Maternal Methyl Supplements in Mice Affect Epigenetic Variation and DNA Methylation of Offspring

Craig A. Cooney,*†3 Apurva A. Dave,*† and George L. Wolff*,**

*Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205 and Divisions of †Molecular Epidemiology and **Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079

ABSTRACT This study was designed to determine if maternal dietary methyl supplements increase DNA methylation and methylation-dependent epigenetic phenotypes in mammalian offspring. Female mice of two strains were fed two levels of dietary methyl supplement or control diet prior to and during pregnancy. Offspring of these mice vary in phenotype, which is epigenetically determined and affects health and 2-y survival. Phenotype and DNA methylation of a long terminal repeat (LTR) controlling expression of the agouti gene were assayed in the resulting offspring. Methyl supplements increase the level of DNA methylation in the agouti LTR and change the phenotype of offspring in the healthy, longer-lived direction. This shows that methyl supplements have strong effects on DNA methylation and phenotype and are likely to affect long-term health. Optimum dietary supplements for the health and longevity of offspring should be intensively investigated. This should lead to public policy guidance that teaches optimal, rather than minimal, dose levels of maternal supplements.


KEY WORDS: • diet • methylation • epigenetic • maternal • supplement

Epigenetic variation in populations

Health and life span within cohorts of mammals can depend on many factors, including diet, lifestyle, genetics and epigenetics. In humans, all of these factors are no doubt important, although difficult to assess individually. However, in many rodent studies, some of the variation in these parameters has been minimized, if not eliminated, and yet considerable health and life span variation remains. For example, C57BL6 mice and Fischer 344 rats are highly inbred and contain very little genetic variation, yet these species show substantial variation in life span with the earliest deaths often occurring at about 18 mo of age. However, as many as 10% of the animals may survive to 30 mo or longer (Fig. 1). In these studies, considerable credible efforts were made to assure that animals received the same food and were maintained at the same temperature, humidity, light and dark cycle, etc. (1–5). Although there may have been some variations in the amount of food eaten, experiments to control this variable still yield a considerable spread in longevity (1–5).

Epigenetic variation has been described in several mammalian species including the mouse (6), fox (7) and rat (8). Even though epigenetic variation is likely a key factor in mammalian health and life span variance, it remains uncontrolled and unaccounted for in most populations. As with other traits, it seems likely that epigenetically determined ones vary across a range (Fig. 1), even though they rarely present themselves in ways that are readily observed and quantified. Epigenetic and environmental interactions can be studied with minimal confounding genetic and environmental variation by using well-characterized inbred mice.

DNA methylation and epigenetics

In mammals, as well as higher plants, birds, reptiles, fish and some fungi, several aspects of genome control are affected by
DNA methylation [i.e., 5-methyldeoxycytidine (5-MC)]. These aspects of genome control include suppression of expression by intragenic parasites such as endogenous retroviruses (ERVs) (10–12); inactivation of X chromosomes (13,14); and silencing of some, possibly many, genes including many showing genomic imprinting and epigenetic inheritance (12,15–19). ERVs contain viral genes and promoter enhancer sequences [long terminal repeats (LTRs)] and can express their viral genes as well as drive expression of nearby housekeeping or tissue-specific genes (6,10–12). 5-MC is the best-characterized epigenetic mechanism in mammals, and both DNA methyltransferase (Dnmt) and one of the chromatin proteins that bind methylated DNA [methyl-cytosine guanine dinucleotide (CpG)-binding protein 2 (MeCP2)] are necessary for mammalian development (20,21). Inheritance of 5-MC patterns, by dividing cells in vivo or in vitro, can occur with imperfect fidelity. The mechanism appears to hinge on a marked (10- to 20-fold) substrate preference of the maintenance methylase for methylated DNA, which probably is due to DNA methylation [methyl-cytosine guanine dinucleotide (CpG)-binding protein 2 (MeCP2)] are necessary for mammalian development (20,21). Inheritance of 5-MC patterns, by dividing cells in vivo or in vitro, can occur with imperfect fidelity. The mechanism appears to hinge on a marked (10- to 20-fold) substrate preference of the maintenance methylase for methylated DNA, which probably is due to DNA methylation.

Somatic 5-MC patterns from one generation of animals to the next have been demonstrated (17,18,30).

**Methyl metabolism and 5-MC**

The methyl groups of 5-MC are either synthesized de novo in one-carbon metabolism or are supplied preformed in the diet. These processes rely on essential dietary folates or folic acid (for one-carbon metabolism), essential dietary methionine or dietary or endogenous betaine and choline (preformed methyl groups). Likewise, methionine, zinc and vitamin B-12 (cobalamin) are all dietary essentials and are used as intermediates and enzymatic cofactors to transport and transfer methyl groups in methyl metabolism (31,32) (Fig. 2). Dnmts use S-adenosylmethionine (SAM) as a donor to methylate cytosines in DNA. At least some Dnmts are inhibited by the reaction product S-adenosylhomocysteine (SAH), and some or all are zinc-finger enzymes (24,33–35).

Extensive nutritional deficiency studies with adult rodents, as well as recent studies of adult humans, show that levels of 5-MC are dependent on nutritional factors such as dietary folate, methionine and choline (31,36–38).

Mammals and other animals have been selected for efficient youthful reproduction and not for extended longevity (31). There is no a priori reason for thinking that nutritional requirements adequate for growth and reproduction are optimal for long-term health and longevity. Likewise, maternal nutrition adequate for producing offspring with normal growth and reproduction is not necessarily the optimal balance for offspring’s long-term health and longevity.

To address questions of optimal nutritional balance, it is necessary to study supplementation as well as deficiency. Few studies address the role of methyl supplementation in DNA methylation or in determination of epigenetic phenotype (6).

**The yellow agouti mouse: epigenetics linked to coat color**

Epigenetically determined characteristics produce interesting developmental patterns in many animals, yet detection of these patterns may be indirect and require special assay techniques that may obscure the complexities of the patterns. Some animals have epigenetically determined coat color patterns where considerable complexity can be readily observed. In particular, epigenetic variation in expression of the agouti gene is seen in mice with the viable yellow allele (A\(^{\text{Avy}}\)) of agouti. Expression of agouti in this A\(^{\text{Avy}}\) allele is driven by an ERV LTR, specifically by the LTR of an intracisternal A particle (IAP) sequence. Due to epigenetic regulation, as indicated by an animal’s coat color (6) and 5-MC (18), this A\(^{\text{Avy}}\) gene is expressed to different degrees in genetically identical mice. Failure to epigenetically suppress the A\(^{\text{Avy}}\) gene during development causes the agouti gene to be ectopically overexpressed later in life. This high level of agouti expression in essentially all tissues causes numerous downstream metabolic and endocrine effects that ultimately affect gross biological end points such as obesity and survival. This agouti overexpression and its physiological effects have been termed the yellow agouti obese mouse syndrome. This syndrome includes a yellow or mottled yellow coat color, altered metabolism and obesity from a young age. It also results in adult diabetes, increased cancer susceptibility and, by 24 mo of age, twice the mortality seen in normal mice (6,39).

In mice carrying the A\(^{\text{Avy}}\) allele, a continuous spectrum of variegated coat color patterns of agouti areas (eumelanotic mottling) on a yellow background characterizes the majority of A\(^{\text{Avy}}\)/a mice. Their phenotypes are defined by the degree of this

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**FIGURE 1** (Upper panel) Survival declines over a considerable range in mammals. Survival for adult humans declines between about 40 and 100 y of age. For some typical inbred rodent strains, survival declines between about 18 and 33 mo of age. Note that scales are approximate. (Lower panel) As with other traits, those that are epigenetically determined are probably distributed unevenly and over a considerable range in populations.
mottling. Thus a Y5 "clear yellow" mouse (Fig. 3a) is not mottled and is at one extreme of this spectrum, whereas the Y0 pseudoagouti mouse (Fig. 3b) has essentially normal (agouti) hair and occupies the other extreme of the spectrum. The Y0 phenotype resembles the species-type "agouti" (A"y") phenotype with essentially normal agouti coat color pattern. Y0 mice do not become obese or diabetic and, in comparison with Y2-Y5 mice, are relatively resistant to liver tumorigenesis (9).

Mice of the genotype A"y"/a are used, rather than A"y"/A"y" mice, because A"y"/a mice show a complete spectrum of coat color and epigenetic variation whereas A"y"/A"y" mice are almost entirely yellow. Mice of the A"y"/A"y" genotype do not show sufficient coat color variation to be useful for observation of overt epigenetic characteristics.

Coat color and other characteristics of A"y"/a mice are determined by the level and tissue specificity of agouti gene expression. In animals with a partial or full yellow coat, agouti is overexpressed both ectopically and in the hair follicles. An agouti coat indicates cyclic expression of agouti limited to the hair follicles (Fig. 4).

Viable yellow mice provide an excellent model in which to manipulate epigenetics and observe epigenetic phenotype (e.g., coat color) and associated molecular events (e.g., DNA methylation of the A"y" allele). Very few studies have quantified epigenetic variation in populations (6,40), much less used maternal treatments to affect this variation (6). Here we describe how maternal diet affects the distribution of epigenetically determined phenotypes and DNA methylation in offspring.

METHODS

Chemicals and reagents

Restriction enzymes (HaeIII and HpaI) were from New England Biolabs (Beverly, MA). DNAase I was from the Molecular Research Center (Cincinnati, OH). Taq polymerase (AmpliTaq) and various polymerase chain reaction (PCR) reagents were from PE Applied Biosystems (Foster City, CA). All other chemicals were of analytical grade or molecular biology grade.

Animals and epigenetic phenotypes

All animal experiments were approved by and conducted in accordance with the Animal Care and Use Committee of the National Center for Toxicological Research. Animals were housed and bred as described previously (6). Two inbred mouse strains that carry both the a and A"y" mutant alleles at the agouti locus (41) were used. These strains, VY/WifC/H/Nctr-A"y" (VY) and YS/WifC/H/Nctr-A"y" (YS) have been described previously (39). The a and A"y" mutant alleles are of great utility because the level and pattern of agouti gene expression can be determined from each animal's coat color pattern (6) as illustrated in Figure 3. The a allele is virtually null, leaving expression of agouti and determination of coat color in A"y"/a mice to the single copy of the A"y" allele.

Ectopic agouti gene expression produces the yellow agouti obese mouse syndrome. The associated coat color is visually apparent just 7 d after birth (6,41). Offspring were produced by A"y"/a x A"y"/a matings.
and the degree of mottling (Y0–Y5) of their coats was visually estimated. The estimated ranges of coat color patterns for the degrees of mottling are as follows: Y0, no yellow, 100% agouti; Y1, <3% yellow, >95% agouti; Y2, 5–33% yellow, 67–95% agouti; Y3, 33–67% yellow, <33–67% agouti; Y4, 67–99% yellow, 1–33% agouti; Y5, >99% yellow, <1% agouti. Y1 mice specifically have a few thin yellow lines or tiny yellow spots, mainly in the rump area, on a Y0 background (Fig. 3b).

Maternal diet studies

Dams of the inbred mouse strains VY and YS were fed diets with methyl supplementation at two different levels during pregnancy as described previously (6). Methyl-supplemented diets were designed to provide substantially increased amounts of cofactors and methyl donors for methyl metabolism (31) and Dnmt (24,34,35). Methyl-supplemented diets were prepared by fortifying the control diet (NIH-31) with the above two supplements at the University of Arkansas for Medical Sciences (UAMS) Sequencing Facility using a Model 377 DNA Sequencer and Big Dye terminator chemistry (PE Applied Biosystems).

DNA methylation

Sites in the LTR containing CpGs were used as targets to assay DNA methylation. One HaelI site (PuGC/GPy) and three HinP1I sites (GCGC) are found in the LTR. An additional HinP1I site is found just outside the LTR but within the IAP sequence. Both HaelI and HinP1I are methyl-sensitive restriction enzymes; i.e., they will not cut DNA if the sequence is PuGC/GPy or GCGC, respectively. Note that all HaelI sites are also HinP1I sites but not conversely.

LTR methylation in A^v/a mice was determined by cutting DNA with either HaelI or HinP1I, heat inactivating the enzyme (95°C for 20 min) and amplifying the resulting DNA using PCR. A control was run in which half the sample was set aside before restriction digestion and water was added in place of restriction enzyme. In this control, no diminution of PCR amplification will take place as a result of unmethylated (cleaved) sites in the DNA. In the restriction-digested samples, PCR amplification is attenuated as a result of cleavage at unmethylated sites in the A^v/IAP-LTR sequence.

The PCR products from the control and restricted DNA samples were run side by side on an agarose gel, which was then stained with ethidium bromide, viewed under ultraviolet light and digitally photographed. This provided a permanent record of the pattern and intensity of the DNA bands. These digital images were then quantified with the aid of the above two primers at the University of Arkansas for Medical Sciences (UAMS) Sequencing Facility using a Model 377 DNA Sequencer and Big Dye terminator chemistry (PE Applied Biosystems).

DNA preparation

DNA was isolated from tissues using DNAzol (Molecular Research Center) (42). Tissue (e.g., liver or kidney) was homogenized in DNAzol and put through a series of centrifugations, ethanol precipitation and washes according to the DNAzol protocol with slight modifications. The main modifications were that tissues were often left to dissolve in DNAzol for 1 h or more after homogenization and, at the end of the procedure, DNA was dissolved and stored in Tris-EDTA buffer [TE; 10 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4]. After full dissolution, a small aliquot of each DNA was diluted 50-fold with TE, and the ultraviolet spectrum was determined for subsequent calculation of DNA concentration.

PCR and DNA sequencing

A set of DNA primers for PCR was designed to span a region including the A^v/IAP-LTR proximal to the agouti gene. One of these primer sequences (5'-GAGTTTACGACATACTCT-3') lies in an agouti exon (43), and the other (5'-AATTTTCGACCCTATCTCTTAA-3') was designed to match an established IAP sequence (44). PCR conditions were 94°C for 60 s; 30 cycles of 94°C for 30 s, 56°C for 30 s and 69°C for 30 s; then 72°C for 10 min and 4°C indefinitely. A "hot start" procedure was used in which the polymerase (with some buffer) was separated from the other components by a wax layer that melts at 80°C (AmpliWax PCR genes; PE Applied Biosystems). A PCR product of 870 base pairs (bp) was identified by agarose gel electrophoresis and recovered using a QiAquick Gel Extraction Kit (Qiagen, Valencia, CA). This product was sequenced in both directions with the aid of the above two primers at the University of Arkansas for Medical Sciences (UAMS) Sequencing Facility using a Model 377 DNA Sequencer and Big Dye terminator chemistry (PE Applied Biosystems).

<table>
<thead>
<tr>
<th>Dietary component</th>
<th>MS diet supplement per kg</th>
<th>3SZM diet supplement per kg</th>
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<tbody>
<tr>
<td>Choline, g</td>
<td>5</td>
<td>15</td>
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<tr>
<td>Betaine, g</td>
<td>5</td>
<td>15</td>
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<tr>
<td>Folic acid, mg</td>
<td>5</td>
<td>15</td>
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<tr>
<td>Vitamin B-12, mg</td>
<td>0.5</td>
<td>1.5</td>
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<tr>
<td>L-Methionine, g</td>
<td>—</td>
<td>7.5</td>
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<tr>
<td>Zinc, mg</td>
<td>—</td>
<td>150</td>
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1 The above supplements were added to NIH-31 diet to give 1000 g of the respective final diet. See Wolff et al. (6) for details of supplements, components and diets.
Northampton, MA) software for comparison of group means and by Spearman rank-order correlation (Microcal Origin software) for measures of individuals. Differences were considered significant when \( P < 0.05 \). Comparisons of groups for their epigenetic phenotypes were analyzed as described previously (6).

RESULTS

Maternal diet and the distribution of epigenetically determined phenotypes of offspring

Animals born to dams fed different maternal diets were evaluated for epigenetic phenotype, according to the degree of agouti in their coats, using the criteria described above and previously (6). Distributions are plotted in Figure 5. These histograms indicate a broad distribution of epigenetic variation in these populations of mice. There is a shift in distribution toward phenotypes with more agouti coat (combined black and yellow pigment in the hair) as increasing levels of methyl supplement are added to the diet. These trends toward agouti phenotype with increasing maternal methyl supplement are highly significant [\( P < 0.001 \) for VY strain and \( P < 0.05 \) for YS strain (6)].

DNA methylation

The contiguous sequence for this region of the \( A^\alpha \) gene was determined and found to contain the expected IAP-LTR and agouti sequences (Fig. 6). The homology to agouti (46) is 100% and the homology to IAP-LTRs is also very high (97–100% identity match with several other known IAP-LTRs in GenBank; e.g., Ref. 44 and GenBank accession no. M99279). Figure 6 also shows a schematic diagram of the DNA and restriction sites used.

Methylation was determined at specific sites of this LTR for the two most yellow phenotypes (Y4 and Y5) and two most agouti phenotypes (Y0 and Y1) of mice. An example is shown of a gel with PCR products of restriction enzyme-digested DNA compared with PCR products from equal amounts of uncleaved control DNA (Fig. 7a); scans of the lanes of this gel are presented in Figure 7b. In each case, the restriction enzyme-digested DNAs from Y0 and Y1 mice give a strong band after PCR with intensity \( \geq 58\% \) of that produced after PCR uncleaved controls (Figs. 7 and 8). This indicates a high level of methylation on the \( Hae \) site and/or all four of the \( HinP11 \) sites in the LTR. In contrast, restriction-digested DNAs from Y4 and Y5 yellow mice produced \(<15\% \) of the control signal (Figs. 7 and 8), indicating low methylation on the \( Hae \) site and/or at least one of the four \( HinP11 \) sites in the LTR. These levels have been quantified from numerous gels, and some of these results are presented in Figure 8. The methylation levels of agouti proximal-LTR \( Hae \) sites of the least yellow phenotypes (Y1 and Y0) average \( 79\% \) of controls (\( n = 8 \)) while those of the most yellow phenotypes (Y5 and Y4) average only \( 11\% \) of controls (\( n = 4 \)); the difference is significant at \( P < 0.000001 \). Additional qualitative \( Hae \) digest data and quantitative \( HinP11 \) digest data are consistent with these results. The Spearman rank-order correlation coefficient \( (r_s) \) for a regression of methylation extent (the percentage of intact \( Hae \) site) against yellow pigmentation (Y class) is 0.979, with \( P < 0.03 \) (Fig. 8). This correlation coefficient represents a subset of data based only on the two least yellow (Y0 and Y1) and the two most yellow (Y4 and Y5) phenotypes.

These results show a strong effect of maternal methyl supplements on the distribution of epigenetically determined phenotypes in offspring and show that these epigenetic phenotypes are strongly correlated with LTR DNA methylation.

DISCUSSION

Epigenetic variation in mammals

Epigenetic variation, a likely key factor in health and life span variance, remains an uninvestigated factor in most studies of health and longevity. Just as natural populations exhibit distributions of trait values, diets and lifestyles, it seems likely that epigenetically determined traits vary across a broad range as well; however, they may only rarely present themselves in ways that we can readily observe or quantify in populations.

Epigenetic variation affecting health and life span could contribute significantly to individual variation in longevity, as seems to be the case in experimental animals with identical genetic and environmental backgrounds. Similarly, epigenetic variation could account for a significant portion of the health and life span variation seen in humans, although parsing these influences from other variables in humans would be a daunting task without guidance from well-defined examples in animal systems. The epigenetic variations that affect adult health and life span are probably established during embryonic and fetal development (Fig. 9). Many likely involve the incomplete methylation of genes and ERVs. Using well-characterized in-
bred mice, epigenetic and environmental interactions can be studied with a minimum of confounding genetic and environmental variation.

Viable yellow mice show a broad spectrum of coat variations in each population and thus make a highly suitable system for investigating maternal and other factors that affect epigenetic variation. Furthermore, the molecular genetics of this system have been well described, as have been many neuroendocrine effects of agouti overexpression (39,43,47).

Some intermediary steps have been identified between agouti overexpression and long-term adverse health effects in yellow mice (39,47). Furthermore, many of these effects bear similarities to the effects of agouti homologues in humans (47).

Shifting the distribution of epigenetic variation

We show a shift in the distribution of epigenetic variation in offspring after maternal methyl supplementation. Although a substantial range of variation remains in these offspring, the ability to shift the distribution of epigenetic variation raises the possibility that the distribution of health variation in populations could be shifted and, on balance, improved. Further treatments, or cumulative multigenerational treatments, might further shift the distribution of epigenetic phenotypes. From a health perspective this might greatly improve the health and longevity of the population.

A useful display of epigenetic variation would be the level and distribution of methyl groups on specific functional sequences such as LTRs associated with cellular or viral genes. This may not have a 100% correspondence with phenotype or gene expression because a preponderance of methylation is in...
many cases sufficient to suppress expression (48). Thus, some variation could exist in DNA methylation that would nevertheless result in apparent homogeneity in gene expression or overt phenotype. As identification of human sequences involved in epigenetic variation increases, it may be possible to determine epigenetic phenotype and even predict disease susceptibility by measuring DNA methylation of specific sequences.

Many human diseases may have significant epigenetic components. Epigenetics plays an important role in many cancers that appear late in life (49) and may have roots in epigenetic inheritance or epigenetic variation established in early development. Rare conditions such as Prader-Willi and Angelman syndromes have significant inherited or developmental epigenetic components (50). Likewise, some childhood cancers (51) and much more common diseases such as diabetes (52) and some psychiatric disorders (53) may have significant inherited or developmental epigenetic components. The tendency of disease and some psychiatric disorders to appear late in life (49) and may have roots in epigenetic variation increases, it may be possible to identify the UAMS Sequencing Facility, and drawings and Robert J. S. Reis, DNA sequence determination by Alan Gies of the DNA methylation variation in mammals

Insufficient methylation of many parts of the genome can lead to disease in humans (54–56), and, in mice, lowered survival (6). Although some levels of 5-MC have been deemed insufficient for particular purposes, there is little information available concerning optimal levels of 5-MC. It might be assumed that as yet to be defined “normal” levels of 5-MC are “adequate”; however, there are few studies that address whether increased levels of 5-MC on particular sequences might be protective against disease or might extend life span beyond normalcy. It has long been known that DNA methylation declines with age in mice (31,57–59). It has been recently shown that expression of the MeCP2 gene drops substantially with age in mice (60). It has been proposed for some time that higher densities of DNA methylation on certain sequences allow for greater surety of health (10,12) and greater life span (31,61).

Numerous nutritional and biochemical studies (37,38,62) as well as epidemiological studies (63) have shown that nutrients for methyl metabolism are essential for maintaining DNA methylation and preventing cancer in rodents and humans. However, few studies have addressed optimal levels and combinations of nutrients with respect to DNA and gene regulation, the application of such findings in mice could lead to improved maternal nutritional balances to improve the health and life span of humans.

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LITERATURE CITED


