Maternal epigenetics and methyl supplements affect *agouti* gene expression in A^{vy}/a mice

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ABSTRACT **'Viable yellow' (A^{vy}/a) mice are larger, obese, hyperinsulinemic, more susceptible to cancer, and, on average, shorter lived than their non-yellow siblings. They are epigenetic mosaics ranging from a yellow phenotype with maximum ectopic *agouti* overexpression, through a continuum of mottled agouti/yellow phenotypes with partial *agouti* overexpression, to a pseudoagouti phenotype with minimal ectopic expression. Pseudoagouti A^{vy}/a mice are lean, healthy, and longer lived than their yellow siblings. Here we report that feeding pregnant black a/a dams methyl-supplemented diets alters epigenetic regulation of *agouti* expression in their offspring, as indicated by increased agouti/black mottling in the direction of the pseudoagouti phenotype. We also present confirmatory evidence that epigenetic phenotypes are maternally heritable. Thus A^{vy} expression, already known to be modulated by imprinting, strain-specific modification, and maternal epigenetic inheritance, is also modulated by maternal diet. These observations suggest, at least in this special case, that maternal dietary supplementation may positively affect health and longevity of the offspring. Therefore, this experimental system should be useful for identifying maternal factors that modulate epigenetic mechanisms, especially DNA methylation, in developing embryos.—Wolff, G. L., Kodell, R. L., Moore, S. R., Cooney, C. A. Maternal epigenetics and methyl supplements affect *agouti* gene expression in A^{vy}/a mice. *FASEB J.* 12, 949–957**

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THE MATERNAL REPRODUCTIVE TRACT, arguably, is the environment most critical to the developing mammalian embryo. Its metabolic and physiologic characteristics modulate the zygote's development through all embryonic stages until birth. Indeed, the conditions in the embryo's immediate milieu seem to determine many characteristics and susceptibilities of the adult organism (1–4).

Mammalian development is dependent on DNA methyltransferase (MTase)[2] and its product 5-methylcytosine (5MC) to help establish, define, or stabilize the various cell types that constitute the developing embryo (5, 6). In mammals, 5MC is a major epigenetic mechanism, with some 5MC patterns being inherited epigenetically (6–8). DNA MTase requires S-adenosylmethionine (SAM) (9) and uses zinc as a cofactor (10).

Synthesis of the chief methyl donor SAM is dependent on dietary folates, vitamin B_{12}, methionine, choline, and betaine (11), which are also available as nutritional methyl supplements. In the human maternal diet, folic acid is important for the prevention of neural tube birth defects, where it may act via methyl metabolism (12, 13) and possibly via 5MC. Little is known about how maternal dietary methyl supplements affect epigenetic regulation of the developing mammalian embryo or whether high levels of certain methyl supplements are toxic. Cooney (11) proposed that such supplements could affect gene expression and 5MC levels in adults and that the level of gene-specific 5MC in young mammals could affect their adult health and longevity.

The mouse *agouti* alleles, A^w and A, regulate the alternative production of black (eumelanin) and yellow (pheomelanin) pigment in individual hair follicles. Transcription of the gene occurs only in the skin during the short period when the yellow subapical band is formed at the beginning of each hair growth cycle. This cyclic expression results in the 'agouti' coat pattern.

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[2] Abbreviations: EM, eumelanic mottling; HS diet, contains half of the supplement level in the MS diet; IAP, intracisternal A particle; LTR, long terminal repeat; 5MC, 5-methylcytosine; MS diet, standard methyl-supplemented diet; SAM, S-adenosylmethionine; 3SZM diet, contains 3× as much methyl supplement as MS diet plus zinc plus methionine; MTase, DNA methyltransferase.

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Due to mutations in the regulatory region of the *agouti* locus, mice bearing the dominant 'viable yellow' (A^{vy}), 'IAPyellow' (A^{iapy}), or 'hypervariable yellow' (A^{hvy}) alleles synthesize much more pheomelanin than eumelanin. These mutations arose through spontaneous insertions of single intracisternal A particle (IAP) sequences in different regions of the *agouti* gene, all preceding the first coding exon (1, 2, 14). In these yellow mice, the gene is under control of the IAP promoter/enhancer and is transcribed continuously in essentially all tissues. This results not only in yellow hair but also in obesity, hyperinsulinemia, diabetes, increased somatic growth, and increased susceptibility to hyperplasia and tumorigenesis (15).

In mice of the non-agouti genotype, *a/a*, no pheomelanin is synthesized, except in hair follicles in the ears and perineal area, due to insertions of non-IAP retroviral sequences in the intron preceding the first coding exon of the *agouti* gene (16). Because the *a* allele produces neither yellow nor pseudoagouti phenotypes, it is often used as the second allele in studies with the dominant yellow mutants.

Expression of A^{vy}, A^{iapy}, and A^{hvy} can be down-regulated epigenetically. In yellow A^{iapy}/a and A^{hvy}/a mice, the proximal IAP long terminal repeat (LTR), containing promoters and enhancers, is hypomethylated, whereas in pseudoagouti A^{iapy}/a mice and their black A^{hvy}/a homologs, the LTR is methylated (1, 2). Thus, in pseudoagouti mice the IAP promoter is inactive, allowing the *agouti* promoters to regulate transcription of the gene. The mouse genotypes A^{vy}/a and A^{iapy}/a are expressed in almost identical phenotypes. Since the agouti protein is continuously and ectopically expressed in both mutants, it is likely that in A^{vy}/a mice, a regulatory mechanism is operative similar to that reported for A^{iapy} (2) and A^{hvy} (1).

A continuous spectrum of variegated patterns of eumelanic mottling (EM), i.e., agouti/black areas, on a yellow background characterizes A^{vy}/a mice. Their phenotypes are defined by the degree of EM (**Table 1**). Thus, a 'clear yellow' mouse is at one extreme of the EM spectrum and the 'pseudoagouti' mouse (**Fig. 1**) occupies the other extreme. The latter resembles the agouti ($A/-$) coat color phenotype, does not become obese, is normoinsulinemic, and is less susceptible to tumorigenesis (17).

In A^{vy}/a mice, there is partial maternal epigenetic inheritance of phenotype. In general, maternal epigenetic inheritance occurs when the epigenetic phenotype and/or allelic expression of the mother is a determinant of the epigenetic phenotype and/or allelic expression of the offspring. For A^{vy}/a dams, the proportion of pseudoagouti offspring depends on the mother's *agouti* locus epigenetic phenotype. The data in **Table 2** confirm and extend previous observations (2, 18) that pseudoagouti dams produce a considerably higher proportion of pseudoagouti offspring than do yellow phenotype dams. The epigenetic phenotype affects not only epigenetic inheritance in the A^{vy}/a genotype, but also may confer the potential for multigenerational inheritance of epigenetically determined characteristics (**Table 3**).

TABLE 1. *Proportions of high and low degrees of eumelanic mottling (EM) among A^{vy}/a offspring from a/a dams of two inbred strains fed methyl-supplemented diets*

	High EM[a]	Low or no EM[b]
Strain and diet	% (N)	% (N)
Strain VY		
NIH-31 (control)	42.6 (75)	57.4 (101)
MS	59.8 (98)[c]	40.2 (66)
3SZM	65.8 (52)[c]	34.2 (27)
Strain YS		
NIH-31 (control)	65.6 (99)	34.4 (52)
MS	66.3 (67)	33.7 (34)
3SZM	78.4 (69)[d]	21.6 (19)

[a] Pseudoagouti + almost pseudoagouti + heavily mottled + 1/2 of the mottled pups. [b] Slightly mottled + clear yellow + 1/2 of the mottled pups. [c] $P < 0.001$ for VY strain and MS diet compared to control NIH-31 diet, as well as for the trend, NIH-31 < MS < 3SZM. [d] $P < 0.05$ for YS strain with 3SZM diet compared to MS or NIH-31 diet and for the trend NIH-31 < MS < 3SZM.

In both A^{vy}/a and A^{iapy}/a mice, the proportions of zygotes differentiating into pseudoagouti phenotype offspring are determined by the gender of the parent contributing the mutant allele, as well as by the dam's strain genome. These gender and strain effects demonstrate, respectively, genomic imprinting and strain-specific modification of the A^{vy} and A^{iapy} alleles.

Genomic imprinting occurs when the level of allelic expression in offspring depends on the gender of the parent contributing the allele (6). Imprinting is a parental gender effect on gene expression in offspring but is *neither* the inheritance of a parent's epigenetic somatic characteristics *nor* the inheritance of a parent's somatic allelic imprint (although these may happen to coincide between parent and offspring). The data in Table 2 also confirm and extend previous observations (18) on genomic imprinting and strain-specific modification in A^{vy}/a mice.

Here we report that differentiation toward the pseudoagouti phenotype of A^{vy}/a embryos is favored by feeding the mothers methyl-supplemented diets during pregnancy. Thus, epigenetic regulation of the A^{vy} allele is modulated not only by imprinting, strain-specific modifier effects, and maternal epigenetic inheritance, but also by the maternal diet.

MATERIALS AND METHODS

Animals

Inbred mouse strains YS/WffC3Hf/Nctr-A^{vy} and VY/WffC3Hf/Nctr-A^{vy} were used for dietary studies at F_{116}-F_{120} of

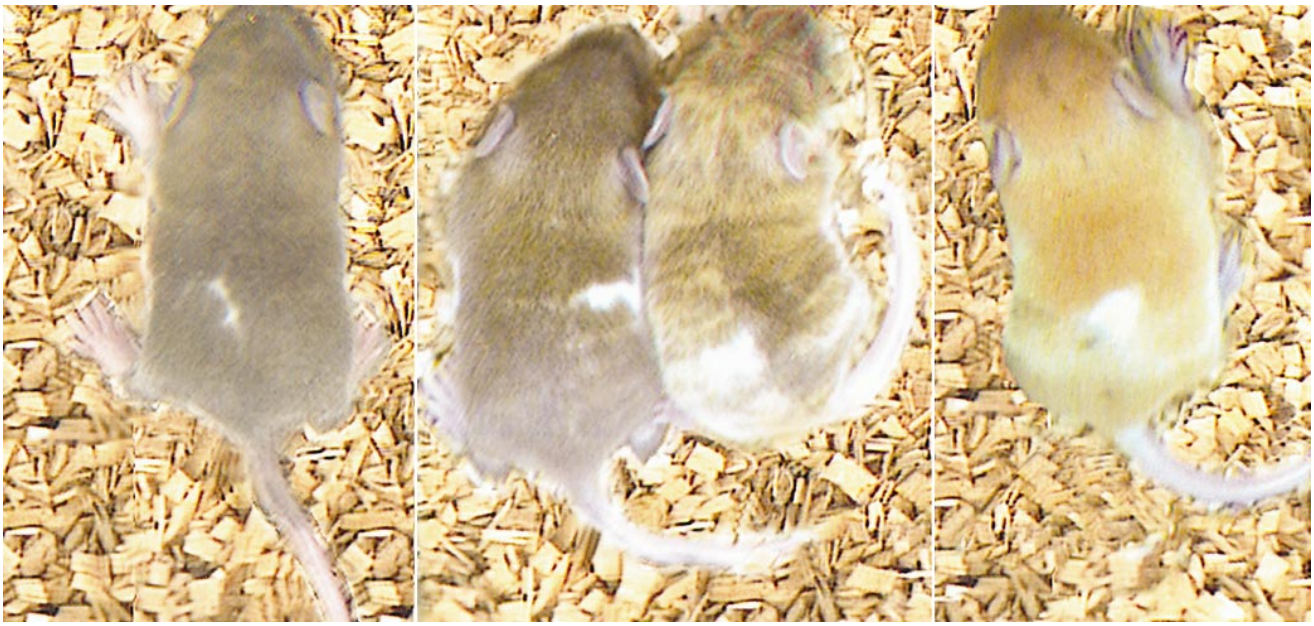

Figure 1. Strain YS A^{vy}/a phenotypes. From left to right: pseudoagouti, almost pseudoagouti, heavily mottled, and slightly mottled. The heavily and slightly mottled phenotypes are found at opposite ends of a continuum in which each mouse has a unique pattern. Note the characteristic faint yellow stripes on the right rear quadrant of the almost pseudoagouti mouse; the yellowish tinge of this mouse is a lighting artifact. The white spot on each mouse is due to recessive spotting (*s*), for which the YS strain is homozygous. The very light area on the rump of the heavily mottled mouse is a lighting artifact.

YS and F_{107}-F_{111} of VY and for maternal epigenetic inheritance studies. In dietary studies, black *a/a* females were date-mated at 6–8 wk of age to the same strain of mottled yellow or pseudoagouti A^{vy}/a males to produce litters of black *a/a* and various phenotypes of A^{vy}/a mice. The litters were phenotyped at 8 and 21 days of age. The spectrum of mottling necessitated the definition of six phenotypic classes (Table 1) for the purpose of grading offspring in dietary studies.

Maternal epigenetic inheritance and imprinting data (Table 2) were obtained from phenotypes of offspring produced by *1)* clear yellow, mottled yellow, or pseudoagouti A^{vy}/a females mated by black *a/a* males, and *2)* black *a/a* females mated by clear yellow, mottled yellow, or pseudoagouti A^{vy}/a males. The proportions of clear/mottled yellow, pseudoagouti, and black phenotypes among their offspring were determined. Maternal epigenetic inheritance and imprinting data were obtained from animals on control diet only.

Diets

Methyl-supplemented diets were designed to provide substantially increased amounts of cofactors and methyl donors for methyl metabolism (11) and, in one diet, to provide additional zinc, a cofactor for the mouse DNA MTase (10). The methyl-supplemented diets, viz., standard methyl-sup-

TABLE 2. *Proportion of pseudoagouti mice among A^{vy}/a offspring produced by yellow A^{vy}/a, pseudoagouti A^{vy}/a, and black a/a dams given control diet*

DAM *Geno*(Pheno) *type*	SIRE *Geno*(Pheno) *type*	Pseudoagouti A^{vy}/a offspring			
		1972–1977[a]		1991–1997	
		%	N	%	N
Strain VY[b]					
A^{vy}/a (mottled yellow)	*a/a*	1.0*	17/1706	1.0**	31/3015
A^{vy}/a (pseudoagouti)	*a/a*	5.3*	15/281	6.3**	51/809
a/a	A^{vy}/a (mottled yellow)	11.8	272/2296	10.2	237/2323
a/a	A^{vy}/a (pseudoagouti)	10.1	60/593	9.3	62/669
Strain YS[c]					
A^{vy}/a (mottled yellow)	*a/a*	0.3	2/607	0.2**	5/2579
A^{vy}/a (pseudoagouti)	*a/a*	1.2	4/329	2.3**	5/216
a/a	A^{vy}/a (mottled yellow)	16.8	49/292	7.0	181/2576
a/a	A^{vy}/a (pseudoagouti)	16.9	72/427	8.4	19/225

[a] From ref. 18, with permission. [b] VY/Wf-A^{vy} 1972–78; VY/WffC3Hf/Nctr-A^{vy} 1978–1997. [c] YS/ChWf-A^{vy} 1972–78; YS/ChWffC3Hf/Nctr-A^{vy} 1978–1997. * $P < 0.001$ compared to other mating combinations using the same strain and time period. ** $P < 0.0001$ compared to other mating combinations within the same strain and time period.

TABLE 3. *Some determinants of epigenetic inheritance*

Specific class	General category	Possible mechanisms
Maternal epigenetic phenotype (gene specific)*	Epigenetic inheritance	5MC or chromatin marks carried through to the next generation.
Possible multigenerational effects		Uterine metabolic or hormonal factors leading to recapitulation of a specific pattern of gene expression in offspring.
		These mechanisms could be influenced by maternal environmental factors.
Paternal epigenetic phenotype (gene specific)	Epigenetic inheritance	Initiated by nuclear transplantation in early embryos. Altered cytoplasmic environment in very early development leads to 5MC changes and possibly other chromatin marks.
Established multigenerational effects		Once established, these marks are directly or indirectly carried through to the next generation (33).
Maternal genotype**	Strain-specific modifiers	Specific genes acting in *trans* to produce gene products that effect epigenetic silencing, e.g., could be genes for transcription factors, chromatin factors, metabolism, or signaling pathways (31, 32, 42, 43, 44, 45).
Gender of parent contributing a particular allele**	Genomic imprinting	Gender-specific marking of germline genomes during germ cell development. Genomic imprinting can be complete (6, 46) or partial (3, 18, 47).
Maternal methyl-supplemented diets*	Maternal nutrition	MS diet: SAM and SAH levels, membrane fluidity, intracellular signaling, early embryo growth rate (11, 38).
Possible multigenerational effects		3SZM diet: Same as for MS diet plus greater zinc saturation of DNA MTase or other zinc finger chromatin proteins (10, 41, 48).
IAP LTR expression**	Transposition, *cis*-activation	Gene-regulating DNA sequence that is a target for 5MC or other epigenetic silencing mechanism (1, 2, 14, 27).
Possible multigenerational effects		
Transgene inactivation	Gene silencing	Provides a DNA sequence that is a target for silencing and becomes epigenetically modified, often by 5MC (31, 43, 44, 45, 49).
Variety of multigenerational effects		
Monoallelic expression	Gene dosage adjustment	Sequence copy number in diploid cells (50). Parts of counting mechanisms to regulate gene dosage such as in X inactivation, e.g., 5MC, chromatin, and specific RNAs such as Xist (50, 51, 52, 53).

* Observed in $A^{vy}/-$ mice. ** Observed in both $A^{vy}/-$ and $A^{iapy}/-$ mice.

plemented diet (MS), HS (contains half of the supplement level in the MS diet), and 3SZM (contains 3× as much methyl supplement as MS diet plus zinc plus methionine), were prepared by fortifying the control 'methyl-sufficient' NIH-31 diet (**Table 4**).

These supplements contain folic acid, vitamin B_{12}, betaine, and choline in the same proportions but in different absolute amounts. The relative level of components in HS, MS, and 3SZM supplements is 0.5, 1.0, and 3.0, respectively. 3SZM supplement also contains methionine and zinc (in addition to threefold the MS level of folic acid, vitamin B_{12}, betaine, and choline). The absolute amounts of all components in the HS, MS, and 3SZM supplements are given in Table 4. All diets were pelleted after addition of the supplements and were fed ad libitum.

Dams were taken off their supplemented diets after giving birth; however, to avoid health problems in dams or pups from abrupt diet change after giving birth, the dams were given

TABLE 4. *Composition of dietary methyl supplements*[a]

MS diet supplement	HS diet supplement	3SZM diet supplement
5 g Choline	2.5 g Choline	15 g Choline
5 g Betaine	2.5 g Betaine	15 g Betaine
5 mg Folic acid	2.5 mg Folic acid	15 mg Folic acid
0.5 mg Vitamin B_{12}	0.25 mg Vitamin B_{12}	1.5 mg Vitamin B_{12}
		7.5 g L-methionine
		150 mg Zinc

[a] The above are added to NIH-31 diet to give 1000 g of the respective final diet. The final total amounts in 3SZM represent, in terms of the NIH-31 levels, ~9 times choline, ~9 times folic acid, ~60 times vitamin B_{12}, ~3.1 times methionine, and ~4.7 times zinc. Betaine not determined in NIH-31. Choline is from choline chloride, betaine is anhydrous, zinc is from $ZnSO_4 \cdot 7H_2O$. All components were obtained from Harlan Teklad (Madison Wis.) except betaine which was from Finnsugar Bioproducts (Schaumburg Ill.), and zinc, which was from Fluka (Milwaukee Wis.).

lower dose diets for 1 or 2 wk before being placed solely on NIH-31 diet again. Two different schedules were followed: #1) MS diet for 2 wk before first date mating and through pregnancy, HS diet for the first week after birth, NIH-31 diet thereafter; #2) 3SZM diet for 2 wk before first date mating and through pregnancy, MS diet for first week after birth, HS diet for second week after birth, NIH-31 diet thereafter. Males were given control diet only.

Statistical analyses

For statistical analysis of the dietary studies, data from animals representing those with a majority of yellow in their coats (clear yellow, slightly mottled, and one-half of the mottled animals) were combined and compared with combined data from animals with a relatively high degree of EM in their coats (pseudoagouti, almost pseudoagouti, heavily mottled, and the other one-half of the mottled mice) (Table 1). The 'mottled' category included mice with intermediate degrees of EM that could not be designated as either 'lightly' or 'heavily' mottled. For the statistical analysis, one-half of these mottled mice were assigned to the 'high EM' category and the remaining one-half to the 'low or no EM' category.

To detect a trend in the ratio of high to low degrees of EM across dietary groups, Rao-Scott tests (19) were conducted separately for the YS and VY strains. To determine whether the two strains differed in these ratios, a stratified analysis, using the Cochran-Mantel-Haenszel test (20), was performed, with each dietary group serving as a stratum.

Whether methylated diets influenced the number of pups born or weaned was determined by analyses conducted separately for each strain using the SAS procedure GLM for single factor analysis of variance. Dunnett's test was used to compare each diet group to its appropriate control (21).

To analyze maternal epigenetic inheritance and imprinting data, comparisons of the proportions of pseudoagouti mice among A^{vy}/a offspring were made for each strain between dams of various phenotypes, using chi-square tests for differences in proportions (22).

RESULTS

Data from two types of studies are presented here. In dietary studies, the effects on offspring of maternal diet during pregnancy were determined. In maternal epigenetic inheritance and imprinting studies, the effects on offspring of maternal epigenetic phenotype and of the gender of the parent contributing the A^{vy} allele were determined in animals fed only the control diet.

Dietary studies

Dams of two inbred mouse strains were fed methyl-supplemented diets during pregnancy and the degrees of EM of their offsprings' coats were estimated. Maternal dietary methyl supplementation (Table 4) increased EM of the offspring in both mouse strains (Table 1). In strain VY mice, the MS diet produced a strong effect on offspring phenotype, with the proportion of offspring with high EM increasing from 42.7% with control diet to 59.8% on MS diet ($P<0.001$). In these mice, the 3SZM diet had the additional effect of inducing a new phenotype, 'almost pseudoagouti' (see below).

In strain YS mice, the MS diet produced no significant effect on offspring phenotype, whereas the 3SZM diet increased the proportion of offspring with high EM from 65.5% on control diet to 78.4% on MS diet ($P<0.05$). The 3SZM diet also induced the new 'almost pseudoagouti' phenotype in these mice.

The Rao-Scott test indicated statistically significant differences in means and trend in phenotypic classifications among the three diet groups for the VY strain ($P<0.001$) and between the 3SZM and other diet groups in the YS strain ($P<0.05$). Methyl supplementation increased the ratio of high to low EM in the predicted direction (Table 1).

A phenotype not previously observed by the grader (G.L.W.) in 30 years, designated 'almost pseudoagouti', was found *only* in litters from dams fed the 3SZM diet (Fig. 1). These mice have a few thin yellow lines or tiny spots, mainly in the rump area, on a pseudoagouti background and were found in ~13% (N=10) and ~20% (N=18) of VY and YS offspring, respectively. Thus, the 3SZM diet greatly increased the proportion of mice that had 'almost' or entirely agouti coat color patterns.

The Cochran-Mantel-Haenszel test demonstrated a statistically significant difference between strains in the proportion of mice with high EM. This proportion was larger in all three dietary groups among YS mice than among VY mice ($P<0.001$) (Table 1).

Despite very high levels of supplementation (especially in the 3SZM diet, with over 4% w/w supplement), none of the diets exerted any detectable adverse effects on litter size, mortality between birth and weaning, health of the dams or that of the offspring until they were at least 6 wk old, or the ratio of a/a to A^{vy}/a offspring (data not shown).

Maternal epigenetic inheritance and imprinting studies

Analyses of breeding data from two time periods, separated by 20 years, revealed partial maternal epigenetic inheritance and imprinting with respect to epigenetic regulation of the A^{vy} allele among animals on control diets (Table 2).

Maternal epigenetic inheritance (23, 24) may be due either to the direct inheritance of epigenetic factors affecting gene expression from dam to offspring or to recapitulation in the offspring of the dam's pattern of gene expression and epigenetic allele modification due to metabolism, hormonal balance, or other factors in her uterine microenvironment.

The degree of maternal epigenetic inheritance can be measured by comparing the percentage of pseudoagouti offspring from pseudoagouti dams with that from mottled dams. For example, if the proportion of pseudoagouti A^{vy}/a offspring from pseudoagouti

dams is P_p and the proportion of pseudoagouti $A^{vy}/$ a offspring from mottled yellow dams is P_m, then the ratio P_p/P_m gives a value E, which is a measure of the degree of maternal epigenetic inheritance (i.e., P_p/P_m=E). There is maternal inheritance of epigenetic phenotype when $E > 1$. In strain VY, the percentage of pseudoagouti A^{vy}/a offspring from pseudoagouti dams was 6.3% in 1991–1997, whereas the percentage of pseudoagouti A^{vy}/a offspring from mottled dams was 1.0% (Table 2). Thus P_p/P_m was 6.3/1.0; E = 6.3 and there was maternal epigenetic inheritance ($E>1$; $P<0.0001$). Therefore, in this case, pseudoagouti dams produced about 6.3-fold the proportion of pseudoagouti offspring as did yellow dams.

Pairwise comparisons between yellow, pseudoagouti, and black dams of the proportions of pseudoagouti offspring were highly significant ($P<0.001$). For both strains, the proportions of pseudoagouti offspring varied for dams of different phenotypes as follows: black > pseudoagouti > yellow.

Genomic imprinting occurs in A^{vy}/a mice and can be measured by comparing the percentage of pseudoagouti offspring when the A^{vy} allele is *paternally* inherited with that when the A^{vy} allele is *maternally* inherited. For example, if the proportion of pseudoagouti A^{vy}/a offspring from sires is P_s and the proportion of pseudoagouti A^{vy}/a offspring from dams is P_d, then the ratio P_s/P_d gives a value G, which is a measure of the degree of genomic imprinting (i.e., P_s/P_d=G). There is genomic imprinting when $G \neq 1$.

When comparing mottled yellow parents, the percentage in strain VY of pseudoagouti A^{vy}/a offspring from mottled A^{vy}/a sires (dam was black a/a) was 10.2% in 1991–1997, whereas the percentage of pseudoagouti A^{vy}/a offspring from mottled A^{vy}/a dams (sire was black a/a) was 1.0% (Table 2). Thus, P_s/P_d is 10.2/1.0, $G_m \cong 10$, and there is genomic imprinting ($G_m \neq 1$; $P<0.0001$).

When comparing pseudoagouti parents, the percentage in strain VY of pseudoagouti A^{vy}/a offspring from pseudoagouti sires (dams were black a/a) was 9.3% in 1991–1997, whereas the percentage of pseudoagouti A^{vy}/a offspring from pseudoagouti dams (sires were black a/a) was 6.3% (Table 2). Thus, P_s/P_d is 9.3/6.3, $G_p \cong 1.5$, and there is genomic imprinting ($G_p \neq 1$; $P<0.02$).

That the A^{vy} allele is imprinted is indicated by the greater proportion of pseudoagouti offspring when the A^{vy} allele is contributed by the sire than when contributed by the dam. The degree of genomic imprinting depends on epigenetic phenotype, i.e., whether the parental gender comparison is of pseudoagouti mice or mottled yellow mice. In strain VY (in 1991 to 1997), $G_m \neq G_p$ ($P<0.001$).

Similar calculations can be made using the remaining data in Table 2 from both strains and both time periods. Thus, there is maternal epigenetic inheritance of alterations in A^{vy} expression and an epigenetic influence on the degree of imprinting at A^{vy}.

DISCUSSION

As indicated by increased proportions of the more strongly mottled phenotypes, methyl-supplemented diets affect expression of A^{vy}. With the control diet, we confirm important previous observations (18) about the effects of maternal epigenetic phenotype and parental gender on A^{vy} expression in offspring: *1)* the epigenetic phenotype of A^{vy} is, in part, maternally heritable, i.e., a pseudoagouti dam is more likely than a yellow dam to produce pseudoagouti offspring; *2)* the gene is partly imprinted, i.e., if A^{vy} is derived from the sire, the epigenetic phenotype of the offspring is much more likely to be pseudoagouti than when the gene comes from the dam. The current data suggest that diet and/or metabolic effects may modulate IAP LTR expression, strain-specific modifiers, maternal epigenetic inheritance, and imprinting—all factors in the epigenetic regulation of transcription of A^{vy}.

IAPs are endogenous retrovirus-like transposons with about 1000 copies each, widely dispersed in the mouse genome. IAP expression is regulated by DNA methylation (25, 26) and IAP activation of nearby genes is regulated by methylation (1, 2) or other epigenetic factors (27).

Similar sequences (HIAPs) are found in the human genome associated with a number of immunological diseases (28). IAP expression is associated with adverse effects in both mice and humans due to activation of neighboring genes or transcription of the IAP itself; epigenetic suppression of IAPs and other repetitive sequences may broadly affect genome integrity, and thus health (29). In the cases where IAP expression adversely affects health, certain diets and supplements may suppress IAP expression and in turn exert apparent positive effects on health.

Since hair follicle cells develop clonally from single precursor cells (30), there may be an inverse correlation between the degree of eumelanic mottling and the developmental stage at which the A^{vy} IAP LTRs in the affected cells were epigenetically down-regulated. For example, if the IAP LTR of a precursor cell is down-regulated by methylation, the whole clone will produce eumelanin, except for normal yellow band production. This results in a large agouti/black area. If the methylation occurs later during clone formation, the eumelanic areas will be smaller, their specific size depending on the stage of clone development at which the presumed IAP LTR methylation took place. The few yellow lines and spots that characterize the 'almost pseudoagouti' phenotype may represent a small number of hair follicle cells in which, for unknown reasons, the IAP LTRs were de-

methylated relatively late in clone formation. Alternatively, they may represent small clones derived from progenitor cells in which the IAP LTRs were never methylated or otherwise epigenetically downregulated.

Epigenetic regulation of A^{vy} expression is initiated during gametogenesis and development. A^{vy} expression is also partly inherited maternally, suggesting that either epigenetic information is retained during gametogenesis and development or, if once lost, is recapitulated during these processes. This maternal influence, as well as the capacity to recapitulate, is evident from the modulation of expression of the paternal A^{vy} allele by the maternal genotype. Paternal phenotype (yellow or pseudoagouti) has little or no effect on offspring phenotype, whereas maternal genotype and phenotype exert major effects (18). We show here that the maternal influence on expression is influenced strongly by maternal diet, is partly independent of genotype, and appears to be dependent on reproductive tract microenvironment and maternal metabolism. By analogy with other sequences whose epigenetic regulation is modified by the maternal genotype (31, 32), regulation of A^{vy} apparently is also modulated by strain-specific modifiers. This suggests that strain-specific modifiers may act via metabolism or at least are dependent on diet and metabolism for their effects.

Important factors affecting A^{vy} penetrance in the offspring are A^{vy} expression in the mother and the sex of the parent contributing the A^{vy} allele. Depending on the former, there can be large differences in penetrance among the offspring. Suppression of A^{vy} expression in the pseudoagouti dam is partly inherited by some of her offspring (Table 2). This demonstrates that a specific epigenetic alteration of gene expression, resulting in the pseudoagouti phenotype, can be passed through the maternal germline to produce the same specific alterations in the offspring. Such epigenetic inheritance is not limited to a single gender, as Roemer et al. (33) reported partial *paternal* inheritance of epigenetic phenotypes of specific genes produced by specific embryo manipulations.

This, the first report of an effect of dietary methyl supplements on gene imprinting and specific gene expression, demonstrates that diet influences mechanisms of epigenetic regulation, imprinting, and development. Assays of 5MC in the IAP promoter in mottled yellow and pseudoagouti A^{vy}/a mice are currently under way to determine whether 5MC is a likely participant in these epigenetic effects of diet in A^{vy}/a mice.

Others have shown neurodevelopmental or biochemical effects of choline supplementation (34, 35). Meck et al. (34) demonstrated a lifelong improvement in the memory of rats whose mothers were given choline supplements during pregnancy. Several metabolic parameters in dam and embryo have been changed by maternal choline supplementation (35); however, no specific gene effects were identified. Most other examples of phenotypic change reported in offspring based on maternal treatments reflect prevention of pathology caused by dietary deficiency, e.g., neural tube defects or adverse drug effects such as those induced by diethylstilbestrol (36) or alloxan (37).

Cooney (11) proposed that dietary methyl supplements could affect gene expression and 5MC levels in adult animals and that the level of gene-specific 5MC in young mammals might affect their adult health and longevity. The present study reveals that specific methyl supplements in the diets of pregnant mouse dams can affect the expression of a specific gene, *agouti*, even in the offspring. At least in this special case of A^{vy}/a offspring, maternal supplementation may have beneficial health effects in adulthood for the offspring by preventing some of the health problems associated with ectopic agouti expression.

The most obvious mechanisms for these effects of methyl supplements on epigenetic phenotype are changes in methyl metabolism that extend to the embryos. These changes may affect DNA MTase activity by increasing the substrate SAM or decreasing the inhibitor SAH in early embryos, and thus increase the level of DNA methylation in early embryos.

There is also an interplay with strain background genome in that MS diet has a clear effect on strain VY but not on strain YS mice. Again, a likely mechanism would be differences in aspects of methyl metabolism between these two strains. Note that these two strains also differ in the proportion of EM in offspring from dams on control diet. Apparently, one or more of the MS supplement components are limiting in the control diet for dams of the VY strain, but not for those of the YS strain.

Both strains respond to 3SZM supplement, which indicates that one or more of the 3SZM supplement components are limiting in the control diet for VY strain mice and in the MS diet for YS strain mice. In addition, the appearance of the almost pseudoagouti phenotype in both strains on 3SZM diet indicates that one or more of the 3SZM supplement components are limiting with respect to one or more aspects of methyl metabolism in the MS diet for strain VY mice, at least for inducing this phenotype in offspring. The most obvious mechanisms for the effects of 3SZM supplement are on methyl metabolism and/or on zinc availability. Saturation of the DNA MTase with zinc may also affect its activity or specificity and thus affect the methylation level or pattern on DNA (10).

There are other less direct or obvious mechanisms for the effects of these supplements on the epigenetics and development of offspring. Choline, directly from the diet or synthesized via methyl metabolism, is known to affect cell membrane fluidity, membrane-

bound enzyme activity, and intracellular signal transduction (38). Likewise, methionine and folates have essential functions in development including protein and DNA synthesis (39, 40). Effects on these parameters could be important in embryonic development and epigenetics. Similarly, zinc has numerous biological functions and could act via other mechanisms to affect epigenetics in development. For example, numerous zinc finger proteins besides DNA MTase are important for cellular differentiation in development (41).

In humans, folic acid supplements are important for preventing some neural tube birth defects. Because present supplementation levels in humans do not prevent all such defects, it is useful to know what supplementation levels are toxic in mammalian development. In this study, we found no evidence of toxicity at high levels of supplementation in mice.

Whereas most mottled yellow A^{vy}/a mice become fat, are hyperinsulinemic, and are more susceptible to tumor formation, pseudoagouti A^{vy}/a mice remain lean, are normoinsulinemic, and have significantly lower lindane-associated liver tumor prevalence (17). Previously unpublished data from the latter study (17) reveal that, between about 17 and 24 months of age, only 24% of pseudoagouti A^{vy}/a (YS x VY)F_1 females died compared with 50% of mottled yellow A^{vy}/a and 23% of black a/a female mice; no differences in mortality between the lindane-treated and untreated control mice were observed. Thus, by increasing the proportion of the phenotype with a down-regulated A^{vy} allele, the maternal methyl-supplemented diets direct the differentiation of more A^{vy}/a embryos toward a relatively healthier and longer lived phenotype. These effects on health in A^{vy} mice may be a special case; however, similar ectopic expression of other genes in other animals may also have adverse health effects that remain unrecognized simply because they are not accompanied by obvious changes in coat color.

Because coat color pattern is indicative of future health and longevity in these A^{vy}/a animals and is identifiable in mice once the coat appears at 7 days of age, adult characteristics of this epigenetic phenotype can be predicted at 1 wk of age. Therefore, several generations of animals, categorized by their predicted relative long-term health and longevity, can be produced within about 2 years to determine the multigenerational effects of diet or drugs on relative health and longevity.

Overall, the dietary supplement effects and the maternal epigenetic inheritance effect reported here are moderate. Although the effects are significant, they apparently did not interfere with normal biological development or with the high degrees of methylation, demethylation, and other epigenetic changes required in the development and growth of embryos. These moderate effects and the partial expression and penetrance of A^{vy} make this experimental system with visual markers well suited for study of the effects of diet and drugs on epigenetic modulation of gene expression. In contrast, systems in which epigenetic control is complete do not provide the sensitivity to monitor subtle to moderate changes in gene expression. The coat color markers reflecting A^{vy} expression in development are important because, even with PCR techniques, evaluation of mosaic phenotypes in animal populations is difficult; however, coat color markers such as those induced by A^{vy} make identification easy. For these reasons as well as the maternal epigenetic inheritance of phenotype, this A^{vy}/a experimental system should be useful for identifying factors that modulate epigenetic mechanisms, e.g., DNA methylation in developing embryos and over multiple generations. FJ

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