converted which all unmethylated cytosines are deaminated to uracil. Methylated cytosines remain methylated. PCR amplification of the bisulfite converted DNA effectively replaces all uracils with thymine. TaqI recognizes and restrict TCGA sites. Therefore, TaqI will only restrict the PCR product if the cytosine was originally methylated. This results in 22bp and 315bp digestion fragments. HphI recognizes and restricts GGTGA sites. Therefore, HphI will only restrict the PCR product if the cytosine within the GGCGA site was originally unmethylated. Through bisulfite conversion the site becomes GGTGA and is restricted by HphI resulting in 119 and 218bp fragment sizes.



Appendix Figure 4 Methylation Status of the TaqI Site Within a Targeted Region of LINE-1 Methylation status of a TaqI TCGA site was analyzed following bisulfite conversion of the DNA and PCR amplification of a 337bp region containing the recognition sequence near to the transcriptional start site of LINE-1. Control (C) samples (n=7)were compared to 8wk, 27mg CSC (CSC) promoted samples (n=6) (a), or tumor (T) samples (n=9) (b). An undigested control (UD) PCR product was run on each gel for comparison. Visualization of the 337bp and 315bp digestion fragments with equal intensities indicates that the site is 50% methylated and 50% unmethylated in all samples tested.



Appendix Figure 5 Methylation Status of the HphI Site Within a Targeted Region of LINE-1 Methylation status of an HphI GGTGA site was analyzed following bisulfite conversion of the DNA and PCR amplification of a 337bp region containing the recognition sequence near to the transcriptional start site of LINE-1. Control (C) samples (n=7)were compared to 8wk, 27mg CSC (CSC) promoted samples (n=6) (a), or tumor (T) samples (n=9) (b). An undigested control (UD) PCR product was run on each gel for comparison. Visualization of the 218bp and 119bp digestion fragments indicates that the site is partially hypomethylated. The average pixel intensity of the 7 control 218bp digestion fragments within Box A was calculated to be 5% of the average pixel intensity of the 7 control 337bp PCR products. The average pixel intensity of the 6 8wk, 27mg CSC 218bp digestion fragments within Box B was calculated to be 5% of the average pixel intensity of the 6 8wk, 27mg CSC 337bp PCR products. The average pixel intensity of the 9 tumor 218bp digestion fragments within Box C was calculated to be 19% of the average pixel intensity of the 9 tumor 337bp PCR products indicating a statistically significant decrease in methylation. Statistical significance was determined by Student's *t*-test, $p<0.05$.



Appendix Figure 6 Expression of LINE-1 Changes in the gene expression of LINE-1 as detected by real-time PCR are expressed as fold change over control. A statistically significant increase (Student's *t*-test, $p<0.05$) over control was observed in response to 27mg CSC promotion. LINE-1 expression was not increased in tumor tissue.

DISCUSSION

Changes in methylation were observed during recovery suggesting that altered methylation extends past the promotion stage. However there could be numerous causes of these changes. In general, new methylations and hypermethylations were only observed following withdrawal of 3 and 9mg CSC or 27mg CSC (4wk). If *de novo* methylases were down regulated during promotion, withdrawal of the stimulus could result in an overcompensation or upregulation of methylase activity leading to transient hypermethylations or new methylations. Another possibility would be that upregulation of active demethylation in response to the hypermethylation during promotion could cause transient hypomethylation of unintended regions of the genome. In addition, the exact length of time needed to fully restore normal patterns of methylation is unknown. Numerous time points following cessation of the promoting stimuli might be needed to estimate full recovery. Hypomethylation is a prominent alteration observed during recovery. This hypomethylation could be linked to increased apoptosis of abnormal cells. The DNA methyltransferase inhibitor 5-AZA which results in hypomethylation of DNA has been shown to induce apoptosis in colorectal cancer cells via induction of 15-lipoxygenase-1 (15-Lox-1) (His *et al.*, 2005). 15-Lox-1 has been linked to the modulation of apoptosis and the differentiation of colorectal carcinoma cells (Ikawa *et al.*, 1999). Therefore, the interesting increase in hypomethylations during the recovery period could lead to the upregulation of a number of proteins involved in apoptosis contributing to the restoration of normal cell populations.

Analysis of the methylation status of 2 CpG sites around the transcriptional start site of the LINE-1 element revealed slight hypomethylation of one of the two sites only

in the tumor samples. Hypomethylation of repetitive sequences is a commonly reported feature of various cancers. Various gastric cancer cell lines exhibited LINE-1 hypomethylation in conjunction with global hypomethylation. For example, repetitive element hypomethylation was not seen in gastric cancer cell lines without global hypomethylation (Kaneda *et al.*, 2004). These results are consistent with global hypomethylation exhibited by skin tumors (Waston *et al.*, 2003) and evidence for hypomethylation in a region very near to the transcriptional start site of the LINE-1 elements. Prostate cancer also bears some similarity to observations made with precancerous and cancerous skin samples. Coordinate hypermethylation of several genes (e.g. APC, GSTP1, RARB2, and RASSF1A) may occur early in prostate carcinoma followed by LINE-1 hypomethylation as was observed in humans. This hypomethylation has been associated with the progression of prostate cancer (Florl *et al.*, 2004). Hypomethylation of LINE-1 elements has been postulated to lead to increases in expression which would contribute to genetic instability. This has been demonstrated in human urothelial carcinomas. Decreased methylation of LINE-1 elements was demonstrated in conjunction with increases in RNA levels suggesting increased expression (Florl *et al.*, 1999). Our data did not show an increase in LINE-1 expression in tumor tissue, however, a slight increase in expression was noted with 27mg CSC promotion for 8wk. This indicates that the upregulation of LINE during promotion might be controlled by regions of the promoter which were not analyzed. Therefore, genomic instability created by the upregulation of LINE during promotion could contribute to tumor formation, even though LINE expression is not sustained in the skin tumors.

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SUMMARY

In Chapter 1, the ability of diethanolamine (DEA) to alter patterns of methylation in GC-rich regions in B6C3F1 mouse hepatocytes was tested. DEA is hypothesized to upset choline homeostasis by inducing a choline deficient state. This choline deficiency (CD) leads to methyl deficiency which could disrupt 1-carbon metabolism and decrease the capacity of a cell to maintain patterns of methylation resulting in the facilitation of liver tumorigenesis. A choline devoid, methionine deficient diet has previously been shown to lead to global hypomethylation of DNA in the livers of B6C3F1 mice (Counts *et al.*, 1996). The fundamental observation that DEA induces a large degree of hypomethylation in hepatocytes is significant given that decreases in methylation have been associated with the activation of oncogenes and transposable elements. The large extent of hypomethylation combined with the fact that DEA- and CD-induced patterns of altered methylation were 72% similar is suggestive of common targets and pathways shared between them. This provides some basis to support the notion that DEA indirectly depletes the pool of methyl groups needed for methylation of cytosine by inhibiting choline uptake into cells. PB was also demonstrated to alter methylation with high similarity (70%) to CD. Therefore, I concluded that altered methylation, specifically hypomethylation, likely is a non-genotoxic mode of action underlying the abilities of DEA, PB and CD to promote liver tumorigenesis.

In Chapter 2, mice with variable susceptibilities to liver tumorigenesis were assessed in terms of their ability to maintain patterns of methylation during promotion with PB. Additionally, I tested the hypothesis that a progressive accumulation of non-random changes in methylation are involved in tumorigenesis. Analysis of altered

methylation in GC-rich regions was key to addressing this multi-part hypothesis. First, specific regions of altered methylation (RAMs) were seen to carry forward from the 2wk to 4wk time point, a key component of the promotion stage of tumorigenesis. The number of accumulated changes was directly related to the relative sensitivities of the B6C3F1 and C57BL/6 mice. Secondly, by comparing specific RAMs in the relatively resistant C57BL/6 mice to those in the relatively sensitive mice in addition to liver and kidney tissue I demonstrated that PB-induced patterns of altered methylation are highly unique to B6C3F1 mouse liver. Finally, gene-specific analysis in the B6C3F1 mice revealed a non-random pattern of altered methylation in which Ha-*ras* hypomethylation was associated with selective increases in gene expression and the heavily methylated LINE-1 elements were unaffected by PB. This provided the first direct experimental evidence for progressive, non-random changes in methylation as an important component to tumorigenesis. My hypothesis also reinforces the conclusions of previous researchers in this field (Counts *et al.*, 1996; Watson and Goodman, 2002). The data suggest that altered DNA methylation is an epigenetic mechanism underlying the ability of PB to cause liver tumorigenesis. Therefore instability of the epigenome likely contributes to susceptibility to tumorigenesis.

Use of the SENCAR 2-stage mouse initiation/promotion model of skin tumorigenesis in Chapter 3 was suitable for analyzing altered methylation in GC-rich and gene-specific regions in response to promotion. This chapter is highly complementary to chapter 2 in that the notion of progressive changes in methylation could be tested in a distinct model system. Patterns of DNA methylation in tumor tissue provided a highly valuable comparison to pre-cancerous tissue. Similar to Chapter 2, RAMs carried

forward with time and, importantly, were identified in tumor tissue. With 4, 8 and 29wk (tumor) time points, I was able to depict a more concrete example of progressive changes in methylation. I also established a dose-response relationship for increases in methylation and demonstrated reversibility. The observed upregulation of LINE during promotion may be controlled by regions of the promoter which were not analyzed for altered methylation. However, the genomic instability created by the upregulation of LINE during promotion could contribute to tumor formation, even though LINE expression is not sustained in the skin tumors. Based on decreased methylation in addition to significant increased expression of Ha-*ras*, I proposed a role for altered methylation during promotion which might contribute directly to the activation of Ha-*ras* during skin tumorigenesis.

The specific aims, as previously presented, are individually addressed within the context of the experimental observations.

Specific Aims

Each of the three chapters in this dissertation focused on testing the outlined hypotheses and objectives. Each specific aim has been experimentally examined and is separately addressed.

A) Examination of DEA-induced altered methylation in GC-rich regions as a contributor to the development of liver tumors in B6C3F1 mice.

1) To assess global and GC rich methylation alterations in response to DEA, CD, and PB in B6C3F1 mice.

Global methylation status was unchanged in response to DEA, CD, and PB strengthening the need to specifically examine GC-rich regions for a more detailed picture of overall altered methylation. GC-rich methylation was assessed via methylation sensitive restriction digestion, arbitrarily primed PCR, and capillary electrophoretic separation of PCR products. Treatment with DEA, CD and PB resulted in altered patterns of methylation, predominantly hypomethylations, suggesting this is an epigenetic mechanism which contributes to the facilitation of mouse liver tumorigenesis.

2) Compare changes in methylation in GC-rich regions following DEA and PB treatment to those observed after choline deficiency.

Regions of altered methylation were highly similar between DEA and CD and between PB and CD. This suggests that all three treatments share common pathways or targets. The high similarity between DEA- and CD-induced RAMs supports a mechanism where by DEA disrupts choline homeostasis to induce a methyl deficient state which leads to altered patterns of methylation.

B) Effects of phenobarbital (PB) on gene specific and GC rich regional methylation status of hepatic DNA in tumor prone and tumor-resistant mice.

1) To determine if cancer susceptibility in mice is related to differences in the ability to maintain normal patterns of methylation in response to PB

Patterns of methylation in the B6C3F1 tumor prone mice were less stable during promotion with PB in relation to the C57BL/6 mice indicating a decreased ability to maintain patterns of methylation. This was supported by the observation that the total number of RAMs at 4wks in B6C3F1 were more numerous than C57BL/6 at 4wks. Key to answering this specific aim was the observation that, a much higher percentage of RAMs carried forward from 2wk to 4wks in B6C3F1 mice.

2) Characterize progressive changes in methylation by specifically identifying regions of altered methylation that carry forward with time in the B6C3F1 and C57BL/6 mice.

The arbitrarily primed PCR method is unique in that specific RAMs can be tracked from one time point to the next. Therefore, I was able to collect experimental evidence for progressive changes in methylation which carry forward with time during promotion. The tumor prone-B6C3F1 mice showed a relatively large percentage of changes which carried forward in relation to the C57BL/6.

3) Identify changes in methylation which are unique to B6C3F1 liver when compared to altered DNA methylation observed in C57BL/6 liver or B6C3F1 kidney.

In the B6C3F1 mice, 57 unique (as compared to the C57BL/6) RAMs, predominantly hypomethylation, were observed in liver after 2 wk of treatment with PB and this

increased to 86 at 4wk. When comparing B6C3F1 RAMs in liver and kidney only 5 RAMs were in common and equivalent. Therefore, 81 total RAMs were unique to B6C3F1 mouse liver.

4) To determine the effects of PB on the methylation status of the promoter regions of Ha-*ras* and LINE-1 elements and subsequently analyze changes in their gene expression.

One particular CpG site 1137nt upstream of the transcriptional start site showed PB-induced hypomethylation only in the B6C3F1 mice. In addition, the decreases were observed close to the transcriptional start site. The observed hypomethylation, although limited, could be linked to the observed selective increases in gene expression. As expected LINE-1 elements were heavily methylated and gene expression was unchanged in response to PB.

5) To test the reversibility of PB-induced altered methylation in GC-Rich regions and gene-specific promoter regions (*i.e.* Ha-*ras* and LINE-1 elements).

Reversibility was demonstrated for numerous RAMs in GC-rich regions, however, low reversibility of hypomethylations was a critical observation that was characteristic of both B6C3F1 and C57BL/6 mice. One site in the promoter region of Ha-ras was hypomethylated only in the B6C3F1 mice in response to PB. Upon cessation of the promoting stimuli this site was largely reversible. Methylation of this site in C57BL/6 was stable during promotion and recovery periods.

C) Characterization of GC-rich and gene specific methylation changes in tumor and precancerous skin tissue during the promotion stage of the 2-stage, initiation/promotion SENCAR mouse model.

1) Evaluate the methylation status of GC-rich regions following 8wk promotion with increasing doses (3, 9, 18, 27mg) of CSC.

Changes in the methylation status of GC-rich regions in the SENCAR mouse model were treatment related. Specifically, the number of increases (hypermethylations and new methylations) increased in a dose-dependent manner. This is consistent with previous findings indicating a role for hypermethylation in the silencing of tumor suppressor genes during promotion with CSC in the SENCAR mouse model (Watson et al., 2003 and 2004). In addition, dose-dependent changes in methylation were consistent with tumor incidence reported for each dose of CSC.

2) Evaluate the methylation status of GC-rich regions following 4wk and 8wk promotion with 27mg of CSC.

The total number of changes in the methylation status of GC-rich regions in response to 27mg CSC promotion for 4 and 8wks increased with time.

3) Evaluate the methylation status of GC-rich regions in tumor tissue (29wk) and compare to precancerous tissue.

Hypomethylated RAMs were predominant in tumor tissue, in contrast to the clear increase in hypermethylations and new methylations in precancerous tissue. This finding is consistent with the previous observation in which global hypomethylation was only seen in tumor tissue (Watson et al., 2003). The total number of changes was comparable to the 18mg and 27mg CSC promoted precancerous tissue.

4) Characterize progressive changes in methylation by specifically identifying regions of altered methylation that carry forward with time from 4wk to 8wk and from 8wk to tumor tissue (29wk)

GC-rich RAMs induced by CSC were seen to carry forward from 4 to 8wks and from 8wks to tumor in a manner that was consistent with the multistage carcinogenesis model for a progressive accumulation of changes during promotion. Importantly, RAMs originating with the 4wk promotion were identified in the tumor tissue.

5) Assess the reversibility of changes in methylation in GC-rich regions upon cessation of CSC application.

Reversibility was demonstrated for the large majority of RAMs in GC-rich regions following the 4wk promotion-4 or 8wk recovery and 8wk promotion-8wk recovery dosing regimens. In addition, GC-rich regions of altered methylation were also observed following the recovery periods and consisted mainly of hypomethylations, an important consideration for discerning the mechanisms during recovery.

6) Relate changes in the methylation status of the promoter regions of Ha-*ras* and LINE-1 elements to changes in gene expression in both precancerous and tumor tissue.

Promotion with CSC for 8wk resulted in a generalized dose-dependent increase in the incidence of hypomethylation at 2 CpG sites surrounding the transcriptional start site. The incidence of hypomethylation was 100% in tumor tissue. Expression of Ha-ras increased significantly with 18mg and 27mg CSC and even more so in tumor tissue, supporting a role for decreased methylation in activating Ha-ras in skin tumorigenesis. This activation of Ha-ras with 18 and 27mg CSC fit nicely and likely is associated with

the observed increase in tumor incidence at these two doses. Hypomethylation of one CpG site in the promoter region of LINE-1 elements was seen only in tumor tissue. However, gene expression was mildly increased in response to 27mg CSC but not in tumor tissue suggesting my characterization of altered methylation in the promoter region was not sufficient to link changes in methylation to changes in gene expression.

Support for Hypotheses

My overall hypothesis states that susceptibility to carcinogenesis is related inversely to the capacity to maintain normal DNA methylation patterns. Research, presented in Chapters 1-3 was directly related to this hypothesis and largely focused on demonstrating that changes in methylation are progressive and non-random during the promotion stage of tumorigenesis in addition to connecting changes in the methylation status of promoter regions to changes in gene expression. These characteristics do not prove cause and effect but do strengthen the importance of altered methylation as a contributor to the development and progression of tumors as opposed to being a consequence of this process. My findings from three distinct model systems were complementary and consistent indicating the power and significance of this work.

In Chapter 1, B6C3F1 mouse hepatocytes were treated in culture with DEA, PB, and CD resulting in highly similar patterns of altered methylation. The characteristic disruption of methylation patterns in the B6C3F1 mouse *in vivo* was extended *in vitro* and illustrates that instability of the epigenome is a key feature of susceptibility to tumorigenesis. The ability to induce highly reproducible changes in methylation with three different treatments indicates that changes in methylation are not entirely random. Instead, the observed changes are likely the end result of a mis-directed chain of factors responsible for maintaining homeostasis. Chapter 2 was centered on experimentally demonstrating that the tumor prone and tumor resistant mice are fundamentally different in their abilities to maintain patterns of DNA methylation. Compelling experimental evidence was provided to show that specific regions of altered methylation in response to PB, carried forward with time, only in the B6C3F1 mice. Unique regions of altered

methylation as compared to C57BL/6 mice and B6C3F1 kidney were identified as likely critical to the development of tumors. In the context of the multistage model of tumorigenesis, DNA methylation in B6C3F1 mice is less stable and alterations are more apt to accumulate with continued promoting stimuli. With Chapter 3, changes in methylation occurring in precancerous tissue were directly compared to changes occurring in tumor tissue. This was a critical link to connecting destabilized patterns of methylation and, hence susceptibility, to tumor formation. SENCAR mice exhibited a failure to maintain normal methylation which likely resulted in the observed step-wise accumulation of changes during promotion. In addition, dose-dependent changes in methylation were consistent with tumor incidence reported for each dose of CSC. These critical changes were observed in tumor tissue which connects early (precancerous) changes in methylation to the neoplastic state. The complementarities of Chapters 2 and 3 shows that progressive changes in methylation are possibly a universal feature of tumorigenesis. Also key to supporting the hypothesis was to demonstrate that changes in methylation could lead to altered gene expression. Hypomethylation of the Ha-*ras* oncogene was identified to varying degrees in both Chapters 2 and 3. This hypomethylation was shown in conjunction with increases in gene expression suggesting that up-regulation of oncogenes by way of decreased methylation could facilitate tumorigenesis.

DISCUSSION

In my opinion, the following 4 points are the most important and significant aspects of this research to the field of DNA methylation and carcinogenesis.

1. Development of AP-PCR/CE has proven to be a consistent and reproducible method for detecting changes in methylation in GC-rich regions regardless of organ type or model system.

Methylation sensitive restriction digestion, arbitrarily primed PCR, and capillary electrophoretic separation of PCR products is a novel approach to measuring changes in methylation. One very unique feature of the method is the ability to simultaneously measure treatment-related increases, decreases and new methylations in a high throughput highly reproducible manner. The fundamental method originated with Gonzalgo, *et al.*, 1997 and was further developed by Watson and Goodman, 2002b. By targeting CCGG sites, the recognition site of both MspI and HpaII, I was able to gauge the extent of altered methylation in ~7.45% of all CpG dinucleotides estimated for the mouse genome (Frazzari and Greally, 2004). This portion of the genome includes gene coding and promoter regions, CpG islands, and transposable elements. A parallel study performed with BfaI/BssHII more directly targeted CpG islands and expanded the scope of the method. Additionally, assessment of both CpG and CpCpG methylation via HpaII and MspI enzymes respectively was instructive for examining the contribution of CpNpG methylation. Comparatively, other commonly employed genome-wide methods focus only on altered methylation within CpG islands (e.g. RLGS and AIMS). Alternatively, a gene by gene approach can be applied in which single CpG sites or limited regions are

examined. Therefore, this method is less restricted allowing for a more comprehensive approach to addressing treatment related disruption of DNA methylation.

With the ability to more accurately separate the PCR products and quantify the amount of PCR product amplified, the level and depth of analysis was overwhelmingly increased. This allowed for very specific comparisons over time, between mice with different genetic backgrounds and between target and non-target tissues. This very feature is the sole basis of the majority of my experimental findings.

2. Treatment induced changes in methylation are not purely random, but show defined and reproducible patterns of disruption that accrue with time.

Changes in methylation on a genome-wide scale during promotion could be perceived, in general, as a result of random "hits" throughout the genome which, by chance, cause the activation of oncogenes and transposable elements or the suppression of tumor suppressor genes to facilitate tumorigenesis. This is supported by seemingly indiscriminate patterns of altered methylation and the varying extent to which particular sites are altered from one model system to the next. In addition, global genome wide hypomethylation is a common feature of tumor tissue, but the exact cause of each one of those hyomethylation events might be impossible to gauge, at least by today's technology, leading to the conjecture that the majority are random events.

From another perspective supported by my research, and one that I am more inclined to believe, "random" patterns of altered methylation are instead "complex". This implies a multi-factorial basis which may be complicated but can not be considered stochastic. Support for this is first seen in chapter 1, in which patterns of altered

methylation in response to DEA, CD, and PB share a ~70% similarity. Highly similar patterns of altered methylation indicate that some of the same pathways are disrupted, mis-directed, or mis-regulated to result in the highly consistent altered patterns of methylation. This is hypothesized for DEA and CD. DEA induces a choline deficient-like state, therefore, methyl deficiency is the end result of both treatments. Two separate treatments which upset homeostasis in the same way lead to the same changes in methylation in nearly identical regions of the genome.

Chapter 2 also demonstrated and supported the idea of non-random patterns of methylation. Highly methylated LINE-1 elements, a very large portion of the genome (~33%), were unaffected by PB promotion however, changes in methylation were seen in two separate regions of the promoter of Ha-*ras*. Therefore, multiple key factors involved in maintaining stable states of methylation were likely misdirected and thus, targeted to specific genes (e.g. Ha-*ras*). The idea of susceptible or targeted regions of the genome has been suggested previously in terms of methylation-prone and methylation resistant CpG islands (Feltus *et al.*, 2003). The hypothesis states that CpG islands differ in their inherent susceptibility to aberrant methylation and assumes that there are cis-acting factors which distinguish methylation-prone and methylation-resistant CpG islands. The data showed that CpG islands differ in their intrinsic susceptibility to *de novo* methylation, and suggested that the propensity for a CpG island to become aberrantly methylated can be predicted based on its sequence context (Feltus et *al.*, 2003). Therefore, the characteristics of the genome as well as the specific pathways and factors regulating patterns of methylation result in the complex, but not random alterations.

3. Hypomethylation, occurring simultaneously with hypermethylation, is a predominant and highly significant contributor to the development and progression of tumors.

Hypermethylation has received strong focus with good reason as a dominant force in the silencing of tumor suppressor genes. Methylation related gene silencing has been demonstrated for genes involved in apoptosis, angiogenesis, differentiation, DNA repair, metastasis and invasion, drug resistance, and signal transduction, among others (Costello and Plass, 2001). Research by Waston *et al.*, 2003 and 2004 outlined and demonstrated the importance of hypermethylation events in the progression of skin tumorigenesis with the finding that HOXA5, p16, and MGMT are all hypermethylated in response to CSC promotion. These hypermethylation events are critical to the development of tumors however, this approach is very much one sided.

Consistently, both increases and decreases in methylation are observed on a genome-wide scale in precancerous tissue. Therefore, in light of previous work, I concentrated more on the contribution of hypomethylation events to tumorigenesis. In chapters 1 and 2, the predominant alteration induced by DEA, CD and PB was hypomethylation within GC-rich regions. In chapter 3 a fairly dramatic increase in the number of hypomethylated regions was seen in tumor tissue as compared to precancerous tissue. This indicates that hypomethylation potentially plays a variety of roles in the development of tumors and maintenance of the neoplastic phenotype. As previously discussed, I have shown decreased methylation in the promoter region of Ha-*ras* and selective increases in expression in two separate model systems by examining both precancerous and tumor tissue. I believe, the true significance of this is really gained by coupling both the hypomethylation events of the current research with the previously

identified hypermethylation. One very intriguing concept based on the complementary actions of activation and silencing events is the possibility that upregulation of an oncogene can lead to down regulation of a tumor suppressor gene through promoter hypermethylation (Sung *et al.*, 2004). This brings together the importance of considering simultaneous changes as a cumulative series of epigenetic alterations leading to deregulated cell growth.

4. As outlined by the classic multi-stage model of carcinogenesis, the progressive accumulation of critical changes in DNA methylation, both increases and decreases, appears to be a universal feature of tumor promotion.

As presented by the working model of multi-stage carcinogenesis, the promotion stage of tumorigenesis involves the step-wise accumulation of heritable changes which are critical for the selection and clonal expansion of initiated cells (Dragan *et al.*, 1993). Chapters 2 and 3 specifically addressed this issue and demonstrated that regions of altered methylation seen at early time points were also identified at later precancerous and cancerous time points. The nature of the initiated cells could be one or more of the following three types of cells, 1) stem cells, 2) early stem cell progenitor cells or 3) dedifferentiated cells (Bjerkvig et al., 2005). In two distinct experimental models with two separate promoting agents, I have observed that regions of altered methylation carry forward with time. Our method is based on detecting the average change in methylation within a region. This implies that the majority of DNA within the whole liver or skin tissue across numerous animals had to be exhibiting either an increase or decrease within the same region. A small minority of cells would not be able to change the statistical

significance in comparison to control levels. Based on that concept I was likely measuring changes acquired by both mature hepatocytes and stem cells. This opens up the possibility that altered methylation, a heritable genomic change, accumulating in a progressive manner in the differentiated cells of the liver or stem cells could contribute to their evolution into the cancer stem cell-like state.

In either case, the ability to experimentally measure changes in methylation within fairly well defined regions of the genome that carry forward with time is significant. In chapter 2, this was demonstrated at very early time points (2 and 4wks) in relation to the appearance of foci and tumors in B6C3F1 mouse liver. Of particular interest in this model was the observation that a large proportion of the regions of altered methylation in B6C3F1 mouse liver carried forward in comparison to C57BL/6. This is a highly significant observation and shows that B6C3F1 mice accumulate changes much quicker and earlier than C57BL/6 which is in direct agreement with their relative sensitivities to tumor formation. Complementary to this observation was the fact that 81 unique regions of altered methylation were induced by PB in B6C3F1 mouse liver. Again in chapter 3, the same effect was seen in SENCAR mice at 4 and 8wk time points. Here, tumor tissue contained many of the changes generated by both 4 and 8wks of promotion. This is substantial evidence to support not only the heritability of altered patterns of methylation but the persistence and contribution of those changes to the tumor phenotype.

The SENCAR mouse model, was characterized by initiation with DMBA and promotion with CSC. B6C3F1 and C57BL/6 mice were promoted with PB and had no prior chemical initiation event. Although these two models are very distinct, I was able

to consistently show that changes in methylation were progressive. Importantly, this suggests that the accumulation of critical changes in DNA methylation, both increases and decreases, appears to be a universal feature of tumor promotion.

Conclusions

The multi-step (*i.e.* multi-mechanism) and multi-stage process of carcinogenesis is considered the operational framework for experimentally testing and explaining events leading to cell proliferation and tumor formation. Preserving normal patterns of DNA methylation is an essential part of maintaining homeostasis within a cell. The majority of methylation occurs at cytosines which are 5' to guanine and these CpG dinucleotides are located in gene coding and promoter regions (Costello and Plass, 2001) as well as in promoter-like regions of transposable elements (Liang et al., 2002) Therefore, altered methylation in the form of hypomethylation or hypermethylation in relation to control could contribute to the initiation and progression of tumorigenesis through the aberrant regulation of gene expression and the creation of genomic instability (Jones and Baylin, 2002).

Both a gene by gene approach to measuring changes in DNA methylation and a genome-wide approach are necessary for extending the understanding of altered patterns of methylation in the process of tumorigenesis, and its role in defining the susceptibility of an organism to form tumors. Key to my research was the development of a high-throughput and quantitative method for measuring changes in GC-rich regions of the genome. This method provided a means for comparing treatment induced regions of altered methylation over time, between mice with different genetic backgrounds, and

between target and non-target tissues. I was then able to combine this analysis with a targeted and specific approach to examining the promoter regions of the Ha-*ras* oncogene and LINE-1 elements. This was a powerful strategy which served to address my overall hypothesis from multiple angles.

I have characterized patterns of methylation in B6C3F1 mouse hepatocytes in culture in response to treatment with DEA, CD, or PB, all of which result in an increase in the incidence and multiplicity of liver tumors. This established that altered methylation is a fundamental consequence of all three treatments and patterns of methylation are highly similar among them supporting a non-random disruption of the genome. In addition, the simultaneous analysis of the Ha-*ras* oncogene and transposable elements showed that the characteristics of the genome as well as the specific pathways and factors regulating patterns of methylation likely result in the complex, but not random alterations.

I have demonstrated using two separate model systems that changes in methylation accumulate in a progressive manner with time. As seen with B6C3F1 and C57BL/6 mice, the extent and frequency with which changes in methylation accrue seems to strongly relate to their relative susceptibilities to liver tumorigenesis. Susceptibility may also directly relate to the number and identity of the unique regions of altered methylation observed in the B6C3F1 mice. In addition, with the SENCAR mouse skin initiation/promotion model, regions of altered methylation generated by promotion with CSC for 4 and 8wks of promotion carried forward to tumor tissue. This is substantial evidence to support not only the heritability of altered patterns of methylation but the persistence and contribution of those changes to the neoplastic state. In addition,

the large majority of the observed changes were reversible, consistent with the major hallmark of tumor promotion.

My research represents a logical extension of previous work by Counts *et al.*, 1996, Watson *et al.*, 2002b, and Watson *et al.*, 2003 and 2004. Importantly, I have presented novel and detailed data to support altered patterns of DNA methylation as a contributor to tumorigenesis. Overall, my data illustrate that instability of the epigenome is a key feature of susceptibility to tumorigenesis.

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