Epigenetic inheritance at the agouti locus in the mouse

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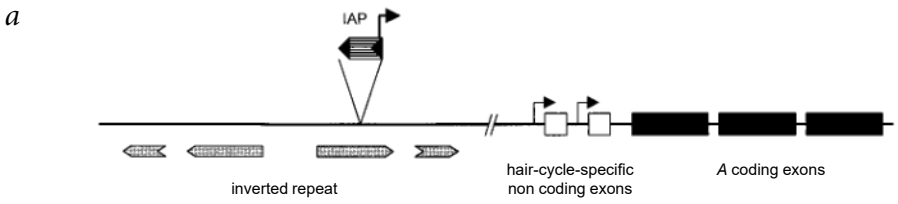
Epigenetic modifications have effects on phenotype, but they are generally considered to be cleared on passage through the germ line in mammals, so that only genetic traits are inherited. Here we describe the inheritance of an epigenetic modification at the agouti locus in mice. In viable yellow (A^{vy}/a) mice, transcription originating in an intra-cisternal A particle (IAP) retrotransposon inserted upstream of the agouti gene (*A*) causes ectopic expression of agouti protein, resulting in yellow fur, obesity, diabetes and increased susceptibility to tumours[1]. The pleiotropic effects of ectopic agouti expression are presumably due to effects of the paracrine signal on other tissues. A^{vy} mice display variable expressivity because they are epigenetic mosaics for activity of the retrotransposon: isogenic A^{vy} mice have coats that vary in a continuous spectrum from full yellow, through variegated yellow/agouti, to full agouti (pseudoagouti). The distribution of phenotypes among offspring is related to the phenotype of the dam; when an A^{vy} dam has the agouti phenotype, her offspring are more likely to be agouti[2,3]. We demonstrate here that this maternal epigenetic effect is not the result of a maternally contributed environment. Rather, our data show that it results from incomplete erasure of an epigenetic modification when a silenced A^{vy} allele is passed through the female germ line, with consequent inheritance of the epigenetic modification. Because retrotransposons are abundant in mammalian genomes, this type of inheritance may be common.

A is responsible for the wild-type coat colour of mice, as it encodes a paracrine signalling protein that causes hair follicle melanocytes to switch from the synthesis of eumelanin (black) to phaeomelanin (yellow). In mice that carry the *A* allele, transcription during the mid-portion of the hair growth cycle produces a sub-apical yellow band on a black hair. A ventral-specific promoter is responsible for a yellow pelage on the belly of the mouse[4]. The A^{vy} allele is one of four (the others being A^{iapy}, A^{iy} and A^{hvy}) alleles carrying IAP (Fig. 1*a*; refs 1,5,6). Mosaic activity of the IAP is responsible for the range of phenotypes in these mice. In A^{iapy} and A^{hvy} mice, the phenotype correlates with methylation of the IAP (refs 6,7). In A^{vy} mice, a yellow maternal phenotype was reported to shift the proportion of phenotypes in offspring, producing fewer pseudoagouti pups[2,3]. This was attributed to an effect of maternal metabolism on the phenotype of offspring. Alternatively, the maternal effect might result from the persistence of epigenetic modifications through meiosis, and we designed experiments to distinguish the two possibilities.



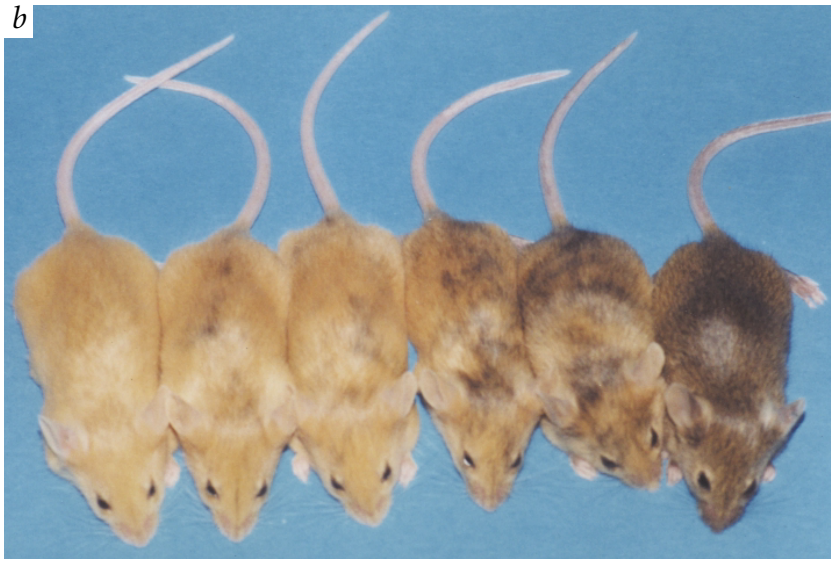


Fig. 1 The A^{vy} allele: map of the *A* locus and range of phenotypes in isogenic A^{vy} mice. ***a***, A^{vy} has an IAP (subtype IΔI, striped box) inserted in the pseudoexon 1A of the locus, with the direction of transcription from the LTRs (arrowhead) opposite to that of the *A* promoters. Hair-cycle–specific non-coding exons (open boxes), coding exons (filled boxes) and an interrupted inverted repeat (grey bar arrow) are indicated. The locus is not shown to scale (100 kb separates the insertion site and hair-cycle–specific promoters). Transcription originating in a cryptic promoter (arrowhead) in the 3′ LTR of the IAP in the A^{vy} allele results in constitutive expression of agouti in multiple tissues[1,2,4,25]. ***b***, Isogenic C57BL/6 A^{vy}/a mice show a continuum of phenotypes ranging from completely yellow, through degrees of yellow/agouti mottling, to completely agouti (termed pseudoagouti because the mice are isogenic with fully yellow mice and not genetically agouti). The extent of the yellow coat colour correlates closely with adult body weight. Yellow mice have pancellular agouti expression driven by the inserted IAP. Mottled mice are mosaics of cells that have or lack ectopic expression driven by the IAP. Pseudoagouti mice lack expression from the cryptic promoter, so that *A* is regulated by its hair-cycle promoters, and these mice have the wild-type coat colour and normal body weight[1–3].

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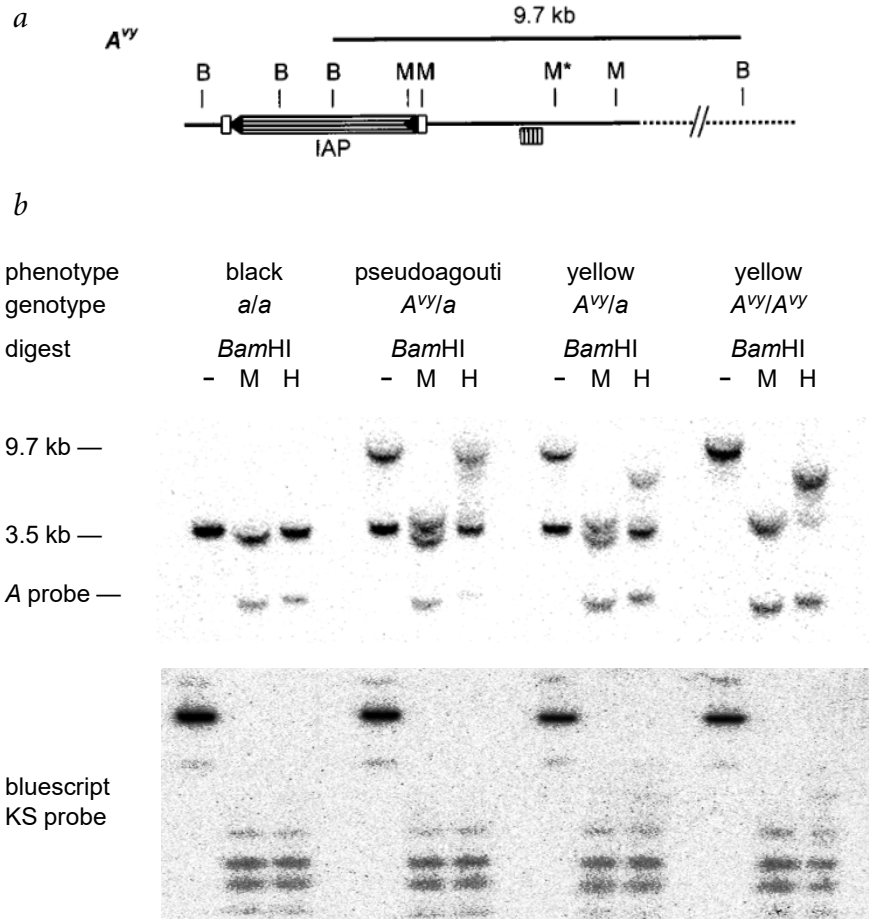


Fig. 2 Increased methylation of A^{vy} in pseudoagouti mice. ***a***, The region of the IAP insertion (horizontal striped box) in the pseudoexon 1A of the locus (open box), including LTRs (arrowheads), *Bam*HI (B) and *Msp*I (M) sites, and a unique DNA fragment (vertical striped box) used as a probe in (*b*) (refs 1,19). A^{vy} is not sequenced over the region indicated (dotted line). ***b***, Top, tail DNA digested with *Bam*HI and either *Msp*I or its isochizomer *Hpa*II, transferred and hybridized with the DNA fragment marked in (*a*). Digestion with *Bam*HI alone produces a 3.5-kb fragment at the *a* allele, whereas the A^{vy} allele appears as a 9.7-kb fragment[1]. *Hpa*II is sensitive to methylation at the internal cytosine of the CCGG site. *Hpa*II digestion of the 9.7-kb *Bam*HI fragment shows differential methylation: in pseudoagouti A^{vy}/a mice, this band is not digested by *Hpa*II, indicating complete methylation of this fragment in most molecules; in yellow A^{vy}/a and yellow A^{vy}/A^{vy} mice the band is reduced in size, indicating that some *Hpa*II sites are not methylated. This shows that the region is more heavily methylated in pseudoagouti mice than in yellow mice, and is consistent with methylation analysis of the A^{iapy} and A^{hvy} alleles[4,6,7]. Bottom, aliquots of the tail DNA digests were spiked with the plasmid Bluescript KS and the membrane was hybridized with the plasmid, showing complete digestion in all tracks. Equivalent digestion of all sets of samples was also seen with a mouse α-globin probe (data not shown). M*, a *Msp*I site resistant to digestion in a proportion of molecules, presumably as a result of methylation of the outer cytosine in a proportion of cells.

We characterized the inheritance of agouti phenotypes in A^{vy}/a mice in a C57BL/6 background. The strain (obtained from the Life Sciences Division, Oak Ridge National Laboratory) has been maintained by brother-sister matings for over 30 generations; thus genetic background effects are discounted. C57 *a*/*a* mice used in these studies are congenic to the A^{vy}/a mice; because *a*/*a* mice carry only the recessive agouti null allele (*a*) they have black coat colour. We found a continuous spectrum of variegated yellow/agouti fur among A^{vy}/a littermates (Fig. 1*b*) as reported in other strain backgrounds[2,3], although the proportions of phenotypes observed differ. We analysed the locus with methylation-sensitive restriction endonucleases and a unique radiolabelled probe that lies close to the IAP insertion (Fig. 2*a*). The A^{vy} allele is more extensively methylated in pseudoagouti than in yellow mice (Fig. 2*b*). This is consistent with findings that the LTR in the IAP of A^{iapy} and A^{hvy} alleles is more highly methylated in pseudoagouti mice[6,7].

The phenotype of a sire contributing an A^{vy} allele is not related to the phenotypes of the offspring. A^{vy}/a sires of all phenotypes produce approximately 40% yellow, 45% mottled and 15% pseudoagouti offspring (Fig. 3*a*). The phenotype of a dam contributing an A^{vy} allele, however, is related to the phenotypes of the offspring. Yellow dams produce yellow and mottled offspring, but no pseudoagouti offspring; mottled dams produce approximately 9% pseudoagouti offspring; and pseudoagouti dams produce 20% pseudoagouti offspring (Fig. 3*b*). These differences are highly significant ($P<0.0001$ for both yellow versus mottled and mottled versus pseudoagouti) and consistent with observations in other genetic backgrounds[2,3]. These results exclude mice with pseudoagouti grand-dams; the grandparental allele was inherited from either a grand-sire or a yellow or mottled dam. When both the dam and grand-dam were pseudoagouti, we observed a grand-maternal effect. Offspring inheriting the A^{vy} allele through two generations of pseudoagouti dams had a higher proportion (33%) of pseudoagouti mice (Fig. 3*c*) than those inheriting the allele from a grandparent other than a pseudoagouti female (20%, Fig. 3*b*). This difference is highly significant ($P=0.003$).

The maternal effect has been noted previously, and was attributed to metabolic differences in the intrauterine environments of developing embryos in yellow and pseudoagouti dams[2]. We sought to test this hypothesis by transferring fertilized oocytes from yellow dams (having pancellular *A* expression) to black dams (with no *A* expression, which have an intrauterine environment equivalent to that of a pseudoagouti dam). There were no pseudoagouti offspring born to foster mothers. The proportions of phenotypes of offspring transferred to black foster mothers were not different from the proportions born to yellow dams (Fig. 4), and significantly different ($P^*=0.02$) from the proportions born to black dams mated with A^{vy}/a sires and to pseudoagouti dams (Fig. 3*a*,*b*). This finding demonstrates that the intrauterine environment itself is not responsible for the maternal effect.

The embryo-transfer experiment did not exclude an effect of maternal environment occurring at some stage earlier than the embryo transfer. The oocyte cytoplasm or effects on the germ cells in a yellow mother may cause the shift in proportions of offspring. To exclude these possibilities, we created breeding schemes to produce pseudoagouti offspring from yellow dams. We mated yellow dams with A^{vy}/a males, and found that some A^{vy}/a offspring (pre-

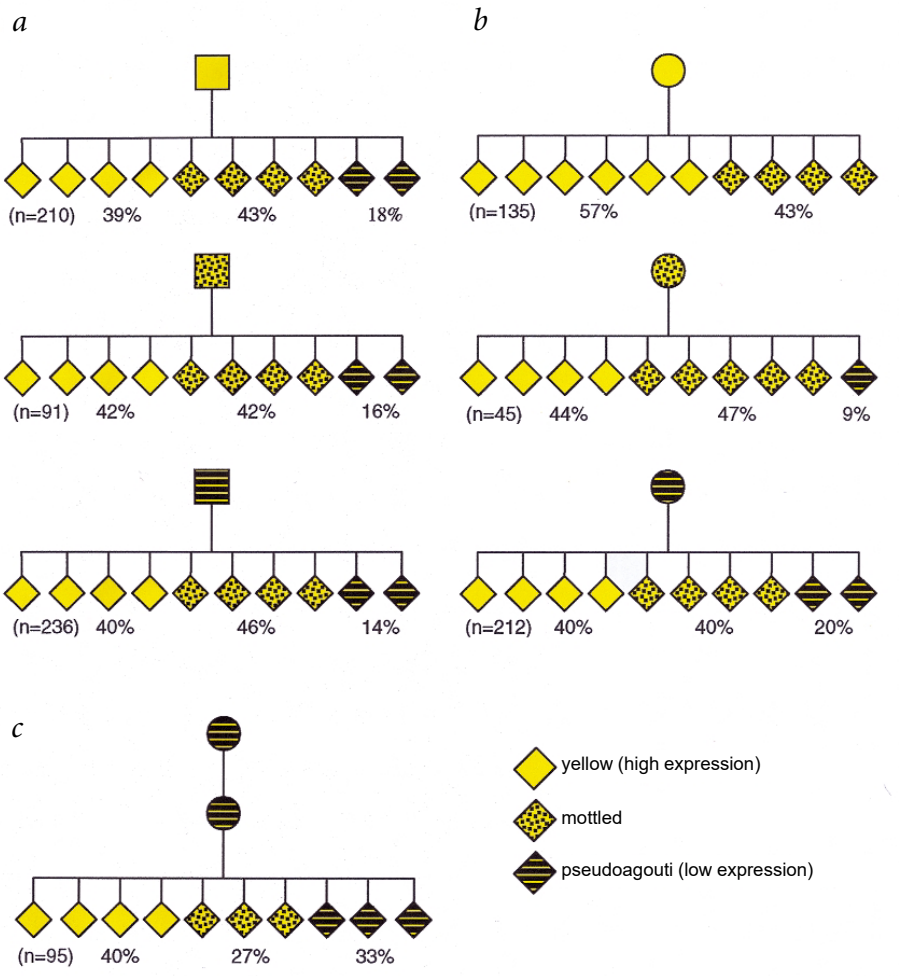


Fig. 3 Inheritance of maternal phenotype. We mated A^{vy}/a C57BL/6 mice of the indicated phenotypes with congenic *a/a* mice, and scored the percentage of offspring of each phenotype. The number of total A^{vy}/a progeny of each cross is indicated (n); *a/a* offspring have been omitted from the pedigrees. ***a***, There is no significant difference in the proportions of phenotypes arising from yellow, mottled and pseudoagouti sires. ***b***, The proportions of phenotypes arising from yellow, mottled and pseudoagouti dams differ significantly ($P<0.0001$ for yellow versus mottled dams and mottled versus pseudoagouti dams). **c**, A grandmaternal effect. Offspring with pseudoagouti grand-dams have been omitted from (*a*) and (*b*). Passage of the allele through two generations of pseudoagouti females produces significantly more pseudoagouti offspring than through only one generation of pseudoagouti dam (*b*; $P=0.003$).

sumably with paternally inherited alleles introduced to the oocyte at fertilization) were pseudoagouti (Fig. 5*a*). This demonstrates that oocyte cytoplasm contributed by a yellow female does not prevent development of pseudoagouti offspring. To exclude an effect on the oocyte at a stage earlier than fertilization, we produced A^{vy}/A^{vy} yellow females carrying a pseudoagouti epiallele (a genetically identical allele that is epigenetically distinct) from matings between yellow dams and pseudoagouti sires. Some of these homozygous yellow females carried a pseudoagouti epiallele masked by the dominant yellow epiallele and produced pseudoagouti pups (Fig. 5*b*). Thus the presence of the allele in an environment contributed by a yellow female does not preclude the development of pseudoagouti offspring. Analysis of the methylation status of the agouti locus in these mice suggests the presence of both the active and the silenced epiallele (Fig. 5*c*).

Our results suggest the inheritance of an epigenetic mark causing silencing of the IAP, rather than a maternal environmental effect on offspring, as the explanation for the maternal effect. Yellow females are able to produce pseudoagouti offspring (Figs 4 and 5), so that an explanation other than the maternal environment is required to explain our data (Fig. 3*b*) and previous observations[2,3]. Because the mice in this study are isogenic, genetic differences cannot explain the effect of maternal phenotype on the offspring. Our results are consistent with complete erasure of the epigenetic modification in the male germ line but incomplete erasure in the female germ line. Stochastic establishment of IAP silencing is also more frequent in

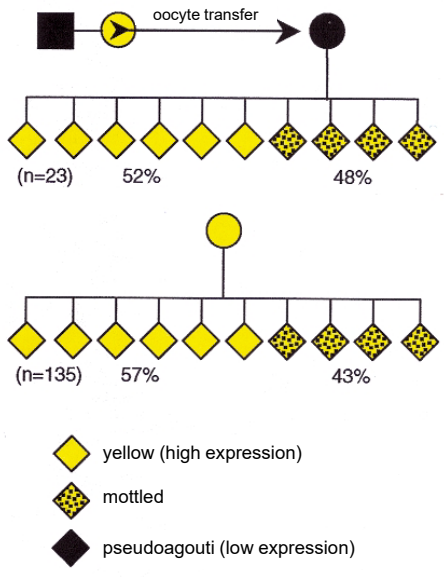
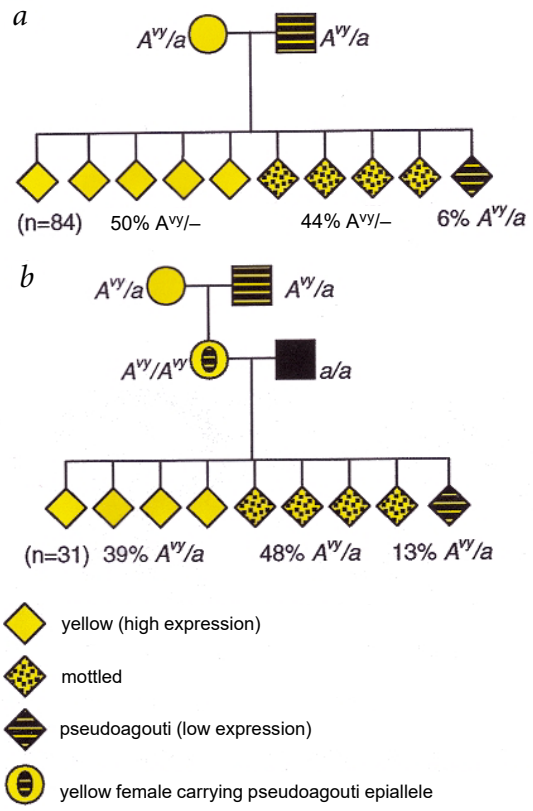


Fig. 4 Oocyte transfer between different intrauterine environments does not affect the phenotypes of offspring. The number of A^{vy}/a progeny is indicated (n), and *a/a* mice have been omitted. Fertilized oocytes were transferred from 0.5-dpc yellow A^{vy}/a dams to pseudopregnant *a/a* dams. The proportion of phenotypes is not different from that produced by yellow A^{vy}/a dams (the lower pedigree is the same as in Fig. 3*b*). The proportion of phenotypes of transferred offspring is significantly different from offspring with *a/a* dams ($P^*=0.02$).



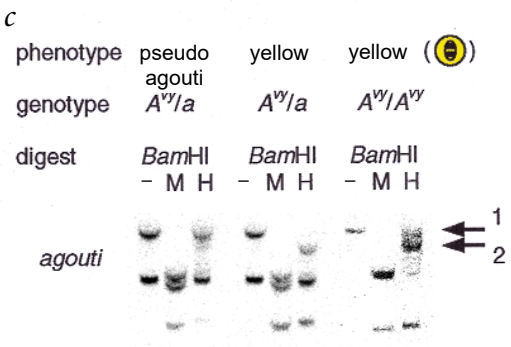


Fig. 5 Breeding schemes used to produce pseudoagouti offspring from yellow dams. ***a***, Yellow A^{vy}/a dams crossed with pseudoagouti A^{vy}/a sires produce pseudoagouti offspring. The proportion of pseudoagouti offspring is approximately half of that observed when a pseudoagouti A^{vy}/a sire is mated with an *a*/*a* dam, because half the paternally inherited A^{vy} alleles are found in A^{vy}/A^{vy} mice with a dominant yellow (or mottled) epiallele. ***b***, Yellow A^{vy}/A^{vy} dams crossed with *a*/*a* sires can produce pseudoagouti offspring when they carry an A^{vy} allele derived from a pseudoagouti grandsire. The striped oval inside the yellow circle indicates that the yellow female is presumably carrying a recessive pseudoagouti epiallele masked by the dominant yellow epiallele. The number of total A^{vy}/a progeny of each type is indicated (n); *a*/*a* mice have been omitted. ***c***, Yellow A^{vy}/A^{vy} dams that produce pseudoagouti offspring carry a hypermethylated A^{vy} allele. Tail DNA digested with *Bam*HI and either *Msp*I or its isoschizomer *Hpa*II (sensitive to CpG methylation), transferred and hybridized with the DNA fragment marked in Fig. 2*a*. Following digestion with *Bam*HI and *Hpa*II, DNA from yellow A^{vy}/A^{vy} dams that give rise to pseudoagouti offspring shows bands corresponding to a heavily methylated epiallele (pseudoagouti, band 1) and a less methylated epiallele (yellow, band 2). (The A^{vy}/a yellow and pseudoagouti data are the same as in Fig. 2*b*.) Equivalent digestion of samples was seen with a mouse α-globin probe (data not shown).

offspring following male transmission than female transmission. The pattern resembles parental imprinting, but with the difference that phenotype is not strictly based on parental sex and genotype. The inheritance of phenotype is presumably based on epigenetic modifications of the IAP that may include DNA methylation or chromatin packaging.

Somatic inheritance of epigenetic modifications underlies cell differentiation and so is typical of complex organisms, but A^{vy} is an example of a mammalian gene that does not undergo complete erasure of all modifications in germ cells. Such epigenetic inheritance is well described in a variety of non-vertebrate species, although in some there is incomplete separation of germ and soma. Paramutation in plants[8,9], mating-type silencing in yeast[10], and some chromatin states in *Drosophila melanogaster*[11,12] are examples of epigenetic inheritance. In maize, the *Suppressor-mutator* (*Spm*) transposon undergoes heritable epigenetic repression[13–15], which is notable because of the resemblance between the effects of *Spm* and those of the IAP at the agouti locus. Heritable silencing of mouse transgenes has been described[16,17], and we have found such effects in approximately 5% of a large series of transgenic lines (unpublished data), but genetic background effects have been difficult to exclude. Inheritance of patterns of gene expression (established by nuclear transfer[18]) and of methylation[19] have been reported in mice and humans, respectively, and a number of articles have focused on the concept of epigenetic inheritance in mammals[20–22]. Thus epigenetic inheritance may not be an uncommon occurrence in mammals, although it may be difficult to distinguish from genetic inheritance: variable expressivity and incomplete penetrance are frequent features of genetic syndromes, but their basis is poorly understood. The behaviour of A^{vy} (variable expressivity and epigenetic inheritance) is based on the activity of a retrotransposon, and there is increasing evidence that retrotransposons make up as much as 35% of mammalian genomes, although the number of these elements that are transcriptionally active is not known[23]. It has been proposed that DNA methylation serves primarily to silence retrotransposons as a genomic defence mechanism to prevent them from transposing and disrupting gene expression[24]. In this regard, it has been shown that a maternal diet rich in methyl donors induces a shift in proportions of phenotypes of A^{vy} mice[3], but it remains to be seen if this environmental establishment of an epigenetic modification is inheritable. A^{vy} provides an example of epigenetic variability and epigenetic inheritance of a pleiotropic trait with pathological effects. If even a small proportion of human genes is subject to similar effects, they may represent a major source of phenotypic variation and sporadic disease.

Methods

Classification of phenotypes. A trained observer classified inbred C57BL/6 mice carrying the A^{vy} allele according to coat-colour (yellow (more than 97% yellow), pseudoagouti (more than 97% pseudoagouti) or mottled (less than 97% yellow and not 97% pseudoagouti)).

Statistics. *P* is the value from the χ^2 distribution for the statistic and the appropriate degrees of freedom. *P** is the probability of 0 pseudoagouti mice of 23 pups given that the expected frequency was 0.15.

Methylation assay. We biopsied 1-cm portions of tail from 3-week-old mice under ether anaesthetic. Following digestion with proteinase K, we purified DNA by phenol/chloroform extraction. We digested DNA (15 g) with *Bam*HI (40 U) or with *Msp*I or *Hpa*II (40 U) overnight. Immediately after setting up the digest, we separated an aliquot of the digest and added Bluescript KS plasmid DNA (10 pg) to the reaction (Fig. 2). We separated the resulting fragments on a 1% agarose gel and analysed the fragments by Southern transfer. We incubated the membrane with a unique 400-bp *Xba*I radiolabelled fragment from a 7-kb *Bam*HI clone of the *A* allele of the agouti locus[25] (agouti probe). Following exposure to a storage phosphor screen, we stripped the membrane and incubated it with a 2.1-kb *Hin*fI/*Sac*I fragment of the mouse α-globin locus to control for equivalent

digestion of samples. We separated the Bluescript KS-spiked digests on a 1.5% agarose gel, analysed the fragments by Southern transfer and hybridized with radiolabelled Bluescript KS.

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1. Duhl, D.M.J., Vrieling, H., Miller, K.A., Wolff, G.L. & Barsh, G.S. Neomorphic *agouti* mutations in obese yellow mice. *Nature Genet.* **8**, 59–64 (1994).
2. Wolff, G.L. Influence of maternal phenotype on metabolic differentiation of *agouti* locus mutants in the mouse. *Genetics* **88**, 529–539 (1978).
3. 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 (1998).
4. Bultman, S.J., Michaud, E.J. & Woychik, R.P. Molecular characterization of the mouse *agouti* locus. *Cell* **71**, 1195–1204 (1992).
5. Perry, W.L., Copeland, N.G. & Jenkins, N.A. The molecular basis for dominant yellow *agouti* coat colour mutations. *Bioessays* **16**, 705–707 (1994).
6. Argeson, A.C., Nelson, K.K. & Siracusa, L.D. Molecular basis of the pleiotropic phenotype of mice carrying the hypervariable yellow (A^{hvy}) mutation at the *agouti* locus. *Genetics* **142**, 557–567 (1996).
7. Michaud, E.J. *et al*. Differential expression of a new dominant agouti allele (A^{iapy}) is correlated with methylation state and is influenced by parental lineage. *Genes Dev.* **8**, 1463–1472 (1994).
8. Brink, R.A. A genetic change associated with the *R* locus in maize which is directed and potentially reversible. *Genetics* **41**, 872–889 (1956).
9. Hollick, J.B., Patterson, G.I., Coe, E.H. Jr, Cone, K.C. & Chandler, V.L. Allelic interactions heritably alter the activity of a metastable maize *pl* allele. *Genetics* **141**, 709–719 (1995).
10. Grewal, S.I.S. & Klar, A.J.S. Chromosomal inheritance of epigenetic states in fission yeast during mitosis and meiosis. *Cell* **86**, 95–101 (1996).
11. Cavalli, G. & Paro, R. The *Drosophila Fab-7* chromosomal element conveys epigenetic inheritance during mitosis and meiosis. *Cell* **93**, 505–518 (1998).
12. Jensen, S., Gassama, M.-P. & Heidman, T. Taming of transposable elements by homology-dependent gene silencing. *Nature Genet.* **21**, 209–212 (1999).
13. Federoff, N.Y. & Banks, J.A. Is the suppressor-mutator controlled by a basic developmental regulatory mechanism? *Genetics* **120**, 559–557 (1988).
14. Martienssen, R. & Baron, A. Coordinate suppression of mutation caused by Robertson's mutator transposons in maize. *Genetics* **136**, 1157–1170 (1994).
15. Barkan, A. & Martienssen, R.A. Inactivation of maize transposon Mu suppresses a mutant phenotype by activating an outward-reading promoter near the end of *Mu1*. *Proc. Natl Acad. Sci. USA* **88**, 3502–3506 (1991).
16. Allen, N.D., Norris, M.L. & Surani, M.A. Epigenetic control of transgene expression and imprinting by genotype-specific modifiers. *Cell* **61**, 853–861 (1990).
17. Hadchouel, M., Farza, H., Simon, D., Tollias, P. & Pourcel, C. Maternal inhibition of hepatitus B surface antigen gene expression in transgenic mice correlates with *de novo* methylation. *Nature* **329**, 454–456 (1987).
18. Roemer, I., Reik, W., Dean, W. & Klose, J. Epigenetic inheritance in the mouse. *Curr. Biol.* **7**, 277–280 (1997).
19. Silva, A.J. & White, R. Inheritance of allelic blueprints for methylation patterns. *Cell* **54**, 145–152 (1988).
20. Jablonka, E., Lachmann, M. & Lamb, M.J. Evidence, mechanisms and models for the inheritance of acquired characters. *J. Theor. Biol.* **158**, 245–268 (1992).
21. Monk, M. Epigenetic programming of differential gene expression in development and evolution. *Dev. Genet.* **17**, 188–197 (1992).
22. Belayev, D.K., Ruvinsky, A.O. & Trut, L.N. Inherited activation-inactivation of the star gene in foxes. *J. Hered.* **72**, 267–274 (1981).
23. Smit, A. The origin of interspersed repeats in the human genome. *Curr. Opin. Genet. Dev.* **6**, 743–748 (1996).
24. Walsh, C.P., Chaillet, J.R. & Bestor, T.H. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature Genet.* **20**, 116–117 (1998).
25. Chen, Y., Duhl, D.M.J. & Barsh, G.S. Opposite orientations of an inverted duplication and alleleic variation at the mouse *agouti* locus. *Genetics* **144**, 265–277 (1996).