Genetic Studies of the Mouse Mutations *mahogany* and *mahoganoid*

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ABSTRACT

The mouse mutations *mahogany* (*mg*) and *mahoganoid* (*md*) are negative modifiers of the *Agouti* coat color gene, which encodes a paracrine signaling molecule that induces a switch in melanin synthesis from eumelanin to pheomelanin. Animals mutant for *md* or *mg* synthesize very little or no pheomelanin depending on *Agouti* gene background. The Agouti protein is normally expressed in the skin and acts as an antagonist of the melanocyte receptor for α-MSH (*Mc1r*); however, ectopic expression of *Agouti* causes obesity, possibly by antagonizing melanocortin receptors expressed in the brain. To investigate where *md* and *mg* lie in a genetic pathway with regard to Agouti and Mc1r signaling, we determined the effects of these mutations in animals that carried either a loss-of-function Mc1r mutation (*recessive yellow, Mc1r^e*) or a gain-of-function *Agouti* mutation (*lethal yellow, A^y*). We found that the *Mc1r^e* mutation suppressed the effects of *md* and *mg*, but that *md* and *mg* suppressed the effects of *A^y* on both coat color and obesity. Plasma levels of α-MSH and of ACTH were unaffected by *md* or *mg*. These results suggest that *md* and *mg* interfere directly with Agouti signaling, possibly at the level of protein production or receptor regulation.

PREVIOUSLY existing mouse mutations collected and characterized over the last several decades are a powerful set of tools for studying mammalian development and disease. In many cases, the ability to construct pathways using double mutant studies and/or transplantation experiments has helped to understand the site of gene action and the type of molecules likely involved (SILVERS and RUSSELL 1955; MCCULLOCH *et al.* 1965; COLEMAN 1973; KAPUR *et al.* 1993).

Among several well-characterized mouse mutations that produce obesity, the *lethal yellow* (*A^y*) and *viable yellow* (*A^vy*) alleles of the *Agouti* gene, are unusual because they result from ectopic expression of a gene product whose normal function is to regulate fur color (SIRACUSA 1994) (reviewed in YEN *et al.* 1994). *Agouti* encodes a novel paracrine factor secreted by dermal papilla cells that causes hair follicle melanocytes to produce yellow pigment, pheomelanin, instead of black or brown pigment, eumelanin (SILVERS 1979) (reviewed in JACKSON 1994). *Agouti* RNA is normally limited to hair follicles, where its transient expression during the midphase of the hair growth cycle gives rise to a subapical yellow band or ticking present in individual hairs (VRIELING *et al.* 1994; MILLAR *et al.* 1995). However, in the *A^y*, *A^vy*, and similar mutations, genomic rearrangements and/or insertions that cause ubiquitous expression of Agouti protein have pleiotropic effects that include a mostly yellow coat, obesity, and insulin resistance (DUHL *et al.* 1994a,b; MICHAUD *et al.* 1994b; MANNE *et al.* 1995).

The biochemical mechanism of Agouti protein action is controversial. In general, its effects are opposite to those observed by activating melanocortin receptors, a group of closely related seven transmembrane domain proteins that respond to alpha-melanocyte stimulating hormone (α-MSH) or to adrenocorticotrophic hormone (ACTH) (CHHAJLANI and WIKBERG 1992; MOUNTJOY *et al.* 1992; GANTZ *et al.* 1993a,b) (reviewed in CONE *et al.* 1996). Indeed, the coat color of mice that carry *A^y* is very similar to that produced by a loss-of-function mutation in the melanocyte receptor for melanocortins (*Mc1r^e*), formerly known as *recessive yellow* (ROBBINS *et al.* 1993). Addition of purified Agouti protein to heterologous cells engineered to express the Mc1r, Mc2r, or Mc4r inhibits the ability of radiolabeled melanocortins to bind these receptors, which indicates that Agouti protein is likely to act as a direct antagonist of α-MSH or ACTH (LU *et al.* 1994; YANG *et al.* 1997). However, several groups have reported effects of Agouti protein *in vitro* that do not require addition of exogenous melanocortins (HUNT and THODY 1995; ZEMEL *et al.* 1995; JONES *et al.* 1996) and there is some evidence to suggest that Agouti protein may alter intracellular calcium levels independently of α-MSH antagonism (reviewed in MANNE *et al.* 1995).

To investigate further the mechanism of Agouti and melanocortin signaling, we have studied the previously

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TABLE 1

Mutations and phenotypes studied

Mutation	Origin	Genetic background	*Agouti* genotype	Coat color phenotype[a]
A^y	Fancy	C57BL/6J	A^y/a	Yellow
mg	Unknown	LDJ/Le	*a/a*	Extreme non-Agouti
		C57BL/6J[b]	*a/a*	Extreme non-Agouti
mg^{3J}	C3H	C3HeB/FeJ	*A/A*	Umbrous[c]
md	C3H	C3H/HeJ	*A/A*	Umbrous[c]
$Mc1r^e$	C57BL/6	C57BL/6J	*a/a*	Sooty yellow

[a] See text and Figure 1 for explanation of phenotypes.
[b] One of us (M.L.L.) has backcrossed the *mg* mutation to C57BL/6J for eight generations; these animals were used for the study depicted in Figure 3C.
[c] On an Agouti background, animals mutant for *mg* are darker than those mutant for *md*.

exisiting mouse coat color mutations *mahogany* (*mg*) and *mahoganoid* (*md*), which lie on mouse chromosomes *2* and *16*, respectively (GREEN 1989). Both mutations alter the balance between pigment types in favor of eumelanin synthesis, and therefore suppress the phenotypic effects of Agouti protein *in vivo* (LANE 1960; LANE and GREEN 1960). To order these genes in a genetic pathway with respect to Agouti and the melanocortin 1 receptor, we generated animals doubly mutant for *mg* or *md* and A^y or the $Mc1r^e$ allele. BEECHEY and SEARLE have previously reported that the *nonagouti curly* mutation, a probable allele of *md* (md^{nc}) is epistatic to the coat color effects of A^y but not to those of the $Mc1r^e$ allele (BEECHEY and SEARLE 1978, 1979). Our results indicate that *mg* and *md* are genetically downstream of *Agouti*, but genetically upstream of the $Mc1r$. Surprisingly, *mg* and *md* suppress the effects of Agouti on obesity as well as on coat color.

MATERIALS AND METHODS

Mouse strains, mutations, and coat color phenotypes: All animals were obtained originally from the Jackson Laboratory (Bar Harbor, ME). The strain of origin, source, genetic background, and relevant coat color genotype for each mutation is listed in Table 1. Most of the breeding studies described below involved a comparison of F_2 animals whose G_0 parents were from different strains. However, linkage between A^y and *mg* limited the number of doubly mutant animals that could be recovered from an F_2 cross. One of us (M.L.L.) has backcrossed the *mg* mutation to C57BL/6J for eight generations, and the studies shown in Figure 2C are based on comparison of non-littermate animals from the same strain backgrounds.

The effects of *mg* and *md* vary according to whether the *Agouti* genotype is *A/A* or *a/a;* therefore interaction between the $Mc1r^e$ and the *md* mutations (whose original backgrounds are *a/a* and *A/A*, respectively) was carried out by first recovering *a/a; md/md* F_2 progeny from a cross to C57BL/6J-*a/a* mice. The coat color phenotypes described in Tables 2–5 as umbrous or dark brown with a yellow ventrum were very uniform among different animals. However, the yellow and sooty yellow phenotypes were more of a continuum than discrete classes; in addition, nonagouti and extreme nonagouti phenotypes are classified together as "black." For measurement of plasma melanocortin levels, isogenic C3HeB/FeJ-mg^{3J}/mg^{3J} animals were compared with C3H/HeJ animals (C3HeB/FeJ was derived from C3H/HeJ by embryo transfer).

Histology: Skin biopsies from the mid-dorsum, mid-ventrum, and the tip of the pinna were obtained from male and female C3H/HeJ, C3HeB/FeJ-mg^{3J}/mg^{3J}, and C3H/HeJ-*md/md* animals between 2 and 4 months of age. Biopsies were fixed in 10% formalin, rinsed with distilled water, and immersed in DOPA reagent (1% D,L-dihydroxyphenylalanine in 0.2 M phosphate buffer, pH 7.4) for 1 hr at 37°. After 1 hr, the biopsies were transferred to fresh DOPA reagent for an additional 3–6 hr, then rinsed with distilled water and fixed in alcohol/formal/acetic acid. Biopsies were embedded in paraffin, sectioned (10 μm), and stained with alcoholic carmine.

Genotyping: Genotype at *mg* or *md* was inferred from the allele sizes of closely linked simple sequence length polymorphisms (SSLPs). D2Mit77 lies within 0.5 cM of *mg* (K. A. MILLER and G. S. BARSH, unpublished results). The positions of *md* and D16Mit9 according to the International Mouse Genome Conference consensus map are 2 and 4 cM, respectively (MGD 1996). Linkage between *md* and SSLP markers has not been measured directly other than what is reported here (see below); therefore in the studies described in Table 2, allele sizes for additional markers [D16Mit182 and D16Mit88, which lie at 3.4 and 9.7 cM, respectively (MGD 1996)] were determined for all animals of the sooty yellow class ($Mc1r^e/Mc1r^e$). In addition, the results shown in Table 4 suggest that no recombination was observed between *md* and D16Mit9 in 42 meioses (22 *Ay/A; +/md* and 10 *Ay/A; md/md* progeny). Oligonucleotide primer pairs for D2Mit77, D16Mit9, D16Mit88, and D16Mit182 were obtained from Research Genetics (Huntsville, AL); allele sizes were determined using denaturing polyacrylamide gel electrophoresis to analyze the PCR products from tail DNA of each animal. Allele sizes in animals carrying *mg* or *md* were identical to those observed in C3H/HeJ mice [174 nucleotides (nt) for D2Mit77; 126 nt for D16Mit9]; allele sizes in animals carrying A^y or $Mc1r^e$ were identical to those observed in C57BL/6J mice (170 nt for D2Mit77; 146 nt for D16Mit9). *Agouti* and $Mc1r$ genotypes were inferred from coat color phenotypes as described in RESULTS; in the one equivocal situation, A^y/A; *md/md vs. A/A; md/md, Agouti* genotype was determined by Southern hybridization using an exon 1A probe that detects the A^y-associated deletion.

Additional studies: Plasma ACTH and α-MSH levels were determined using a commercially available radioimmunoassay kit according to instructions provided by the manufactuer with minor modifications (Incstar, Stillwater, MN). Samples

were obtained between 9 AM and 10 AM after animals had remained undisturbed for at least 3 days. Animals were placed in a non-immobilizing restraining device, a small incision was made in the tail vein, and blood was collected by gravity over a 1–2-min period. In most cases, samples from two to three littermates of identical sex and genotype were pooled to reach a total of 0.3–0.5 ml of plasma required for the assay. Measurements of body weight were made on female animals housed together as littermates and allowed free access to water and standard laboratory mouse chow.

RESULTS

The coat colors of animals homozygous for *md* or *mg* are very similar. On an Agouti background (*A/A* or *A/a*), *md* generally produces less darkening than *mg,* but the phenotype of both *mg/mg* or *md/md* animals is described as umbrous, black hair on the dorsum and dark Agouti hairs with greatly reduced yellow ticking on the flank and ventrum (Figure 1A). On a nonagouti background (*a/a*), small amounts of pheomelanin normally observed in perineal, mammary, and pinna hairs are replaced by eumelanin in *mg/mg* or *md/md* animals. In addition, the glabrous (nonhairy) skin of the ears and tail is much darker than normal (Figure 1C). This so-called extreme nonagouti phenotype is nearly identical to that caused by an *Agouti* null allele, but can be difficult to distinguish from nonagouti alone.

To investigate how *md* or *mg* caused darkening of glabrous areas of the skin, we examined split skin whole mount and paraffin-embedded sections from *md/md, mg/mg, mg^{3J}/mg^{3J},* and control C57BL/6J and C3H/HeJ mice at ages of 2–4 months. Melanocyte counts were made separately for the dermis and epidermis of the pinna and tail, but we found no consistent differences in the number of these cells that correlated with the darkened phenotype (data not shown). However, in both pinna and tail, we consistently observed increased transfer of melanosomes to epidermal keratinocytes (Figure 2).

We first considered whether the genes mutated in *md* and/or *mg* might encode structural or enzymatic components required for pheomelanin biosynthesis, and reasoned that, if so, the mutant phenotypes would be altered little or not at all by a defective Mc1r (Figure 4). The $Mc1r^e$ mutation is a frameshift that produces a premature termination codon before the third transmembrane domain and is thought to be a complete loss-of-function (Robbins *et al.* 1993). A 50% gene dosage reduction in $Mc1r^e/+$ animals has no phenotypic effect, but $Mc1r^e/Mc1r^e$ animals are mostly yellow with some eumelanin in the dorsum and pinna hairs, a phenotype often described as sooty yellow. As predicted, F_1 progeny, *+/$Mc1r^e$; +/md; a/a,* or *+/$Mc1r^e$; +/mg; a/a,* were nonagouti. Two classes of F_2 progeny were obtained whose coat color phenotypes were sooty yellow or black, in a ratio of approximately 3:1, respectively (Tables 2 and 3). The sooty yellow F_2 progeny were identical to $Mc1r^e/Mc1r^e$ animals, and the black F_2 progeny were identical to *md/md* or *mg/mg* (no attempt was made to distinguish extreme nonagouti from nonagouti). The genotype for *md* or *mg* was determined in 111 or 92 F_2 animals, respectively. For *md,* five of 25 sooty yellow animals were found to be *md/md* (Table 2); for *mg,* five of 24 sooty yellow animals were found to be *mg/mg* (Table 3), which indicates that the $Mc1r^e$ is epistatic to *mg* and to *md.* These results suggest that the *md* or *mg* mutant phenotypes are unlikely to be caused by a biochemical defect in pheomelanin synthesis and instead lie genetically upstream of the Mc1r.

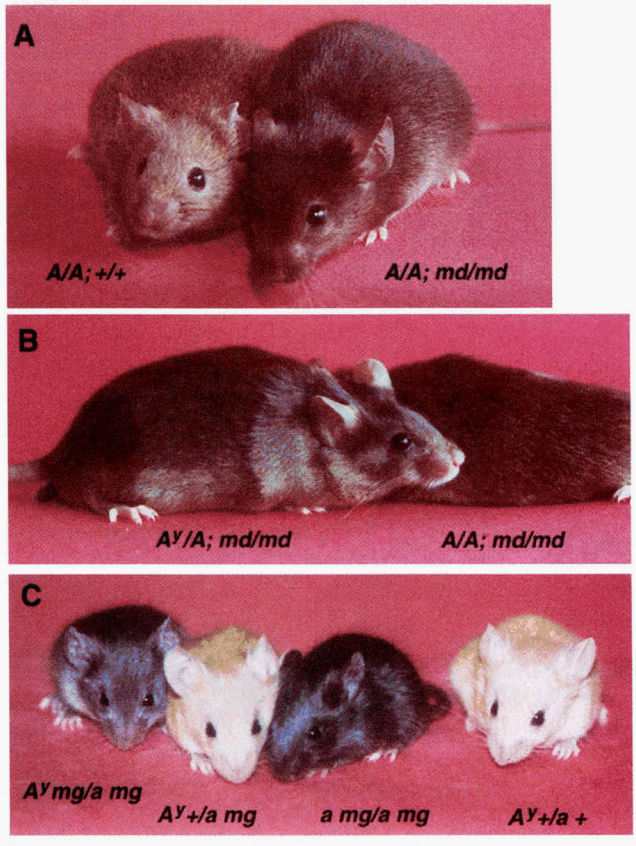

FIGURE 1.—Coat color phenotypes of mutant animals. (A) On an *Agouti* background (*A/A* or *A/a*), homozygosity for *md* produces an umbrous phenotype, black hairs on the dorsum and dark Agouti hairs on the flanks and ventrum. (B) *A^y/A; md/md* animals appear very similar to *A/A; md/md* animals. (C) On a *nonagouti* background (*a/a*), homozygosity for *mg* (or *md,* not shown) produces an extreme nonagouti phenotype, black hairs over the entire animal including the pinna, perineal, and mammary areas. Heterozygosity for *mg* (or *md,* not shown) reveals a semidominant effect only when A^y is present, *A^y +/a mg* animals are sooty yellow rather than completely yellow as in *A^y +/a +* animals. This phenotype also distinguishes the coat color effects of A^y from those of $Mc1r^e$; C57BL/6J-*$Mc1r^e/Mc1r^e$* animals are sooty yellow (not shown) whereas C57BL/6J-*A^y/a* animals are completely yellow. The apparent differences between *md* and *mg* in A and C are due to the *Agouti* background.

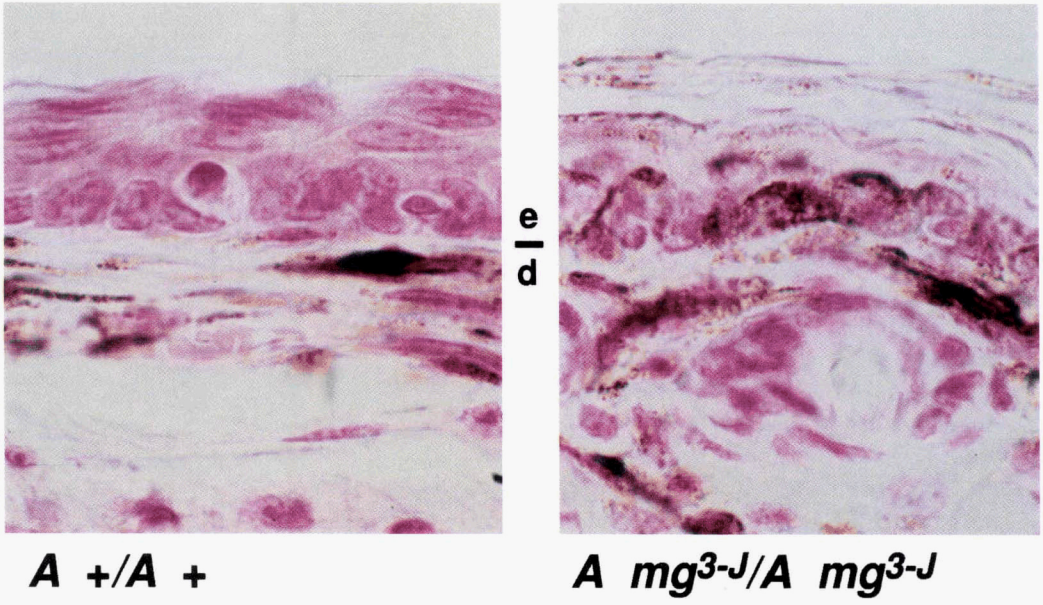

FIGURE 2.—Paraffin-embedded section of DOPA-reacted pinna skin from control (C3H/HeJ-*A* +/*A* +) and mutant (C3HeB/FeJ-*A* $mg^{3\text{-}J}/A\ mg^{3\text{-}J}$) animals. The pinna normally contains many melanocytes located centrally in the dermis (d) whose dendrites lie parallel to the long axis of connective tissue cells of the dermis; in addition, there is a population of melanocytes that lies at the junction between the dermis and epidermis (e). In the tail and pinna of *md*/*md* and *mg*/*mg* animals (not shown) and in the pinna of $mg^{3\text{-}J}/mg^{3\text{-}J}$ animals, basal keratinocytes and the overlying stratum corneum contain many more melanosomes compared to non-mutant animals. Magnification, 1000×.

To determine if *md* or *mg* were genetically upstream of normal *Agouti* transcription, we made use of the A^y allele, in which *Agouti* coding sequences are controlled by a heterologous promoter and, as a consequence, abnormally transcribed in a constitutive manner (DUHL *et al.* 1994a; MICHAUD *et al.* 1994a). The coat colors of A^y/A; +/*md*, or A^y/a; +/*mg* F_1 progeny were similar but, surprisingly, not identical to their A^y/A; +/+ or A^y/a; +/+ parents, which are completely yellow. Instead, the F_1 animals were sooty yellow, which suggested that *md* and *mg* were genetically downstream of *Agouti*, and, further, that *md* or *mg* are semidominant rather than recessive. Both notions were confirmed in the F_2 generation (Tables 4 and 5). For *md*, 59 of 236 F_2 progeny exhibited an umbrous phenotype (Table 4). Based on 10 animals tested for genotype at *md* and *Agouti*, A^y/A; *md*/*md* animals could not be distinguished from *A*/*A*; *md*/*md* animals (Figure 1B), demonstrating that *md*

TABLE 2

F_2 progeny of +/*Mc1r*^{e}; +/*md*; *a*/*a* × +/*Mc1r*^{e}; +/*md*; *a*/*a*

Phenotype	No. observed	No. tested	Genotype at *md*			Combined genotype[a]
			+/+	+/*md*	*md*/*md*	
Sooty yellow	25	25	5	15	5	*Mc1r*^{e}/*Mc1r*^{e}; +/+ *Mc1r*^{e}/*Mc1r*^{e}; +/*md* *Mc1r*^{e}/*Mc1r*^{e}; *md*/*md*
Black	92	87	29	39	19	+/+ or +/*Mc1r*^{e}; +/+ +/+ or +/*Mc1r*^{e}; +/*md* +/+ or +/*Mc1r*^{e}; *md*/*md*
Total	117	112	34	54	24	

[a] All sooty yellow animals were assumed to be homozygous for the *Mc1r*^{e} mutation based on a ratio of sooty yellow:black animals of approximately 1:3. Homozygosity for the *Mc1r*^{e} mutation was confirmed on one animal by direct sequence analysis of PCR-amplified genomic DNA. Among the black animals, no attempt was made to distinguish +/+ from +/*Mc1r*^{e}. Genotype for *md* was inferred from allele size of the closely linked marker D16Mit9 as described in MATERIALS AND METHODS; in addition, all sooty yellow animals were also typed for D16Mit182 and D16Mit88. Of the five sooty yellow animals who typed homozygous for the *md*-associated D16Mit9 allele, all were homozygous for the *md*-associated D16Mit182 allele and three were homozygous for the *md*-associated D16Mit88 allele.

TABLE 3

F_2 progeny of *+/Mc1r^e; +/mg; a/a* × *+/Mc1r^e; +/mg; a/a*

Phenotype	No. observed	No. tested	Genotype at *mg*			Combined genotype[a]
			+/+	*+/mg*	*mg/mg*	
Sooty yellow	28	24	6	13	5	*Mc1r^e/Mc1r^e; +/+* *Mc1r^e/Mc1r^e; +/mg* *Mc1r^e/Mc1r^e; mg/mg*
Black	104	68	16	27	25	*+/+* or *+/Mc1r^e; +/+* *+/+* or *+/Mc1r^e; +/mg* *+/+* or *+/Mc1r^e; mg/mg*
Total	132	92	22	40	30	

[a] Genotype for *mg* was determined as described in MATERIALS AND METHODS. As for the case with *md,* sooty yellow animals were assumed to be homozygous for the *Mc1r^e* mutation, and this assumption was confirmed on one animal by direct sequence analysis of PCR-amplified genomic DNA. Similarly, among the black animals, no attempt was made to distinguish *+/+* from *+/Mc1r^e.*

is epistatic to A^y (Table 4). Of 23 sooty yellow F_2 animals that were tested, 22 were *md/+* and one was *+/+*; of six completely yellow animals that were tested, three were *md/+* and three were *+/+* (Table 4). These results indicate that a weak semidominant effect of *md* is apparent in animals that carry A^y, but also suggest that strain differences between C3H/HeJ-*md/md* and C57BL/6J-A^y/*a* can influence the extent of *Ay*-induced pheomelanogenesis. A similar phenomenon has been described for the DK/Lm-A^y mouse strain (LAMOREUX and GALBRAITH 1986).

For *mg* and A^y, three of 56 F_2 progeny exhibited an unusual coat color phenotype, dark brown dorsum with a yellow ventrum, and were found to be A^y/*a; mg/mg.* (Because *mg* and *A* are linked, ratios of F_2 progeny obtained are not those of independently segregating loci.) Similar to the situation with *md,* the sooty yellow phenotype was strongly but not exclusively associated with heterozygosity for *mg* (Figure 1C; Table 5). Thus, *md* and *mg* each can suppress A^y-induced pheomelanogenesis and therefore are unlikely to interfere with a process that normally regulates *Agouti* transcription.

The animals described above that were doubly mutant for A^y and *md* or *mg* were also used to determine the effects of *md* or *mg* on A^y-induced obesity (Figure 3, A and B). Excess weight gain in mice that carry A^y first becomes apparent between 5 and 8 weeks of age, and can reach 30–60% above control levels depending on strain background (CASTLE 1941; CARPENTER and MAYER 1958). We found that heterozygosity or homozygosity for *md* suppressed A^y-induced obesity in a dose-dependent manner (Figure 3A). The levels of weight gain in A^y/*a; md/md* animals were similar to those observed in *A/A; md/md* animals, but ~20% higher than in *A/A* animals, which suggests that *md* is not a general inhibitor of food intake or growth. For *mg,* only a small number of doubly mu-

TABLE 4

F_2 progeny of A^y/*A; md/+* × A^y/*A; md/+*

Phenotype	No. observed	No. tested	Genotype at *md*			Combined genotype[a]
			+/+	*+/md*	*md/md*	
Sooty yellow	92	23	1	22		A^y/*A; +/md*[b] A^y/*A; +/+*
Yellow	28	6	3	3		A^y/*A; +/+*[b] A^y/*A; +/md*
Umbrous	59	15			15	A^y/*A; md/md A/A; md/md*
Agouti	57	14	10	4		*A/A; +/+* *A/A; +/md*
Total	236	58	14	29	15	

[a] Genotype for *md* was determined as described in MATERIALS AND METHODS. Genotype for A^y was determined in 10 of the umbrous animals by Southern hybridization with a probe that detects the A^y-associated deletion; seven were found to carry A^y.
[b] The sooty yellow phenotype is strongly but not exclusively associated with heterozygosity for *md* as described in the text.

TABLE 5

F_2 progeny of A^y +/*a mg* × A^y +/*a mg*

Phenotype	No. observed	No. tested	Genotype at *mg*			Combined genotype[a]
			+/+	+/*mg*	*mg*/*mg*	
Sooty yellow	25	18		18		A^y +/*a mg*
Yellow	7	2	1	1		A^y +/*a* +[c]
						A^y +/*a mg*
Black	21	14		2	12	*a* +/*a mg*
						a mg/*a mg*
Dark brown[b]	3	3			3	A^y *mg*/*a mg*
Total	56	37	1	21	15	

[a] Genotype for *mg* was determined as described in MATERIALS AND METHODS.
[b] Dark brown dorsum and yellow ventrum; see Figure 1C.
[c] The sooty yellow phenotype is strongly but not exclusively associated with heterozygosity for *mg* as described in the text.

tant F_2 animals were produced because of its linkage to A^y, but in this sample, *mg* inhibited A^y-induced obesity (Figure 3B). As an alternative approach, we compared body weight gain among non-littermate A^y +/*a* +, A^y *mg*/*a mg*, and *a* +/*a* + animals from the same genetic background and found that *mg* suppressed the effects of A^y on obesity to a level similar to that observed in *a* +/*a* + animals (Figure 3C).

Administration of α-MSH or related compounds to mice that carry A^{vy} affects coat color in a manner similar to that caused by *md* or *mg* (GESCHWIND 1966; GESCHWIND *et al.* 1972), and therefore we considered whether one or both mutations might be explained by increased melanocortin production. Among C57BL/6J, C3H/HeJ, C3H/HeJ-*md*/*md*, LDJ-*mg*/*mg*, and C3HeB/FeJ-mg^{3J}/mg^{3J} animals, we found that plasma levels of α-MSH or ACTH varied approximately three- or 1.5-fold, respectively (Table 6). However, this variation did not correlate with mutant genotype and was therefore most likely caused by strain differences other than genotype at *md* or *mg*.

DISCUSSION

Gene interaction studies carried out with previously existing mouse mutations have played a key role in understanding important biological processes including the cellular and physiologic action of endothelin 3, mast cell growth factor, leptin, and the Agouti protein (SILVERS and RUSSELL 1955; MCCULLOCH *et al.* 1965; COLEMAN 1973; KAPUR *et al.* 1993). In the case of Agouti, its effects on coat color though not on obesity require an intact Mc1r, since animals that carry loss-of-function mutations for both genes (*a*/*a*; $Mc1r^e$/$Mc1r^e$) are yellow and non-obese, whereas animals that carry A^{vy} and the constitutively active Mc1r mutation *sombre* ($Mc1r^{som}$) are black and obese (WOLFF *et al.* 1978; SILVERS 1979). By contrast, our results demonstrate that the *md* or *mg* mutations suppress the effects of A^y on coat color and obesity. Taken together with the interactions between *md* or *mg* and the $Mc1r^e$ mutation, these findings suggest a genetic pathway in which *md* and *mg* play key roles in posttranscriptional production or reception of Agouti signaling (Figure 4).

The coat color effects of *md* and *mg* are similar to those caused by intraperitoneal or subcutaneous injection of α-MSH (GESCHWIND 1966; GESCHWIND *et al.* 1972; SHIMIZU *et al.* 1989), but we found that plasma levels of α-MSH and ACTH were not correlated with either mutation. The source of melanocortins that normally stimulates pigment cells is not clear (reviewed in SLOMINSKI *et al.* 1993; WINTZEN and GILCHREST 1996), and it is possible that the coat color effects of *md* or *mg* might still be explained by increased local production of melanocortins by keratinocytes. However, neither a local nor a systemic increase in melanocortin production would provide a simple explanation for the effect of *md* and *mg* on obesity, since injection of α-MSH or related compounds into A^{vy}/*a* or *a*/*a* animals stimulates rather than inhibits weight gain (SHIMIZU *et al.* 1989). Instead, *md* and *mg* are most likely to act by interfering directly with Agouti signaling, either in its processing or secretion, binding to a cell surface receptor, or postreceptor regulation. Each of these possibilities is discussed below in more detail.

Agouti protein is normally secreted by dermal papilla cells; its sphere of action includes melanocytes in overlying but not adjacent hair follicles (POOLE 1975; MILLAR *et al.* 1995). Recombinant Agouti protein produced by insect cells is glycosylated and contains an amino-terminal basic domain that is not required for activity *in vitro* (WILLARD *et al.* 1995), but the form of the mature protein in tissues is not known. A defect in posttranslational modification or secretion could easily explain the effects of *md* and *mg* described here, but would have to be present both in hair follicles and in the tissue or tissues responsible for A^y-induced obesity.

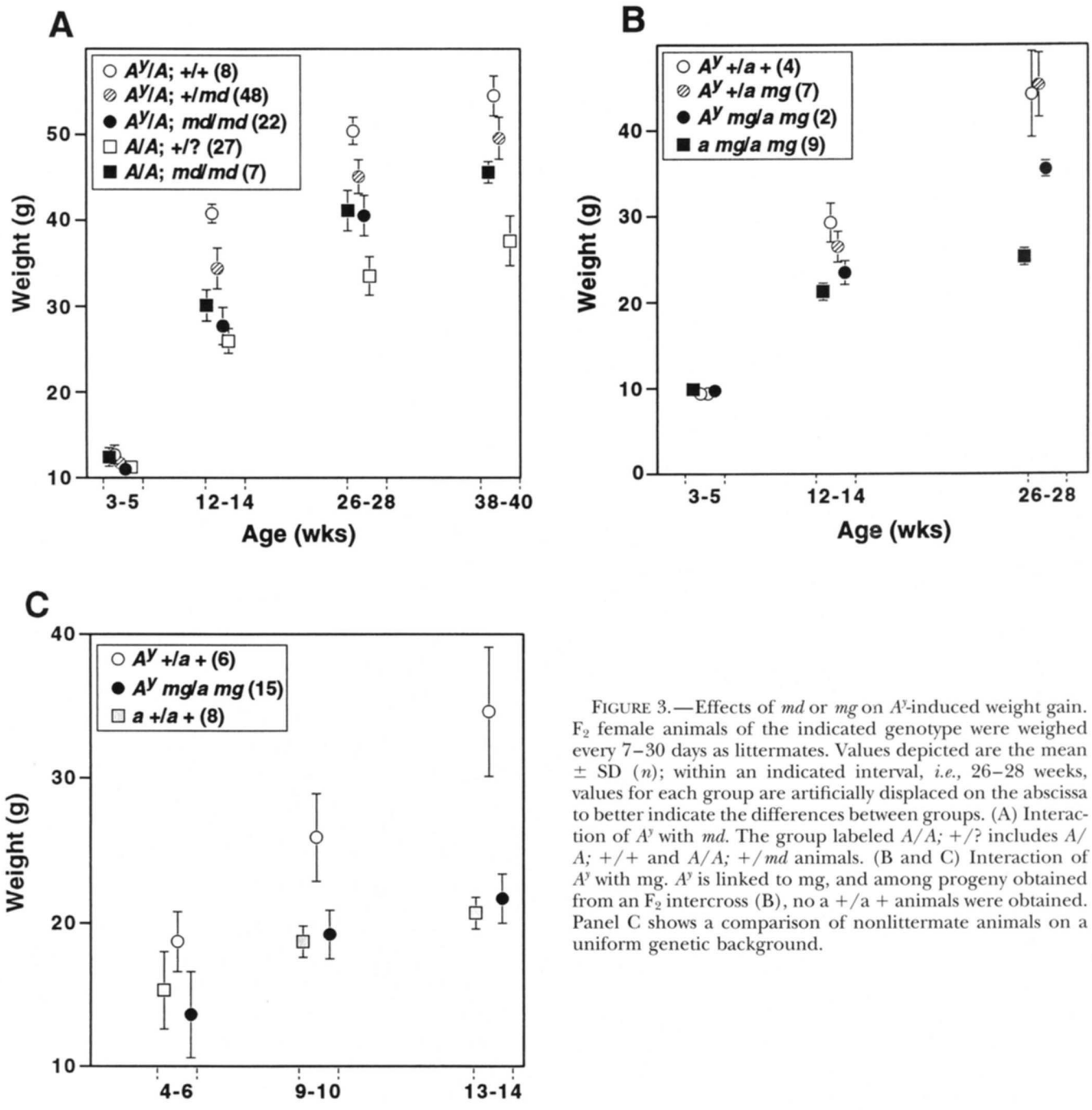

FIGURE 3.—Effects of *md* or *mg* on A^y-induced weight gain. F_2 female animals of the indicated genotype were weighed every 7–30 days as littermates. Values depicted are the mean ± SD (*n*); within an indicated interval, *i.e.*, 26–28 weeks, values for each group are artificially displaced on the abscissa to better indicate the differences between groups. (A) Interaction of A^y with *md*. The group labeled *A/A; +/?* includes *A/A; +/+* and *A/A; +/md* animals. (B and C) Interaction of A^y with mg. A^y is linked to mg, and among progeny obtained from an F_2 intercross (B), no a +/a + animals were obtained. Panel C shows a comparison of nonlittermate animals on a uniform genetic background.

In vitro, recombinant Agouti protein inhibits the binding of radiolabeled melanocortins to cells that express the Mc1r, Mc2r, or Mc4r (LU *et al.* 1994; YANG *et al.* 1997). Because the ability of Agouti protein to inhibit cAMP accumulation *in vitro* requires the presence of a functional melanocortin receptor, these findings strongly suggest that the Mc1r is the normal Agouti receptor. Although structural similarity between Agouti protein and omega-conotoxins has led to speculation that Agouti protein could act as a calcium channel antagonist (reviewed in MANNE *et al.* 1995), experiments in which Agouti protein was reported to affect calcium flux may also be explained by impurities in the preparation or secondary effects (ZEMEL *et al.* 1995; JONES *et al.* 1996). In addition, recent gene targeting studies indicate that animals deficient for the Mc4r mimic many of the metabolic and body-weight regulation abnormalities caused by ectopic expression of Agouti, sug-

gesting that Agouti-induced obesity is caused by Mc4r antagonism (HUSZAR and AL 1997). Thus, neither *md* nor *mg* are likely to encode a specific Agouti receptor that acts independently of melanocortin signaling. However, direct binding of Agouti to a cell surface protein has not yet been demonstrated, and it is possible that *md* or *mg* interfere with formation or assembly of a complex that includes a melanocortin receptor and additional proteins.

Finally, *md* or *mg* might interfere with melanocortin receptor regulation. For example, an increased number of melanocortin receptors at the cell surface or a failure to undergo normal ligand-induced desensitization would lead to increased melanocortin signaling, and therefore suppress the effects of increased antagonist production in mice doubly mutant for A^y and *md* or *mg*. Furthermore, increased melanocortin signaling would be apparent only in the presence of a functional receptor; therefore, *md* or *mg* would have no effect in mice homozygous for the $Mc1r^e$ mutation.

Many mouse coat color mutations have pleiotropic effects because the molecular machinery required for pigment cell development and function is shared by other cells or tissues (reviewed in JACKSON 1994). In the case of Agouti, however, the pleiotropic effects of A^y are apparent only because a signaling protein normally restricted to the skin can elicit a response when expressed abnormally in other parts of the body. Our results indicate that *md* and *mg* have a wider spectrum of action than was previously apparent because both mutations suppress A^y-induced obesity. Furthermore, multiple alleles with nearly identical phenotypes have arisen for *md* and *mg* (GREEN 1989), which suggests that both mutations may represent a loss- rather than gain-of-function. We have suggested previously that variation in the human *Agouti* gene is unlikely to contribute to any human phenotype other than skin or hair color (DUHL *et al.* 1994b; WILSON *et al.* 1995). The same may not be true, however, for human homologues of *md* and *mg*.

TABLE 6

Melanocortin levels in plasma

Animal	α-MSH (pg/ml)[a]	ACTH (pg/ml)[a]
C57BL/6J	63 ± 17	358 ± 47
C3H/HeJ	190 ± 25	228 ± 27
C3H/Hej-*md*/*md*	195 ± 14	271 ± 69
LDJ-*mg*/*mg*	88 ± 18	346 ± 46
C3HeB/FeJ-mg^{3J}/mg^{3J}	121 ± 9	264 ± 41

[a] Plasma levels were determined using a radioimmunoassay as described in MATERIALS AND METHODS. Initial results were obtained on samples pooled together by sex and genotype, but no differences were apparent between males and females and the results shown here include both sexes.

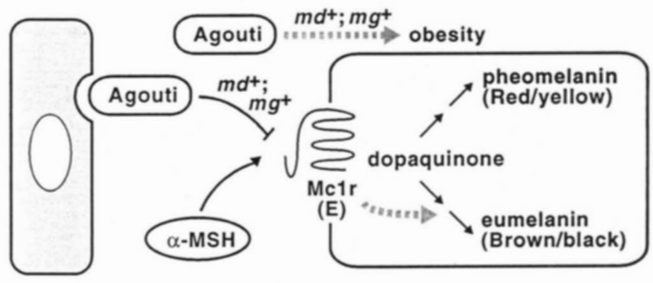

FIGURE 4.—Diagram summarizing the interactions between *md* or *mg*, and A^y or the $Mc1r^e$. The $Mc1r^e$ is epistatic to *md* and *mg*, which, in turn, are epistatic to the effects of A^y on coat color and obesity. Because multiple alleles of *md* and *mg* have arisen with identical phenotypes, they are likely to represent loss-of-function mutations in a process or processes required directly for elaboration or receipt of Agouti signaling. An alternative hypothesis whereby *md* or *mg* could interfere with melanocortin receptor regulation or desensitization is discussed in the text.

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