Genetic Studies of the Mouse Mutations mahogany and mahoganoid

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Manuscript received March 11, 1997
Accepted for publication May 8, 1997

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, Ay) or a gain-of-function Agouti mutation (lethal yellow, A'). We found that the Mc1r mutation suppressed the effects of md and mg, but that md and mg suppressed the effects of A' 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') and viable yellow (A") 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', A", 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 adrenocorticotropic hormone (ACTH) (ChhaIani and Wikberg 1992; Mountjon et al. 1992; Gantz et al. 1993a,b) (reviewed in Cone et al. 1996). Indeed, the coat color of mice that carry A' is very similar to that produced by a loss-of-function mutation in the melanocyte receptor for melanocortins (Mc1r), 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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Genetics 146: 1407–1415 (August, 1997)
living 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'/ or the McI' allele. BEECHFY and Searle have previously reported that the nonagouti curly mutation, a probable allele of md (mdn) is epistatic to the coat color effects of A'/ but not to those of the McI' allele (Beechy and Searle 1978, 1979). Our results indicate that mg and md are genetically downstream of Agouti, but genetically upstream of the McI'. 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 F2 animals whose G0 parents were from different strains. However, linkage between A' and mg limited the number of doubly mutant animals that could be recovered from an F2 cross. 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.

TABLE 1

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Origin</th>
<th>Genetic background</th>
<th>Agouti genotype</th>
<th>Coat color phenotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A'</td>
<td>Fancy</td>
<td>C57BL/6J</td>
<td>A'/a</td>
<td>Yellow</td>
</tr>
<tr>
<td>mg</td>
<td>Unknown</td>
<td>LDJ/Le</td>
<td>a/a</td>
<td>Extreme non-Agouti</td>
</tr>
<tr>
<td>mg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>C3H</td>
<td>C3HeB/FeJ</td>
<td>A/A</td>
<td>Umbrous</td>
</tr>
<tr>
<td>md</td>
<td>C3H</td>
<td>C3H/HeJ</td>
<td>A/A</td>
<td>Umbrous</td>
</tr>
<tr>
<td>McI'</td>
<td>C57BL/6</td>
<td>C57BL/6J</td>
<td>a/a</td>
<td>Sooty yellow</td>
</tr>
</tbody>
</table>

*See text and Figure 1 for explanation of phenotypes.

1 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.

On an Agouti background, animals mutant for mg are darker than those mutant for md.

plasma melanocortin levels, isogenic C3HeB/FeJ-mg<sup>2</sup>/mg<sup>2</sup> 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 pina were obtained from male and female C3H/HeJ, C3HeB/FeJ-mg<sup>2</sup>/mg<sup>2</sup> 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°C. 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/acidic acetic. Biopsies were embedded in paraffin, sectioned (10 μm), and stained with acetic 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 (McI'/McI'). In addition, the results shown in Table 4 suggest that no recombination was observed between md and D16Mit 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'/ or McI'/ were identical to those observed in C57BL/6J mice (170 nt for D2Mit77, 146 nt for D16Mit9). Agouti and McI' genotypes were inferred from coat color phenotypes as described in RESULTS; in the one equivocal situation, A'/A; md/md versus A'/A; md/md, Agouti genotype was determined by Southern hybridization using an exon 1A probe that detects the A'-associated deletion.

Additional studies: Plasma ACTH and a-MSH levels were determined using a commercially available radioimmunoassay kit according to instructions provided by the manufacturer 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 litters 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 litters 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/Au/mg, 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 McIr (Figure 4). The McIr 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 McIr/+ animals has no phenotypic effect, but McIr/+ McIr animals are mostly yellow with some eumelanin in the dorsum and pinna hairs, a phenotype often described as sooty yellow. As predicted, F1 progeny, +/McIr; +/md; a/a, or +/McIr; +/mg, a/a, were nonagouti. Two classes of F2 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 F2 progeny were identical to McIr/McIr animals, and the black F2 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 F2 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 McIr mutation 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 McIr.

![Figure 1](image-url)
FIGURE 2.—Paraffin-embedded section of DOPA-reacted pinna skin from control (C3H/HeJ-A+/A+) and mutant (C3HeB/F6J-A mg3-J/A mg3-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 mg3-J/mg3-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' allele, in which Agouti coding sequences are controlled by a heterologous promoter and, as a consequence, abnormally transcribed in a constitutive manner (DUHLE et al. 1994a; MICHAUD et al. 1994a). The coat colors of A'/A; +/md, or A'/a; +/mg F1 progeny were similar but, surprisingly, not identical to their A'/A; +/+ or A'/a; +/+ parents, which are completely yellow. Instead, the F1 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 F2 generation (Tables 4 and 5). For md, 59 of 236 F2 progeny exhibited an umbrous phenotype (Table 4). Based on 10 animals tested for genotype at md and Agouti, A'/A; md/md animals could not be distinguished from A'/A; +/md/md animals (Figure 1B), demonstrating that md

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. observed</th>
<th>No. tested</th>
<th>Genotype at md</th>
<th>Combined genotype⁴</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+/+</td>
<td>+/md</td>
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<td>Sooty yellow</td>
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<td>5</td>
<td>15</td>
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<td>Black</td>
<td>92</td>
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⁴All sooty yellow animals were assumed to be homozygous for the McIr' mutation based on a ratio of sooty yellow:black animals of approximately 1:3. Homozygosity for the McIr' 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 +/McIr'. 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

F₂ progeny of +/McIr; +/mg; a/a × +/McIr; +/mg; a/a

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. observed</th>
<th>No. tested</th>
<th>Genotype at mg</th>
<th>Combined genotype</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+/+</td>
<td>+/mg</td>
</tr>
<tr>
<td>Sooty yellow</td>
<td>28</td>
<td>24</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
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</tr>
<tr>
<td>Black</td>
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<td>68</td>
<td>16</td>
<td>27</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>92</td>
<td>22</td>
<td>40</td>
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</table>

*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 McIr 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 +/McIr.

is epistatic to A' (Table 4). Of 23 sooty yellow F₂ 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', but also suggest that strain differences between C3H/HeJ-md/md and C57BL/6j-A'/a can influence the extent of A'-induced phaeomelanogenesis. A similar phenomenon has been described for the DK/Lm-A' mouse strain (LAMOREUX and GALBRAITH 1986).

For mg and A', three of 56 F₂ progeny exhibited an unusual coat color phenotype, dark brown dorsum with a yellow ventrum, and were found to be A'/a; mg/mg. (Because mg and A are linked, ratios of F₂ 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'-induced phaeomelanogenesis and therefore are unlikely to interfere with a process that normally regulates Agouti transcription.

The animals described above that were doubly mutant for A' and md or mg were also used to determine the effects of md or mg on A'-induced obesity (Figure 3, A and B). Excess weight gain in mice that carry A' 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'-induced obesity in a dose-dependent manner (Figure 3A). The levels of weight gain in A'/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

F₂ progeny of A'/A; md/+ × A'/A; md/+ *Genotype for md was determined as described in MATERIALS AND METHODS. Genotype for A' was determined in 10 of the umbrous animals by Southern hybridization with a probe that detects the A'-associated deletion; seven were found to carry A'.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. observed</th>
<th>No. tested</th>
<th>Genotype at md</th>
<th>Combined genotype</th>
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<td>Yellow</td>
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<td>3</td>
</tr>
<tr>
<td>Umbrous</td>
<td>59</td>
<td>15</td>
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</tr>
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<td>Agouti</td>
<td>57</td>
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<td>236</td>
<td>58</td>
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</table>

*Genotype for md was determined as described in MATERIALS AND METHODS. Genotype for A' was determined in 10 of the umbrous animals by Southern hybridization with a probe that detects the A'-associated deletion; seven were found to carry A'.

*The sooty yellow phenotype is strongly but not exclusively associated with heterozygosity for md as described in the text.
tant F₂ animals were produced because of its linkage to A', but in this sample, mg inhibited A'-induced obesity (Figure 3B). As an alternative approach, we compared body weight gain among non-littermate A' +/+ , A' +/mg, A' mg/a mg, and a +/a + animals from the same genetic background and found that mg suppressed the effects of A' 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' effects 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-md/md, and C3HeB/FeJ-md/md animals, we found that plasma levels of α-MSH were not correlated with 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 on obesity, since injection of α-MSH or related compounds into A' +/a or A' +/mg 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 post-receptor 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' -induced obesity.

### 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 McIr, since animals that carry loss-of-function mutations for both genes (a/a; McIr'/McIr') are yellow and non-obese, whereas animals that carry A' and the constitutively active McIr mutation sombre (McIr<sup>som</sup>) 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' on coat color and obesity. Taken together with the interactions between md or mg and the McIr mutation, these findings suggest a genetic pathway in which md and mg play key roles in postranscriptional production or secretion 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 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' -induced obesity.
**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 (Huszár 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' 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' 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' 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'-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

<table>
<thead>
<tr>
<th>Animal</th>
<th>α-MSH (pg/ml)*</th>
<th>ACTH (pg/ml)*</th>
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</thead>
<tbody>
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<td>C57BL/6J</td>
<td>63 ± 17</td>
<td>358 ± 47</td>
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<td>C3H/HeJ</td>
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</tr>
<tr>
<td>LDJ-md/mg</td>
<td>88 ± 18</td>
<td>346 ± 46</td>
</tr>
<tr>
<td>C3HeB/FeJ-mg&lt;sup&gt;3&lt;/sup&gt;/mg&lt;sup&gt;3&lt;/sup&gt;</td>
<td>121 ± 9</td>
<td>264 ± 41</td>
</tr>
</tbody>
</table>

*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' or the Mc1r'. The Mc1r' is epistatic to md and mg, which, in turn, are epistatic to the effects of A' 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.

This work was supported in part by grants from the National Institutes of Health to M.L.L. (EY-10223) and to G.S.B. (DK-28506) who is an Assistant Investigator of the Howard Hughes Medical Institute.

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Communicating editor: N. A. JENKINS