Methyl deficiency, DNA methylation, and cancer: Studies on the reversibility of the effects of a lipotrope-deficient diet

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Methylation of C residues in CpG sites in the regulatory regions of a wide variety of genes has been linked to silencing of their expression. During normal mammalian development, loss of methylation at specific sites accompanies tissue-specific activation of genes. Overall decreases in the level of DNA methylation and alterations in the pattern of methylation of specific genes are also closely linked to tumor development in humans and other mammals. Dietary methyl deficiency sufficient to cause hepatocarcinogenesis in male rats induces profound and rapid changes in the morphology and metabolic activity of liver cells. As we have previously reported, these changes include a decrease in the overall level of DNA methylation and alternations in the patterns of methylation and levels of transcripts of specific growth-related genes. These alterations persist as long as the rats are maintained on a methyl-deficient diet.

The starting hypothesis for the studies summarized here is that methyl deficiency induced changes in liver cells that persist, even when dietary sources of methyl groups are restored, are more likely to be critical for establishment of neoplasia than those that are reversible. We find that loss of methylation at specific sites in liver DNA persists for at least 9 weeks after restoration of methionine, choline, folate, and vitamin B_{12} to the diet of rats previously deprived of these nutrients for 4 weeks. Other molecular changes are reversed in less than 3 weeks. This suggests that exposure of rats to alternating periods of dietary methyl deficiency and sufficiency may provide an experimental model for determining whether persistent alterations in methylation of growth regulatory genes allow affected hepatocytes to escape constraints on cell division because they respond to growth stimuli differently than cells in which the genes are normally methylated. (J. Nutr. Biochem. 4:672–680, 1993.)

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Introduction

Enzymatic addition of methyl groups to carbon 5 of cytosine [C] residues in DNA can be considered the equivalent of introducing a fifth base, thus providing a mechanism for postsynthetic alteration of the information content of the genome. In mammalian cells, the most thoroughly documented function of this information is in providing one level of regulation of gene expression.[1,2] The presence of 5-methyl cytosine [5mC] residues in DNA influences interaction between pro-

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teins and DNA. 5mC residues in regulatory regions of genes can directly prevent or enhance binding of transcription factors.[3-5] 5mC residues in DNA also enhance binding of proteins that may serve to alter the accessibility of DNA to transcription factors or to enhance or stabilize the packaging of DNA into "inactive" chromatin.[6-8] Although interactions between 5mC residues in DNA and protein have generally been found to have a negative influence on transcription, there is no a priori reason to rule out the possibility that 5mC residues in specific sites in DNA might also enhance transcription by blocking binding of negative regulatory factors or enhancing binding of positive regulatory factors. A positive correlation between gene expression and methylation has been reported for a few genes.[9,10]

Methylation of DNA appears to play an important role in normal embryonic development.[11] Patterns of methylation of specific genes undergo characteristic modification during development. Loss of methylation at regulatory sites correlates with their activation in specific tissues. There is evidence that inactivation of one of the two X chromosomes in female mammalian somatic cells involves DNA methylation and that allele-specific methylation may be a factor in genomic imprinting.[12-17]

Conversely, disruption of normal tissue-specific patterns of DNA methylation has been implicated in development of cancer. Many carcinogens block DNA methylation either by direct alkylation and inactivation of DNA methyltransferase or by forming adducts with DNA that render it a poor substrate for the methyltransferase.[18-20] The 5mC content of DNA from tumors and tumor-derived cell lines is generally lower than that observed in normal tissues.[20] Hypomethylation of specific sites in proto-oncogenes such as c-*myc*, c-*Ki-ras* and c-*Ha-ras* has been detected in a variety of human and animal tumors.[21-26] Interference with methylation in vivo has also been shown to lead to tumor formation in animals. Feeding of methylation inhibitors such as L-ethionine or 5-azacytidine and induction of decreased ratios of AdoMet:AdoHcy through dietary depletion of methionine and choline have all been shown to induce formation of tumors in the liver and other organs in rats.[27-33]

In one of the best-studied examples of human tumor progression, it has been shown that hypomethylation of DNA is an early event in the development of colon cancer, although the changes observed do not always correlate with changes in gene expression.[22,25,26] In addition, some genes in the colon tumors become "hypermethylated," leading to the suggestion that silencing of tumor suppressor genes by methylation may also play a role in tumor development.[34,35]

The studies summarized here represent our approach to determining how loss of methylation at specific sites in growth regulatory genes is related to the ability of altered hepatocytes to escape the normal constraints on cell division.

Methods and materials

Animals and diet

The diets used were based on a semisynthetic formulation, ADD-A14400, obtained from Research Diets, Inc., New Brunswick, NJ USA. This formulation lacks methionine, choline, vitamin B_{12}, and folic acid. Male Fischer 344 rats were fed either a methyl-deficient diet [MDD-Diet A14403-ADD supplemented with 9 g/kg D,L-homocystine] or an adequate diet [CSD-Diet A14404-ADD supplemented with 5 g DL-methionine, 2 g choline chloride, 5 mg folic acid, and 100 μg vitamin B12/kg]. MDD is more severely deficient in sources of methyl donor than diets referred to by other authors in this series.[36-40] However, during a 4-week course of feeding, the effects of this diet on the liver cannot be distinguished from those of a diet deficient solely in choline and methionine.[41] To compare the effects of MDD with effects of carcinogen exposure, rats were fed ad libitum Purina chow (meal) (Ralston Purina, St. Louis, MO USA) containing a carcinogenic dose of 2-acetylaminofluorene [AAF-0.06% wt/wt] or DL-ethionine [0.5% wt/wt] as indicated. All rats were cared for in accordance with institutional guidelines. The diets, feeding protocols, and methods for DNA and RNA methyltransferase, DNA and RNA isolation, and for assays of methyltransferase activity and evaluation of DNA methylation status of bulk DNA and specific genes have been described.[42-49]

Labeling index

BrdUrd [200 mg/kilo] was injected intraperitoneally 1 hr prior to sacrifice. One × 1.2 × 0.3-cm pieces from each liver lobe were fixed in neutral buffered formalin for 24 hr prior to paraffin embedding. A representative section of ileum was processed on each slide as a positive control for BrdUrd incorporation. For immunohistological staining, 5-μm sections were mounted on slides coated with 0.5% gelatin in 0.05% potassium dichromate. The sections were depariffinized, rehydrated, and sequentially digested with pepsin and a combination of EcoRI nuclease with Exonuclease III as described.[50] After incubation for 30 min in 0.3% H_2O_2 to block endogenous peroxidase, the sections were incubated for 30 min with 0.1% bovine serum albumin in phosphate buffered saline to reduce nonspecific antibody binding. Incubation with monoclonal anti-BrdUrd (M-744, Dako Corp., Carpinteria, CA USA) at a dilution of 1:20 was carried out overnight at 4° C. ABC-peroxidase staining was carried out as described by the supplier of reagents (Vectastain, Vector Laboratories, Inc., Burlingame, CA USA). Liver sections from untreated rats and sections reacted with nonimmune goat IgG were used as negative controls. All sections were lightly counterstained with hematoxylin prior to microscopic examination for labeled nuclei, accumulation of fat droplets, and altered morphology. The labeling index (LI) was determined by counting the number of BrdUrd-labeled nuclei in 10 random X10 fields.

Results

Effects of methyl deficiency on nucleic acid methylation and gene expression

Short-term (1 to 4 week) feeding of MDD to rats leads to significant hypomethylation of DNA and tRNA in

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the liver as determined by the ability of these nucleic acids to serve as substrates for methyltransferases in vitro[36] (*Table 1*). The methyl group acceptance of DNA isolated from the livers of rats fed MDD for 4 weeks is 3 to 4-fold higher than acceptance by DNA from livers of rats CSD for the same period of time. Methyl acceptance of tRNA from the same livers is 5- to 10-fold higher in rats fed MDD compared with CSD. This suggests that during feeding of MDD both DNA and tRNA are being synthesized under conditions that are not optimal for efficient transfer of methyl groups, i.e., that newly synthesized nucleic acids are not being fully methylated in vivo which makes them better methyl acceptors in vitro. It can be concluded that hypomethylation does not result from increased turnover or decreased synthesis of methyltransferase because an increase in the activity of both DNA methyltransferase and N2-guanine tRNA methyltransferase II[51] (*Table 1*) is observed in extracts from the same livers that contain hypomethylated nucleic acids. However, depletion of intracellular pools of the methyl donor AdoMet and an increase in levels of AdoHcy, a competitive inhibitor of AdoMet-dependent methyl transfer reactions, that occur within 1 week of feeding MDD[29] (diet 5) are likely to account for inhibition of nucleic acid methylation.

Feeding of MDD for periods of 1 to 4 weeks also leads to a time-dependent loss of methylation of specific CCGG sites in the c-*Ha-ras*, c-*fos*, and c-*myc* genes that can be detected as increased sensitivity to cutting by the restriction endonuclease HpaII. HpaII cleaves CCGG sites but not C5mCGG sites where 5mC is present in one [hemi-methylated sites] or both strands [fully methylated sites]. The alteration in methylation patterns of these genes is accompanied by an increase in the levels of their mRNA transcripts. It should be noted that the effects of the diet on DNA methylation in the liver are neither random nor universal. None of the genes studied lost methylation at all CCGG sites, as would be indicated by detecting the same cleavage pattern in HpaII- and MspI-digested DNA. CCGG sites in the c-*Ki-ras* genes remain fully methylated, and c-*Ki-ras* mRNA levels do not change in the livers of rats fed MDD for 4 weeks.[44] These results suggest that methylation at some CpG sites is maintained more efficiently than at others when the supply of AdoMet is limiting. It remains to be determined whether this difference is due to an effect of the sequence context of CpG sites on the activity of mammalian DNA methyltransferase or to differences in association with specific nuclear proteins or in chromatin configuration at some CpG sites that limits their ability to serve as substrates for DNA methyltransferase.

It has been well documented that feeding of lipotrope-deficient diets to rats for periods as short as 1 week causes fat accumulation in liver cells and a stimulation of cell proliferation.[27,52,53] Although there is marked variation between animals in the degree of proliferative response, feeding of MDD consistently leads to at least a four-fold increase in the number of proliferating cells within 24 to 96 hr (*Table 2*). The labeling index of liver

Table 2 Effect of dietary methyl deficiency and subsequent restoration of adequate levels of methyl groups on the labeling index in rat liver

Diet	Days	BrdUrd (+) Nuclei*
MDD	1	230 ± 150 (8)
	4	160 ± 100 (8)
	28	200 ± 80 (8)
CSD	1	60 ± 10 (4)
	4	16 ± 5 (4)
	28	8 ± 2 (4)
	56	5 ± 1 (4)
MDD for 28 days followed by CSD for	1	46 ± 10
	2	20 ± 5
	4	16 ± 5
	28	7 ± 2

*Average number of peroxidase (+) nuclei/10 × 10 fields ± SE in tissue slices derived from three different areas of the liver. The number of livers examined is indicated in parentheses.

Table 1 Reversibility of diet-induced alterations in DNA and tRNA methylation and in levels of DNA and tRNA methyltransferase activities in rat liver

	Methyl acceptance in vitro		Methyltransferase activity	
Diet*	DNA (cpm × 10⁻³ $^{3}CH_3$/2μg)	tRNA (cpm × 10⁻³ $^{3}CH_3$/10μg)	DNA (cpm × 10⁻³ $^{3}CH_3$/mg protein)	tRNA (cpm × 10⁻³ $^{3}CH_3$/mg protein)
CSD, 4 weeks	5.1	0.8	32	5
MDD, 4 weeks	15.0	8.1	100	12
MDD, 4 weeks Followed by:				
CSD, 1 week	5.8	1.5	36	6.8
CSD, 2 weeks	4.4	1.4	35	6.6

Data shown are from a representative experiment. Each point is the average value for triplicate determinations in two separate assays on pooled DNA or tRNA or enzyme extract from three animals. Variation between determinations did not exceed ±8%. Adapted from data in Reference 54.

*MDD is a methyl-deficient semisynthetic diet [ADD] supplemented with 9 g/kg DL-homocystine; CSD is an adequate diet, ADD, supplemented with 5 g DL-methionine, 2 g choline chloride, 5 mg folic acid, and 100 μg vitamin B_{12}/kg.

cells remains high throughout 4 weeks of feeding MDD (*Table 2*). Fat accumulation can be detected within 48 hr in the liver cells of all animals fed MDD (*Figure 1a*). Within 96 hr, large fat droplets are present in almost all cells in the liver (*Figure 1b*); the fat content of the liver continues to increase until, by 4 weeks, normal-appearing hepatocytes are rare.

Reversal of effects of MDD feeding

To determine the stability of the metabolic and morphological changes and changes in nucleic acid methylation and gene expression induced in liver cells by short-term dietary methyl deficiency, adequate levels of methionine, choline, folate, and vitamin B_{12} (CSD) were restored to the diet of rats fed MDD for 4 weeks. The rate of cell proliferation, as indicated by incorporation of BrdUrd into DNA, dropped precipitously within 24 hr and returned to the levels found in age-matched animals continually maintained on CSD within a few days (*Table 2*). The overall extent of DNA and RNA methylation were restored to normal levels within 1 to 2 weeks of restoring lipotropes to the diet[54] (*Table 1*). At the same time, activities of DNA and tRNA methyltransferases in the liver dropped to normal values (*Table 1*). mRNA levels for all of the growth regulatory genes studied also returned to control levels within 1 to 3 weeks.[54] Northern analysis of c-myc mRNA levels is shown as an example (*Figure 2*). In contrast, MDD-induced hypomethylation of specific HpaII sites in c-*myc*, c-*fos*, and c-*Ha-ras* genes persisted for up to 3 weeks after refeeding an adequate diet.[54]

We are in the process of examining the extent of methylation of HpaII sites in these genes during an extended period after restoring lipotropes to the diet of rats fed MDD for 4 weeks. Southern blot analysis of HpaII digests of the c-*myc* gene in DNA isolated from rat liver during a course of feeding of MDD indicates that fragments of ~1.7 and 1.5 kbp become more abundant, and that there is progressive loss of HpaII fragments >4.2 to 5 kbp[54] (*Figure 3*, compare lanes 1 and 2 in regions indicated by arrows). Even though sufficient levels of methyl donors are available to allow normal methylation of tRNA and bulk DNA, these alterations in the normal pattern of liver cell c-*myc* gene methylation persist for at least 9 weeks (*Figure 3*, lanes 3–5). During the same period, the normal morphology of the liver is slowly restored (Data not shown). Although little cell proliferation is occurring (*Table 2*), the number of normal-appearing hepatocytes increases in a few days, presumably because secretion of very low density lipoprotein (VLDL) resumes after restoration of methionine and choline to the diet.[56] However, even after 4 weeks on an adequate diet, the livers of rats previously fed MDD still have patches of cells with large fat droplets.

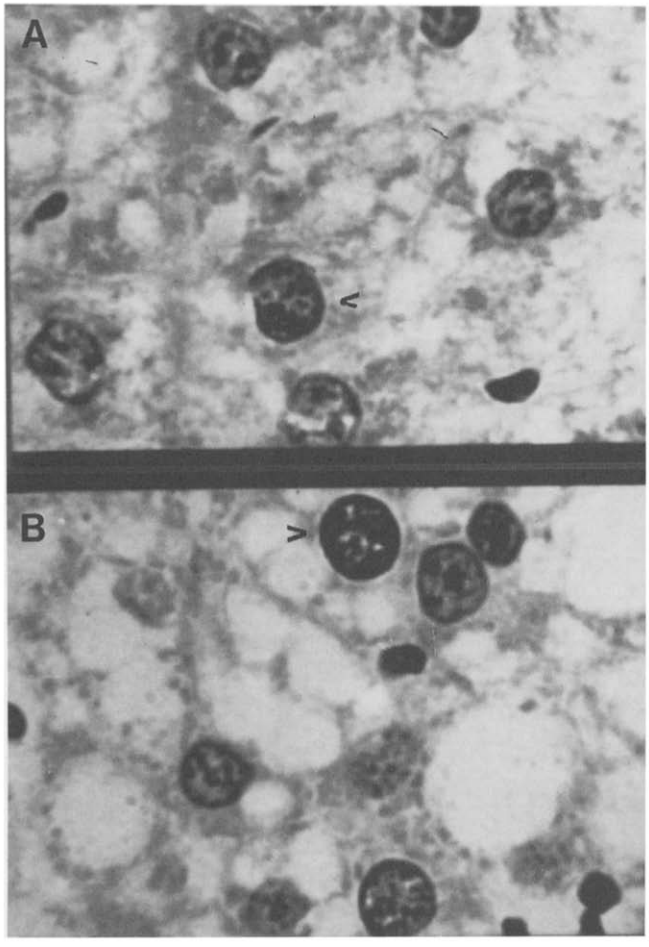

Figure 1 Rapid effect of dietary methyl deficiency on accumulation of lipid in rat liver. A: 48-hr feeding of MDD. Multiple large fat vacuoles (unstained areas) are obvious in most hepatocytes. B: 96-hr feeding of MDD. Fat vacuoles are greatly expanded in size compared with those seen at 48 hr. Normal hepatocyte morphology is severely disrupted. Representative areas from formalin-fixed liver sections processed as described in Methods and materials. Animals were injected i.p. with BrdUrd 1 hr prior to harvesting liver. Solid arrows indicate BrdUrd positive nuclei. Photomicrographs were taken at 1000 × magnification.

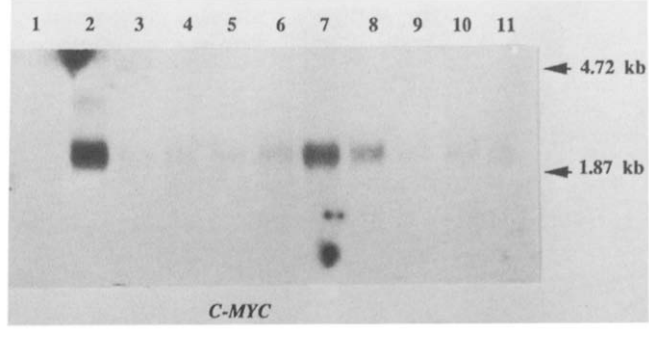

Figure 2 Effect of dietary methyl deficiency and subsequent feeding of an adequate diet on levels of c-*myc* mRNA in rat liver. Northern blot analysis of poly(A⁺) RNA (5 μg/lane) of rats fed: lane 1, control diet (CSD) for 4 weeks; lane 2, methyl deficient diet (MDD) for 4 weeks; lanes 3–5, CSD for 5 weeks; lanes 6–8, MDD for 4 weeks followed by CSD for 1 week. Lanes 9–11, MDD for 4 weeks followed by CSD for 3 weeks. RNAs in each lane were prepared from individual livers. The blot was probed with pSVcmycI, which contains the second and third exons of the mouse c-*myc* gene. All methods have been previously described.[44]

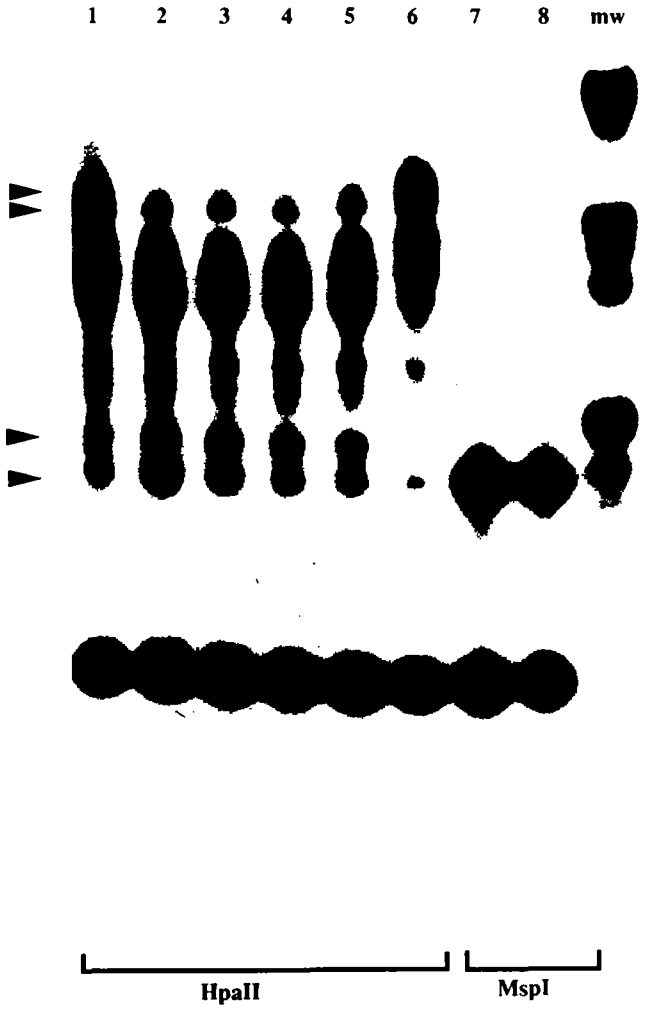

Figure 3 Effect of dietary methy. defic ency and subsequent feeding of an adequate diet on methylation of CCGG sites n the c-*myc* gene. Southern blot analysis of HpaII (lanes 1–6) and MspI (Lanes 7,8) digested DNAs from rats fed anes 1,7 control diet (CSD) for 4 weeks. lane 2, methy' deficient diet (MDD) for 4 weeks ane 3 MDD for 4 weeks followed by CSD for 3 weeks. lanes 4,8 MDD for 4 weeks followed by CSD for 6 weeks. Lane 5, MDD for 4 weeks followed by CSD for 9 weeks. Lane 6 CSD for 13 weeks. Arrows ind.cate fragments whose concentration was altered in digests of DNA from MDD-fed rats as compared with CSD-fed rats. The blot was probed with a purified 2.4 kbp XbaI-HindIII fragment from pSVcmyc1 that includes the second and third exon of the mouse c-*myc* gene.[56]

Effect of MDD on p53 gene methylation and expression

The p53 tumor suppressor gene product, which can act as a transcription factor,[57] appears to negatively regulate passage of cells through the cell cycle.[58–60] Cells lacking functional p53 protein continue through S-phase after DNA damage, while cells with normal p53 protein arrest in G_1.[61] It has been suggested that loss of p53 or functional changes in p53 protein that prevent this block might allow more frequent use of a damaged template and fixation of mutagenic lesions.[61] Expression of abnormal p53 protein has been detected in livers of rats fed choline-deficient diets for 12 months.[62] Although we have not been able to detect increased p53 protein production in rat liver after 4 weeks of feeding MDD by immunohistochemical methods or by Western blotting (data not shown), levels of p53 mRNA increase markedly within 2 weeks (*Figure 4*, lanes 7, 8). This increase is comparable to that found on feeding the hepatocarcinogens AAF or DL-ethionine for 2 to 4 weeks (*Figure 4*, lanes 1, 4, 5) and only slightly lower than that observed in an AAF-induced tumor (*Figure 4*, lane 9). HpaII sites in the p53 gene(s) that become hypomethylated in DNA from livers of rats fed MDD (*Figure 5*, compare lanes 1, 2 with lanes 3, 4) can be detected using full-length human p53 cDNA as probe.[63] Similar to what was observed for c-*myc*, c-*Ha-ras*, and c-*fos* genes, loss of methylation at these sites persists for at least 3 weeks (lanes 5, 6). This suggests that hypomethylation of the p53 gene may also act to enhance its transcription. However, because there are at least two p53 pseudogenes in the rat genome,[64] it will be necessary to localize the unmethylated HpaII

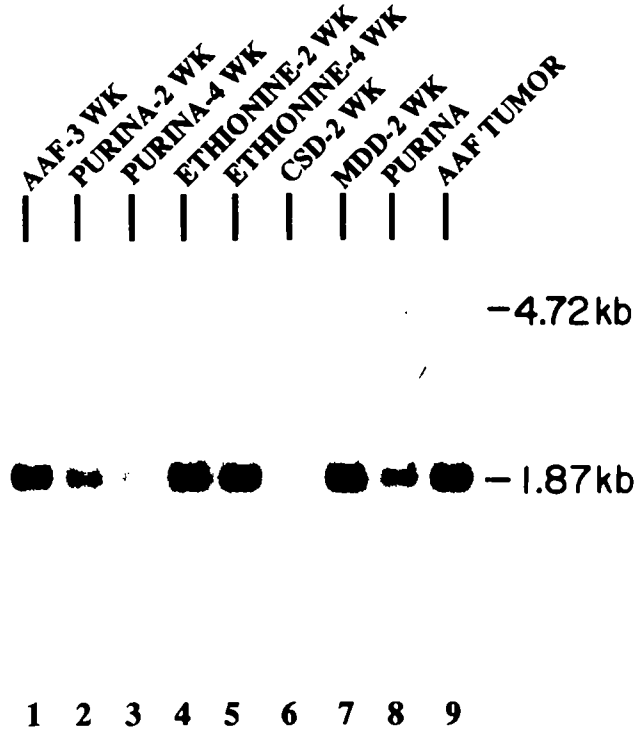

Figure 4 Effect of carcinogen feeding or methyl deficiency on levels of P53 mRNA in rat liver. Northern blot analysis of poly(A+) RNA (5 μg lane) of rats fed: Lane 1, 0.06% wt/wt AAF in Purina chow for 3 weeks. lanes 2,3, Purina chow rats age matched to those fed carcinogens for 2 and 4 weeks respectively. lanes 4,5 0.5% wt/wt DL-ethionine in Purina chow for 2 and 4 weeks, respectively: lane 6 CSD for 2 weeks. lane 7 MDD for 2 weeks. lane 8 Purina chow for 10 mos. lane 9, tumor induced by feeding of AAF for 8 mos. All rats were 6–8 weeks old at the time feeding of experimental diets was started. Female Fisher 344 rats were used for experiments with DL-ethionine feeding because males are not susceptible to DL-ethionine carcinogenesis.[65] Male Fisher 344 rats were utilized for all other experiments. The blot was probed with a purified 2.1 kb BamHI fragment from pnp53c1[66] that contains the entire coding region of human p53 cDNA.

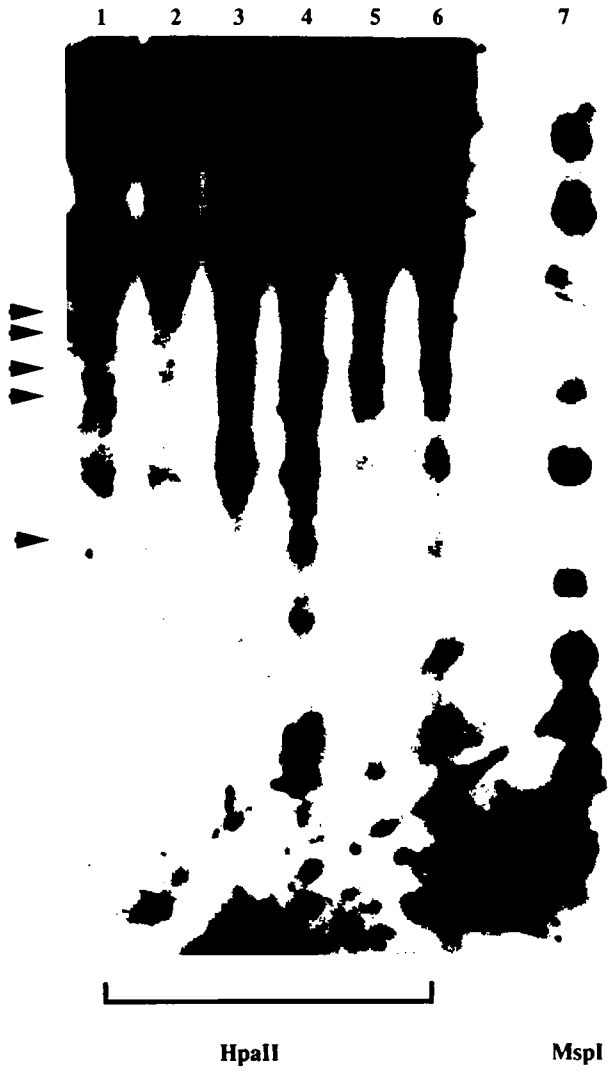

Figure 5 Effect of dietary methyl deficiency and subsequent feeding of an adequate diet on methylation of CCGG sites in the p53 gene. Southern blot analysis of HpaII (lanes 1–6) and MspI (lane 7) digested DNAs from rats fed: lanes 1, control diet (CSD) for 4 weeks; lane 2, methyl-deficient diet (MDD) for 1 week; lanes 3,4, MDD for 4 weeks; lane 5, MDD for 4 weeks followed by CSD for 1 week; lane 6, MDD for 4 weeks followed by CSD for 3 weeks. Arrows indicate fragments whose concentration was increased in digests of DNA from livers of rats fed MDD for 4 weeks [lanes 3,4] or MDD for 4 weeks followed by CSD for 1–3 weeks [lanes 5,6] as compared with animals fed CSD continuously (lane 1). These additional bands indicate that at least some CCGG sites in the p53 gene became completely and persistently unmethylated during the period of dietary methyl deficiency. The blot was probed with a purified 2.1 kb BamHI fragment from php53c1[55] that contains the entire coding region of human p53 cDNA.

sites in the active p53 gene before a link between the pattern of methylation of the p53 gene and its expression can be established.

Discussion

Because of the rapid disruption of normal liver cell morphology and the large number of metabolic changes induced by dietary lipotrope deficiency in rodents, it is difficult to ascribe the diet's ability to promote or cause liver carcinogenesis to any one factor. Rapid fat accumulation in livers with accompanying and continued cycles of cell death and proliferation are likely to have a promoting effect on tumor development, acting to favor the expansion of clones of initiated liver cells.[27,52,65] Potentially mutagenic radicals generated by increased lipid peroxidation may also play the role of initiators of hepatocarcinogenesis.[66–68] Our studies have focused on early changes in transmethylation reactions that are influenced by hepatic levels of AdoMet and AdoHcy. The results of these studies are consistent with the hypothesis that the ability of lipotrope-deficient diets to induce alterations in the pattern of DNA methylation contributes to their promoting and carcinogenic potential by allowing increased expression of some critical gene or genes. However, it is highly unlikely that the massive changes we detect in extent of methylation in specific DNA sequences during MDD feeding would be possible in the absence of the proliferative effect of the diet. The complete disappearance of some large HpaII fragments of the c-*myc* genes in the liver after 4 weeks of feeding MDD (*Figure 3*, lane 2) indicates that at least one HpaII site in these fragments has become completely unmethylated in virtually all copies of this gene in the liver. If loss of methylation occurs primarily as a result of failure to methylate newly synthesized DNA after replication, almost all of the cells in the liver would have to undergo 2 to 3 rounds of DNA replication in the absence of methylation at these sites for such a conversion to occur.[24]

Our aim in studying the effect of restoring lipotropes to the diets of rats fed MDD was to determine which of the changes resulting from short-term exposure to dietary methyl-deficiency are persistent and, thus, more likely to be preneoplastic or neoplastic in nature.[69] Among the parameters we have studied, changes in patterns of methylation of specific genes proved to be the most persistent, with some CCGG sites maintaining their unmethylated status for as long as 9 weeks. Because the percentage of proliferating liver cells returns to a normal level within a few days of restoring lipotropes to rats fed MDD, and overall levels of tRNA and DNA methylation return to normal in 1 to 2 weeks, this persistent hypomethylation cannot be the result of continuing synthesis of hypomethylated DNA. Rather, it indicates that de novo methylation at the completely unmethylated sites recognized by HpaII occurs very slowly, if at all, in adult rat hepatocytes. If this is the case, it can be predicted that the pattern of methylation of specific genes will return to normal only if hepatocytes with hypomethylated sites in their DNA are replaced by cells with normal patterns of methylation during liver cell turnover. This could occur if stem cells in the liver were either better able to maintain normal patterns of DNA methylation during feeding of a lipotrope-deficient diet or better able to methylate completely unmethylated sites de novo than mature hepatocytes.

While persistent hypomethylation of c-*myc*, c-*fos*, and c-*Ha-ras* genes may be required for the sustained

increase in transcript levels observed in the liver during MDD feeding, it is clearly not sufficient to maintain them in quiescent cells. Levels of c-*myc*, c-*fos*, and c-*Ha-ras* mRNAs return to normal within 1 to 3 weeks of restoring an adequate source of lipotropes to the diet while hypomethylation of these genes may persist for up to 9 weeks. This suggests that transcription factors needed for efficient expression of these genes may be present only in mitogenically stimulated or proliferating liver cells. However, if hypomethylated growth regulatory genes are more readily activated than the corresponding genes with normal patterns of methylation for adult liver, cells containing the hypomethylated genes may be more likely to escape normal constraints on cell division when exposed to low doses of carcinogen or to mitogenic stimuli.

Current theories of carcinogenesis postulate that multiple gene alterations are necessary for the development of malignant tumors.[70–73] These include activation of several classes of oncogenes, inactivation or loss of tumor suppressors, and changes in expression of genes regulating metastasis. Activation of oncogenes can be accomplished by mutation, amplification, or increased levels of expression.[70] Loss of the negative influence of tumor suppressors on cell growth can occur either through gene deletion or inactivation or introduction of mutations that cause loss of function in the product of one or both alleles.[58,74]

As described above, feeding of a lipotrope-deficient diet leads to increased levels of p53 mRNA in the liver and decreased methylation of sites in a p53 gene. This result is the opposite of what would be expected if early silencing of p53 expression by increased methylation of the p53 gene plays a role in the carcinogenic effects of lipotrope deficiency. Because increases in both p53 mRNA and protein occur prior to DNA synthesis in nontransformed quiescent cells stimulated to enter the cell cycle,[75] increased p53 mRNA levels may simply reflect the fact that most of the cells in the liver of lipotrope-deficient animals have been recruited from G_0 into active cell division. A second possibility is that the increase in p53 mRNA represents a cellular response to increased oxidative damage to DNA. Levels of p53 protein have been shown to increase in response to DNA damage.[61] This causes cells that produce normal p53 to arrest in G_1 and allows repair of DNA lesions. Cells that do not express p53 protein or express a mutant p53 protein continue through S-phase, presumably allowing more frequent use of damaged DNA templates and fixation of mutagenic lesions.[61] Interestingly, it has been reported that overproduction of normal p53 protein can lead to self aggregation and loss of function; a correlation has been found between the elevated stability of normal p53 that results from self aggregation and cell transformation.[76] Conversely, overexpression of normal p53 protein can inhibit focus formation by transformed cells and cause apoptosis in cells lacking normal p53 function.[77,78]

The low levels of p53 protein found in normal cells are the result of production of p53 protein with a characteristically short half life.[79,80] Our inability to detect increased levels of p53 protein in livers of rats fed MDD for 4 weeks tends to favor the hypothesis that p53 mRNA levels in the livers of methyl-deficient rats are elevated in proportion to the number of cycling cells, but that little p53 protein accumulates because of its rapid turnover. However, we cannot rule out the possibility that overproduction of p53 protein occurs in a small proportion of liver cells undergoing apoptosis, or that with continued feeding of MDD some cells are transformed through overproduction of p53 protein.

There are a number of indications that common alterations in gene expression occur during the process of hepatocarcinogenesis, regardless of the initiating carcinogen or promoting regime. These include increased expression of c-*myc* and one or more members of the *ras* gene family and decreased expression of cell surface receptors such as the epidermal growth factor receptor and the insulin receptor.[81,82] Many of these alterations are still present in liver tumor cells. For example, hypomethylation of CCGG sites in the region of the second to third exon of the c-*myc* gene, the same sites that become persistently hypomethylated after feeding of a methyl deficient diet, are a common feature of tumor-derived cell lines and human hepatomas.[83,84] An increase in the level of p53 mRNA appears to fall in this category of changes. The same elevated levels of p53 mRNA are found in AAF-induced liver tumors; in livers of rats fed methyl-deficient diets; livers of rats fed AAF, a mutagenic carcinogen that forms DNA adducts; and livers of rats fed L-ethionine, a nonmutagenic carcinogen that acts to inhibit transmethylation reactions.

The methyl-deficient diet employed in our studies is so severe that it does not allow survival of rats for the 70 to 80 weeks required for tumor formation.[39] However, feeding MDD for 15 weeks, followed by maintenance on a chow diet, is sufficient to achieve a 24% incidence of neoplastic nodules in the liver,[51] suggesting that the irreversible changes required for tumor development occur fairly rapidly with this diet. Studies are in progress comparing the effects of MDD feeding on liver cell division and gene expression in age-matched rats continually fed CSD and rats that have recovered from a 4-week course of MDD feeding that still have hypomethylated sites in liver DNA. These studies should allow an evaluation of the importance of persistent undermethylation of growth regulatory genes for hepatocarcinogenesis.

Although it is unlikely that human diets are ever as severely methyl deficient as the experimental diet used here, in many parts of the world it is not uncommon to find diets that are marginally deficient in lipotropes.[85] In both rural and industrialized nations, excess intake of alcohol, administration of certain therapeutic drugs, and exposure to mycotoxins or chemical carcinogens are not infrequent.[18,19,86–89] All of these factors are capable of inhibiting DNA methylation either by reducing AdoMet levels, inactivating DNA methyltransferase, or modifying DNA in such a way as to render it a poor substrate for methylation. If unmethylated sites in specific genes induced by exposure to intermittent exposure to dietary methyl deficiency or other factors that inhibit DNA

methylation are as resistant to de novo methylation as the experimentally induced unmethylated sites detected in our studies, it is possible that cumulative hypomethylation of genes could contribute to the causation of liver cancer in humans.

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