Molecular and Phenotypic Analysis of 25 Recessive, Homozygous-Viable Alleles at the Mouse \textit{agouti} Locus

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ABSTRACT

Agouti is a paracrine-acting, transient antagonist of melanocortin 1 receptors that specifies the subapical band of yellow on otherwise black hairs of the wild-type coat. To better understand both agouti structure/function and the germline damage caused by chemicals and radiation, an allelic series of 25 recessive, homozygous-viable \textit{agouti} mutations generated in specific-locus tests were characterized. Visual inspection of fur, augmented by quantifiable chemical analysis of hair melamins, suggested four phenotypic categories (mild, moderate, umbrous-like, severe) for the 18 hypomorphs and a single category for the 7 amorphs (null). Molecular analysis indicated protein-coding alterations in 8 hypomorphs and 6 amorphs, with mild-moderate phenotypes correlating with signal peptide or basic domain mutations, and more devastating phenotypes resulting from C-terminal lesions. Ten hypomorphs and one null demonstrated wild-type coding potential, suggesting that they contain mutations elsewhere in the \textit{agouti} locus that either reduce the level or alter the temporal/spatial distribution of \textit{agouti} transcripts. Beyond the notable contributions to the field of mouse germ cell mutagenesis, analysis of this allelic series illustrates that complete abrogation of agouti function \textit{in vivo} occurs most often through protein-coding lesions, whereas partial loss of function occurs slightly more frequently at the level of gene expression control.

In mice, as in many other mammals, the wild-type pigmentation pattern of the fur is called agouti. Individual hairs of an agouti coat are black, with a narrow ring of yellow pigment just below the tip; this alternative synthesis of eumelanin (black-brown pigment) vs. pheomelanin (yellow-red pigment) is regulated in a paracrine manner by the \textit{agouti} locus in mice (Bultman \textit{et al.} 1992). A short pulse of \textit{agouti} expression at days 3–5 during the hair-growth cycle (Bultman \textit{et al.} 1992) leads to a controlled wave of agouti protein secretion from the dermal papillae (Millar \textit{et al.} 1995; Matsunaga \textit{et al.} 2000). At nearby hair-bulb melanocytes, the agouti protein binds the melanocortin 1 receptor (Mc1r), thereby inhibiting binding by the agonist, \textalpha{}-melanocyte stimulating hormone (\textalpha{}-MSH; reviewed in Dinulescu and Cone 2000). Signaling by \textalpha{}-MSH through Mc1r promotes eumelanin synthesis, whereas agouti binding to Mc1r downregulates this signaling cascade and induces a transient switch from eumelanin to pheomelanin synthesis within hair-bulb melanocytes. Thus, recessive mutations at the \textit{agouti} (\textit{a}) locus, which impair agouti protein activity or reduce the level of \textit{agouti} mRNA synthesis, result in a darker, less-yellow coat due to reduced pheomelanin banding of individual hairs. Conversely, dominant mutations, in which deregulated \textit{agouti} mRNA synthesis leads to greater than normal agouti activity in the skin, give rise to increased yellow pigmentation of the fur. Most dominant \textit{agouti} mutations produce ectopic agouti in tissues additional to the skin, resulting in a pleiotropic, maturity-onset obesity syndrome that is largely due to constitutive antagonism of related melanocortin receptors (Mcrs) in the central nervous system/hypothalamus (Bultman \textit{et al.} 1992; Lu \textit{et al.} 1994; Michaud \textit{et al.} 1994a; Huszar \textit{et al.} 1997; Ollmann \textit{et al.} 1997). Facilitating agouti-mediated signaling through Mcrs in the skin and brain (Miller \textit{et al.} 1997; Gunn \textit{et al.} 1999; Nagle \textit{et al.} 1999) are two other genes, mahogany and mahoganoid. Although the cellular function of mahoganoid is currently unknown, recent evidence suggests that mahogany is a low-affinity receptor for agouti on the surface of melanocytes (He \textit{et al.} 2001).

Five protein structural domains are shared among the highly conserved mammalian homologs of \textit{agouti} (Kwon \textit{et al.} 1994; Leeb \textit{et al.} 2000). Biological function of the various domains has been ascribed largely through analysis of mutations in the 131-amino-acid (aa) mouse agouti protein \textit{in vivo} and through melano-
cortin (Mc) binding inhibition assays in vitro. The N-terminal 22 aa comprise a cleavable signal peptide that is indispensable in vivo (Hustad et al. 1995; Perry et al. 1996), as it provides entry into the secretory pathway (Blobel and Dobberstein 1975). The cysteine-rich C terminus (40 aa), which is believed to form a highly ordered, disulfide-bonded structure (Pallaghy et al. 1994), is both necessary and sufficient for Mc-binding inhibition at McRs in vitro (Willard et al. 1995; Kiefer et al. 1997), suggesting that the C-terminal domain binds directly to McRs in vivo. The mature agouti N terminus (54 aa) contains a conserved and presumably glycosylated arginine that is important for optimal activity in vivo (Perry et al. 1996). Similarly, the central, 29-aa basic domain is also required for full activity in vivo (Perry et al. 1996; Miltenberger et al. 1999). Although the biological role(s) of these domains remain uncertain, the mature N terminus and/or basic domain may mediate a low-affinity interaction with mahogany on the surface of melanocytes (He et al. 2001) and potentially promote a high-affinity interaction between the C terminus and adjacent Mc1Rs. Sandwiched between the basic domain and the cysteine-rich region are several proline residues that contribute mildly to Mcr affinity and selectivity for the agouti protein in vitro (Kiefer et al. 1997). The prolines may provide a flexible “hinge” between the cysteine-rich C terminus and the rest of the protein, thereby better accommodating the possibly disparate protein-protein interactions mediated by these two portions of agouti in vivo.

To investigate further agouti structure/function relationships within its native context (i.e., when synthesized from its normal chromosomal location and acting at its normal target, the hair-bulb melanocyte), we have characterized an allelic series of germline-induced, homozygous-viable, and recessive mutations, using both phenotypic and molecular tools. The 25 alleles analyzed here represent a relatively high percentage of all α-locus mutations that have been recovered in the morphologically specific-locus test (SLT) at the Oak Ridge National Laboratory (ORNL) over several decades (Russell 1951, 2002). The mutant mice exhibit either hypomorphic (reduced function) or amorphic (no function) phenotypes ranging from extremely mild to null, with 56% (8 of the 18 hypomorphs, 6 of the 7 amorphs) encoding aberrant agouti proteins and 44% (10 hypomorphs, 1 amorph) being apparent regulatory mutations. The severity of associated phenotypes indicated the degree to which agouti activity was inhibited at the cellular level, and the visual interpretation of coat phenotypes was verified with a quantitative, chemical analysis of fur melanins. In addition to these data specific to agouti function, correlating the types of heritable DNA damage with various chemicals and radiation utilized in SLTs significantly augments growing toxicological databases and our current understanding of the mutability of mammalian germ cells.

MATERIALS AND METHODS

Mice: All mice originated and were maintained at the Oak Ridge National Laboratory and were fed Purina Laboratory Chow. The C3H/RI (A/A), 101/RI (A*/A*), and C57BL/10Rl (a/a) strains have been maintained by brother-sister inbreeding for >100 generations. All α-locus alleles analyzed arose in SLTs in which (101/RI × C3H/RI)F1, and rarely (C3H/RI × 101/RI)F1, males or females were treated with a mutagen (or were used as untreated controls; see Table 1) and then bred at selected intervals to noninbred T-stock females that were homozygous for recessive alleles (a, Tyrp1, Tyrp4*, p, Mpy5d*, Bmp7*, Ednb*). Such backcrosses at seven visibly marked loci (Russell 1951). The α-locus mutations (A*/a, A*/A*) were therefore originally recovered as G1 progeny (A*/a, A*/A*) carrying one copy of the severe regulatory allele, nonagouti (Bultman et al. 1992). Recessive agouti mutations were identified visually on the basis of reduced pheomelanin in the coat compared to nonmutated, agouti-pigmented littermates (A*/a, A*/A*). Because nonagouti is dominant to null alleles, G1 progeny carrying new amorphic mutations resembled homozygous a/a mice (black dorsal and ventral fur except for a few yellow-pigmented hairs on and behind the ears and around the perineum and mammes). In contrast, hypomorphs are dominant to a, and the primary hypomorphic mutants displayed a range of phenotypes that were intermediate between wild-type agouti (A*/A*, A/A) and nonagouti. Presumed primary α-locus mutants were allelism tested (by crossing to a/a) and were subsequently recovered for propagation and additional genetic tests. Mice carrying hypomorphic alleles were outcrossed to C57BL/10Rl for six or more generations to segregate away induced mutations at other loci and then intercrossed to determine homozygous viability and phenotype. Homozygous, hypomorphic phenotypes for each allele were generally less severe (more yellow) than in mice heterozygous for the same allele (A*/a, A*/A*). Primary mutants that appeared nonagouti were outcrossed to (101/RI × C3H/RI)F1, and 12 homozygous lines (A*/A*, A*/A*, or the marker a/a) were established, revealing whether the mutation was amorphic or α-like. Amorphic α-locus mutations were identified by having uniformly “jet-black” fur, even in areas where nonagouti mice show some pheomelanin. Because all the alleles selected for this study were homozygous-viable (to exclude multi-locus mutagenic events), all were originally propagated as α-locus homozygous stocks. Despite avoidance of brother-sister matings, the small size of the stocks eventually caused decline in vigor in some of them, and six stocks were subsequently bred to be congenic with C57BL/10Rl, yielding α-locus heterozygotes (Table 1). One of these (α6) was successfully restored to homozygous breeding status. Although the 20 α-locus-homozygous stocks do not have identical genetic backgrounds, all of the hypomorphs have considerable contribution from C57BL/10Rl (due to the initial six or more outcrosses), and those propagating amorphs contain 101/RI and C3H/RI contributions.

Chemical analysis of melanins: Hair melanins were analyzed by direct chemical degradation of small hair samples, as described previously (Ito and Fujita 1985; Ozeki et al. 1995). The method is based on the formation of the specific degradation products, aminohydroxyphenylalanine (AHP) from pheomelanin and pyrrole-2,3,5-tricarboxylic acid (PTCA) from eumelanin. Ten to 20 mg of hair was homogenized in water (10 mg hair/ml), using a Ten-Brocke glass homogenizer. Hydrolysis of the hair with hydroiodic acid produced AHP (yield 20%) that was then quantified by HPLC with electrochemical detection. Permanganate oxidation of hair samples produced PTCA (yield 2%) that was quantified by HPLC with UV detection. Due to the consistent yield of these degradation products, 1 ng AHP/mg hair corresponds to 5 ng pheomelanin/mg hair.
Likewise, 1 ng PTC/µg hair corresponds to 50 ng eumelanin/µg hair. Four to six hair samples were plucked from the dorsal midline of a single animal for each allele. Analysis of variance with a 95% confidence level determined that the variance was not equal; therefore, data were analyzed using a two-sample Student’s t-test, assuming equal variance.

**RNA preparation:** Total RNA was prepared from the skin of 4-day-old neonatal mice using standard guandine isothioctanate procedures (Ausubel et al. 1989) or the rapid total RNA isolation kit (5 Prime → 3 Prime). Skin was obtained from the dorsal and/or ventral body region, excluding the head, legs, and tail. For homozygous mutant mice, up to three neonates were used per RNA preparation. For mutant stocks maintained as heterozygotes, no fewer than five neonates were used since the phenotype of a+/a and a/a littermates was not readily apparent at day 4. In the case of a6R, however, a single heterozygous a+/aR neonate was analyzed from an outcross between a homozygous mutant and C57BL/10RI mate.

**RT-PCR analysis:** Total RNA (2 µg) derived from dorsal and ventral skin was reverse transcribed using random hexamer primers (Pharmacia, Piscataway, NJ) and MMLV-reverse transcriptase (Promega, Madison, WI). The RT-PCR products in Figure 3 were obtained by using 1 µl of ventral skin cDNA and one of two forward primers (exon 1A-specific 5’-CACCCAGCTTGATCTTTGAGCC-3’; exon 2-specific 5’-GGACGTTGGAGATGAGAGGAGG-3’) with a common reverse primer (exon 4-specific 5’-AACGGGACTGCGGAGGAGGC-3’). The entire agouti coding region was amplified by PCR using 1–10 µl of dorsal and ventral skin cDNA and primers specific for the 5’ end of exon 2 (5’-CTTCTTCTGGATGGAGGAGG-3’) and the untranslated region of exon 4 (5’-CATTCCCGGTTCGGAAGGC-3’). The PCR products were cloned into the TA vector (Invitrogen, San Diego) and multiple, individual clones were sequenced for each mutant allele.

**Northern analysis:** Selection of poly(A)+ mRNA was performed using the Oligotex mRNA mini kit (Qiagen, Valencia, CA) and total RNA (250 µg) derived from dorsal neonatal skin. For adel, RNA was derived from the whole skin of a single a+/aR neonate, rather than from the dorsal skin only. A Northern blot made by formaldehyde agarose gel electrophoresis was hybridized with a 32P-labeled agouti cDNA probe (Church and Gilbert 1984; Sambrook et al. 1989) and exposed to a phosphorimager plate overnight to quantify the intensity of the agouti band and then to X-ray film for 3 days. The blot was subsequently hybridized with a low-specific-activity probe for Gapdh and exposed to a phosphorimager plate 4 hr for quantification and then to X-ray film overnight.

**Genomic DNA analysis:** Genomic DNA was obtained from tail biopsy and Southern analysis was performed using standard procedures (Church and Gilbert 1984; Sambrook et al. 1989). Individual exons of agouti including splicing junctions were amplified by PCR using the following primers: exon 2 forward (5’-ATCCCTTACCACTCATCTT-3’) and reverse (5’-AGGAACACCATAGCTGCT-3’); exon 3 forward (5’-GTATGGCTTGCTCCCTTCT-3’) and reverse (5’-GTTTCTGAGGCCAGGAA-3’); exon 4 forward (5’-AGGAGGTCTGTCTGACTGTC-3’) and the reverse primer described above that binds within the untranslated region of exon 4. The entire ~5.5-kb genomic region containing all three coding exons was amplified using the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis) and the exon 2 forward and exon 4 reverse primers just described. To amplify the remainder of the 3’ untranslated region (510 bp total) that was not included in previous RT-PCR or genomic clones and that includes the poly(A) signal, the forward primer 5’-GCTTCGGGAACCGGTGCTTG-3’ and reverse primer 5’-TTCCTATGGCAAGAGTGCCG-3’ were used. The hair cycle-specific promoter region and 5’ untranslated exons 1B and 1C (715 bp total) were amplified using the forward primer 5’-GGAGAGCCAGGAGCTTATCC-3’ and reverse primer 5’-AGGAGGTCTCTGAGAGGTCC-3’. All PCR products were then cloned into the TA or TOPO TA vectors (Invitrogen) and multiple individual clones were sequenced.

**DNA sequence analysis:** DNA samples from individual clones were sequenced using the ABI dye terminator ready reaction mix (Perkin-Elmer, Norwalk, CT) or the Big Dye terminator sequencing kit (Perkin-Elmer) and an ABI 377 automated DNA sequencer. The DNA sequence was then analyzed using the following software: Sequence Navigator (version 1.0.0; Applied Biosystems, Foster City, CA), Fasta (version 1.2.0r6; Applied Biosystems), Auto Assembler (version 1.4.0; Applied Biosystems), and Macvector (version 6.5; Oxford Molecular, Palo Alto, CA).

**RESULTS**

**Phenotypic analysis:** Eighteen hypomorphic alleles were grouped into four phenotypic categories (see below) according to the amount of pheomelanin reduction that is visually apparent in the hair, with primary weight given to the appearance of fur along the dorsal midline. Seven alleles with jet-black fur comprised a separate, fifth group. Individual alleles are listed in Table 1, each designated by its superscript symbol. The order of hypomorphic alleles (aR through a308), both between and within the four phenotypic categories, corresponds to the severity of coat phenotypes in adult mice of the indicated genotype (note that some are heterozygotes; see MATERIALS AND METHODS). The mutagens employed, year when each allele was originally identified, and original ORNL stock designation are also shown. The germ-cell stage in which each mutation arose is indicated also, on the basis of the interval between mutagen exposure and conception of the mutant and utilizing existing germ-cell development data (Oakberg 1984).

Figure 1 shows representative homozygous mutant mice from each phenotypic group compared with a wild-type agouti mouse in each panel. Mild hypomorphs exhibit a subtle diminution in adult dorsal fur pheomelanin compared to the wild-type agouti strains of origin C3H/RI (A/A) or 101/RI (A+/A+). Other body regions, such as the sides and ventrum, are not noticeably different from wild type. Moderate hypomorphs comprise the largest phenotype group in which the darkened agouti coat displays reduced pheomelanin in all body regions, although the effect is generally more apparent in dorsal fur. The third category (umbrous-like) is unusual due to preferential darkening of fur along the dorsal midline from nose to tail. In the ventrum and at the dorsal/ventral boundary, however, nearly wild-type levels of yellow-banded fur are apparent, with gradual attenuation in pheomelanin along the sides of the animal toward the dorsal midline. Severe agouti hypomorphs exhibit an obvious reduction in fur pheomelanin in all body regions such that the fur appears very dark but not
TABLE 1
Phenotype and generation of recessive agouti mutations

<table>
<thead>
<tr>
<th>Phenotype group</th>
<th>Mutation</th>
<th>Stock name</th>
<th>Genotype</th>
<th>Mutagen</th>
<th>Germ-cell stage</th>
<th>Date</th>
<th>Note</th>
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<tbody>
<tr>
<td>Mildа</td>
<td>1R</td>
<td>37R145L</td>
<td>a₁R/a₁R</td>
<td>Neutrons</td>
<td>SG</td>
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<td></td>
<td>2R</td>
<td>1FAFye</td>
<td>a₂R/a₂R</td>
<td>X rays</td>
<td>Oocytes</td>
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<td></td>
<td>3R</td>
<td>4MNURh</td>
<td>a₃R/a₃R</td>
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<td>DG</td>
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<tr>
<td>Moderateб</td>
<td>4R</td>
<td>4DThWb</td>
<td>a₄R/a₄R</td>
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<tr>
<td></td>
<td>5R</td>
<td>66CoS</td>
<td>a₅R/a₅R</td>
<td>γ rays</td>
<td>SG</td>
<td>&lt;1984</td>
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<tr>
<td></td>
<td>6R</td>
<td>3THO-IV</td>
<td>a₆R/a₆R</td>
<td>Tritiated H₂O</td>
<td>SG</td>
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<td>12SYNg</td>
<td>a₇R/a₇R</td>
<td>Synthetic fuel</td>
<td>SG</td>
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<td>8R</td>
<td>76G</td>
<td>a₈R/a₈R</td>
<td>X rays</td>
<td>EST</td>
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<td></td>
<td>9R</td>
<td>120ENURd</td>
<td>a₉R/a₉R</td>
<td>ENU</td>
<td>SG</td>
<td>1980</td>
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<tr>
<td>Umbrous-likeс</td>
<td>12R</td>
<td>4FrSb</td>
<td>a₁₂R/a₁₂R</td>
<td>γ rays</td>
<td>SG</td>
<td>1963</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13R</td>
<td>55UTh</td>
<td>a₁₃R/a₁₃R</td>
<td>γ rays</td>
<td>SG</td>
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<td>SG</td>
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<td>Severed</td>
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<td>14R75VH</td>
<td>a₁₆R/a₁₆R</td>
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<td>X rays</td>
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<td>48DTD</td>
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<td>X rays</td>
<td>SG</td>
<td>1962</td>
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<td>Nullе</td>
<td>19R</td>
<td>23ENURhh</td>
<td>a₁₉R/a₁₉R</td>
<td>ENU</td>
<td>SG</td>
<td>1985</td>
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<td></td>
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<td>9ENURh</td>
<td>a₁₀R/a₁₀R</td>
<td>ENU</td>
<td>SG</td>
<td>1980</td>
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<tr>
<td></td>
<td>21R</td>
<td>5FrSb</td>
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<td>γ rays</td>
<td>SG</td>
<td>1963</td>
<td></td>
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<tr>
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<td>22R</td>
<td>207G</td>
<td>a₁₂R/a₁₂R</td>
<td>Spontaneous</td>
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<tr>
<td></td>
<td>23R</td>
<td>14DTTMb</td>
<td>a₁₃R/a₁₃R</td>
<td>X rays</td>
<td>SG</td>
<td>1986</td>
<td></td>
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<tr>
<td></td>
<td>24R</td>
<td>11DT</td>
<td>a₁₄R/a₁₄R</td>
<td>X rays</td>
<td>SG</td>
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<td></td>
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<tr>
<td></td>
<td>25R</td>
<td>15CoS</td>
<td>a₁₅R/a₁₅R</td>
<td>γ rays</td>
<td>SG</td>
<td>1958</td>
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</table>

Phenotypes are based on the appearance of coats in adult mice ~8 weeks of age of the indicated genotypes. MNU, N-methyl-N-nitrosourea; ENU, N-ethyl-N-nitrosourea; EMS, ethyl methanesulfonate; SG, stem cell spermatogonia; DG, differentiating spermatogonia; NA, not applicable; EST, early spermatid; Note 1, prominent demarcation at the dorsal/ventral boundary, as in 101/R1 strain; Note 2, phenotype among littermates is variable; however, the mildest phenotype is indicated; Note 3, nearly null phenotype with pheomelanin present around nipples and perineum.

а Banded hairs are present in the dorsum, sides, and ventrum, with pheomelanin hair around nipples, perineum, and behind ears.

б Banded hairs are present in all body regions, but with reduced pheomelanin. Dorsum is moderately darker than the sides of ventrum. Pheomelanin is prominent around nipples, perineum, and behind ears.

c Pheomelanin is severely reduced along dorsal midline. Banded hair is present on the sides and ventrum but with reduced pheomelanin. Pheomelanin is also reduced around nipples, perineum, and behind ears.

d Pheomelanin is severely reduced in dorsum, sides, and ventrum and moderately reduced around nipples, perineum, and behind ears.

e Hairs are completely eumelanic with no pheomelanin banding. No pheomelanin is present around nipples, perineum, and behind ears. Exception is noted for the nearly null allele, a₁₉R (see Note 3).

The fur of homozygous null agouti mice is entirely black, even in areas where nonagouti mice typically display a few yellow hairs (ears, perineum, and mammae). The single exception in this category is a₁₉R, which is not a true null, as it displays some yellow pigmentation around the mammae and perineum, but no yellow hairs on or behind the ears (or in any other region).

To augment visual description of these mutant phenotypes and to gain a quantifiable measure of agouti gene loss of function, we directly analyzed the amount of pheomelanin and eumelanin in the fur of recessive agouti alleles. In this relatively simple yet sensitive procedure (ITO and Fujita 1985; Ozeki et al. 1995; Ito 1998), chemical treatment of small fur samples yields specific degradation products that are readily detectable by HPLC. The amount of AHP isomers and PTCA is directly proportional to the amount of pheomelanin and eumelanin, respectively, in the original fur sample. This type of analysis has been useful in determining even subtle variations in the distribution of hair melamins induced by various coat color mutations in the mouse (Gunn et al. 2001; Lamoreux et al. 2001).

Figure 2 shows the chemical analysis of melamins from dorsal midline fur of adult agouti mutant mice. AHP...
values varied over a 10-fold range and generally correlated with the severity of visual coat phenotypes in the alleles tested, although a few exceptions were noted ($a^{12R}$, $a^{6R}$, and $a^{20R}$). As a group, mild mutations contained about the same level (102%) of AHP in dorsal fur as did the wild-type control (101/Rl); moderate alleles contained 71%; umbrous-like, 46%; severe, 48%; and nonagouti (C57BL/10Rl), 9%. Mean AHP levels for individual mutations compared to the wild-type control were significantly lower ($P < 0.05$) for the darker mutations (nulls, nonagouti, and hypomorphs $a^{17R}$, $a^{13R}$, $a^{12R}$, $a^{20R}$, $a^{10R}$, and $a^{20R}$), but not for the more temperate hypomorphs ($a^{12R}$, $a^{6R}$, $a^{10R}$, $a^{6R}$, $a^{20R}$, $a^{10R}$, $a^{6R}$, and $a^{20R}$). Heterozygous status of five alleles ($a^{1R}$/a, $a^{2R}$/a, $a^{5R}$/a, $a^{12R}$/a, and $a^{15R}$/a) and mild heterogeneity in the genetic backgrounds of homozygous alleles did not alter the overall trend, nor did age variation among adult mice tested (18–31 weeks) contribute significantly to the variance in mean AHP or PTCA determinations, although age generally enhances the level of visible phaeomelanin in the coat. With respect to PTCA levels, significant differences ($P < 0.05$) were observed for most alleles (except $a^{10R}$) compared to either the 101/Rl (wild type) or the C57BL/10Rl (nonagouti black) control;
however, the variation was <2-fold and did not follow a consistent trend with respect to the agouti allelic series. Slight heterogeneity in genetic background among the various mutant stocks could explain this low yet significant variation in eumelanin content. Altogether, these data demonstrate a fair (albeit imperfect) correlation between chemically determined pheomelanin content and visual determination of the severity of agouti loss of function in the various alleles, suggesting that this method provides a semiquantitative measure of the effect of agouti mutations on the level of pheomelanin synthesis in vivo.

**Molecular analysis:** The 25 recessive alleles were generated in (101/Rl × C3H/RI)F1 hybrid mice (see Materials and Methods), so the mutations arose on either a C3H/RI (A) or a 101/RI (A*) chromosome. The wild-type, parental alleles A and A* differentially express four alternatively spliced transcripts (Bultman et al. 1992, 1994; Vrieling et al. 1994) that encode a common protein sequence (Figure 3A). Whereas both A and A* express the temporally restricted transcripts that give rise to the characteristic subapical band of pheomelanin in dorsal and ventral hairs, only A* expresses the ventral-specific transcripts that uniquely generate a predominantly yellow or cream-colored ventrum in A*-derived alleles. To determine the parental allele of origin for the 25 recessive mutations, and to help explain ventral fur phenotypes among the various alleles, we employed a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy using ventral skin from neonatal mice and two PCR primer sets (Figure 3B). One set of primers (Ex 2–4) amplified the coding region that is common to all transcripts in both A and A*-derived alleles. The other set (Ex 1A–4) is unique to ventral-specific transcripts in A*-derived alleles only and yields a larger-sized product. Findings from this analysis were then confirmed by Southern analysis (Chen et al. 1996) using an exon 1A*-specific probe (data not shown). Summarized in Table 2, 11 hypomorphic and 4 amorphic alleles arose on the C3H/RI (A) chromosome, whereas 7 hypomorphs and 3 amorphs were derived from 101/RI (A*).

Although each of the smaller phenotype groups contained exclusively A- or A*-derived alleles, the distribution appeared random in the larger phenotypic categories (moderate, null), suggesting no significant correlation between phenotype category and parental allele mutated.

To identify the precise nature of DNA damage that produced the agouti coat phenotypes, we analyzed the agouti locus at both the genomic and mRNA expression levels. The entire coding region (exons 2–4) of agouti was amplified by RT-PCR from both dorsal and ventral skin of neonatal mice carrying each mutant allele (data not shown; see Materials and Methods). The RT-PCR products were cloned, and several clones from each allele were sequenced to identify potential mutations within the agouti protein-coding region. The affected exon(s) were then isolated from genomic DNA of the appropriate mutant by PCR, cloned, and sequenced to verify the initial RT-PCR results. Further independent verification of these sequence alterations was gained through RNase protection assay, using the entire agouti coding region as a probe (data not shown). Table 2 summarizes the results of these molecular analyses.

Of the 25 recessive alleles characterized, 14 alleles with phenotypes ranging from mild to null demonstrated aberrant protein-coding potential. Point muta-
Summary of the molecular characterization of recessive agouti mutations

TABLE 2

<table>
<thead>
<tr>
<th>Phenotype group</th>
<th>Genotype</th>
<th>Parental allele</th>
<th>DNA change</th>
<th>Amino acid change</th>
<th>mRNA level</th>
<th>Site/type of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>a16R/a16</td>
<td>A TGA → GTA</td>
<td>stop → Leu112 + 26 aa</td>
<td>~Wt</td>
<td>E4-cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a2R/a2R</td>
<td>A None identified</td>
<td>None</td>
<td>ND</td>
<td>Regulatory</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a20R/a20</td>
<td>A GAA → AAG</td>
<td>Gln65 → Lys</td>
<td>ND</td>
<td>E3-basic domain</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>a13R/a13</td>
<td>A None identified</td>
<td>None</td>
<td></td>
<td>mRNA levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a14R/a14</td>
<td>A None identified</td>
<td>None</td>
<td></td>
<td>mRNA levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a15R/a15</td>
<td>A None identified</td>
<td>None</td>
<td></td>
<td>mRNA levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a16R/a16</td>
<td>A Δ 9 nt</td>
<td>Δ Phe116Leu116Cy516</td>
<td>~Wt</td>
<td>E2-signal peptide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a3R/a3R</td>
<td>A GTC → GAC</td>
<td>Val5 → Asp</td>
<td>~Wt</td>
<td>E2-signal peptide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a10R/a10</td>
<td>A AG → TG</td>
<td>Δ Lys85Lys87</td>
<td>[2.9]</td>
<td>E4-splice acceptor, basic domain</td>
<td></td>
</tr>
<tr>
<td>Umbrous-like</td>
<td>a12R/a12</td>
<td>A GCC GAG → GCA_AAG</td>
<td>Glu68 → Lys</td>
<td>ND</td>
<td>E3-basic domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a13R/a13</td>
<td>A GAG → AG</td>
<td>Glu68 → Lys</td>
<td>ND</td>
<td>E3-basic domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a14R/a14</td>
<td>A TCC → CCC</td>
<td>Ser112 → Pro</td>
<td>~Wt</td>
<td>E4-cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a15R/a15</td>
<td>A None identified</td>
<td>None</td>
<td>~Wt</td>
<td>Regulatory/cell type</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>a16R/a16</td>
<td>A None identified</td>
<td>None</td>
<td>[2.7]</td>
<td>mRNA levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a17R/a17</td>
<td>A None identified</td>
<td>None</td>
<td>[3.6]</td>
<td>mRNA levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a18R/a18</td>
<td>A None identified</td>
<td>None</td>
<td>ND</td>
<td>Regulatory</td>
<td></td>
</tr>
<tr>
<td>Null</td>
<td>a19R/a19</td>
<td>A TGC → AGC</td>
<td>Cy515 → Ser</td>
<td>~Wt</td>
<td>E4-cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a20R/a20</td>
<td>A Δ 11 nt</td>
<td>Δ Phe116Ala117 + fs</td>
<td>~Wt</td>
<td>E4-cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a21R/a21</td>
<td>A TTC → TGC</td>
<td>Phe118 → Ser</td>
<td>~Wt</td>
<td>E4-cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a22R/a22</td>
<td>A Δ CTC → TA</td>
<td>Ser116 → Met + fs</td>
<td>[4.0]</td>
<td>E2-N terminus, fs, premature termination</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>a23R/a23</td>
<td>A None identified</td>
<td>None</td>
<td>ND</td>
<td>Regulatory</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a24R/a24</td>
<td>A Δ 359 nt</td>
<td>Δ Ala54 → Lys54 + fs</td>
<td>~Wt</td>
<td>E3, E4-A basic domain, fs, cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>C3H/R1-A/A</td>
<td>— None</td>
<td>None</td>
<td>Wt</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101/R1-A'/A'</td>
<td>— None</td>
<td>None</td>
<td>Wt</td>
<td>Wild type</td>
<td></td>
</tr>
</tbody>
</table>

Affected nucleotides are indicated by the underlined nucleotides within the context of their codon triplet code. Δ, deletion; aa, amino acids; Wt, wild type; ↓, reduced; ND, not determined; E2, exon 2; E3, exon 3; E4, exon 4; fs.

RNA was prepared from homozygous neonates, unless otherwise noted. Level of agouti mRNA expression was normalized to the level of Gapdh expression in each sample and then compared to the Gapdh-normalized value in C3H/R1 and 101/R1 mice. ~Wt indicates that the normalized expression levels in mutant alleles were ±20% of the normalized level in C3H/R1 and 101/R1 mice.

DNA was obtained from homozygous mutant adults (a20R/a20; a12R/a12), as determined by breeding tests.

When neonates were collected for RNA analysis, these stocks were maintained as heterozygotes. Because agouti expression occurs in neonates prior to a visible fur distinction between a/a and a/a (black, extremely low agouti expression) littermates, RNA was prepared from five neonates of mixed (a/a or a/a) genotype. Consequently, quantification of agouti RNA levels in these mutants is not directly comparable to other mutant or wild-type alleles.

RNA was prepared from a20R/a neonates only.

C57BL/10R1 was included as the relevant heterozygote reference. The primary mutation in nonagouti is a severe regulatory defect (Bultman et al. 1992; Figure 4) that is common to both C57BL/6J and C57BL/10R1 inbred strains. C57BL/6J-a/a is like a' at the ventral-specific promoter region (Bultman et al. 1994; Chen et al. 1996) and wild type in the protein-coding region, whereas C57BL/10R1-a/a carries the A orientation at the ventral-specific promoter and a novel signal peptide mutation.

More details on the tables and figures can be found in the original document.
Gapdh was altered in any of the mutant mice, Northern analysis of the C-terminal region of agouti that is believed to bind in any of the mutants because the hypomorphic phenotype is apparent (e.g., 1R for a^{125}). Arrows to the right indicate the agouti-specific band and the internal control, Gapdh.

In the case of alleles maintained as heterozygotes (a^{2R}, a^{3R}, a^{12R}, and a^{30R}), quantification of RNA levels was not possible since d^{7}/a individuals could not be distinguished from a/a littermates. In addition, RNA analysis was not performed for the a^{2R} allele due to poor stock viability. However, extensive sequence analysis of genomic DNA from a^{3R} adults clearly indicated wild-type sequence in the protein-coding region, 3′ untranslated region, and ∼1 kb surrounding the hair cycle-specific promoter and hair cycle-specific 5′ untranslated exons.

**DISCUSSION**

Agouti structure and function: One of the most challenging aspects of this study was accurate correlation of the severity of agouti mutant phenotypes with the molecular changes in the locus (summarized in Figure 5A). On the basis of earlier studies (Bultman et al. 1991; Perry et al. 1996; Kiefer et al. 1997, 1998), we expected that the more severe mutations would map to the C-terminal region of agouti that is believed to bind Mcrs. In fact, all null mutations except one (a^{125}) altered the coding potential of the C terminus and thus ablated agouti activity at the cellular level, although the a^{3R} allele was nearly null with respect to ventral pigmentation. However, one protein-coding mutation, a^{30R}, demonstrated slightly reduced levels of mRNA expression due to a splicing defect. A single-base change at the normal exon 4 splice-acceptor site prompts usage of a surrogate splice acceptor within the exon 4 coding region, which is apparently less efficient, as indicated by a larger RNA product in the a^{30R} lane. Northern analysis also indicated that 2 (a^{12R}, moderate; a^{30R}, umbrous-like) of the 11 putative regulatory alleles express normal to high levels of agouti message, which is wild type in sequence.
Loss-of-Function agouti Mutations

Figure 5.—Summary of molecular analysis. (A) Schematic of the ≥125-kb agouti locus. The thick horizontal line represents the genomic DNA, with solid boxes representing protein-coding exons, open boxes representing noncoding exons (not drawn to scale), and arrows above the noncoding exons representing transcriptional start sites. Two promoter regions are separated by ~100 kb, as indicated by a break in the line. Location of regulatory elements is postulated by the double-headed arrows above the gene, both 5′ (Bultman et al. 1992, 1994; Vrieling et al. 1994) and 3′ (Miller et al. 1994) to the coding exons. Specific alleles believed to carry regulatory mutations are indicated at left, beneath the transcriptional promoters. Allele designations (e.g., 2R for a2R) for the presumed regulatory mutations are aligned in columns that refer to the phenotype categories, from the mildest at the left to the most severe at the right. Dotted lines extending downward from the coding exons show an expanded view of the agouti protein, with the various structural domains indicated. The positions of hypomorphic (1R–14R) and amorphic (19R–25R) coding mutations are indicated below the protein schematic. The position of the 25R mutation also corresponds to the exon 2/3 boundary, and the 10R mutation corresponds to the exon 3/4 boundary. (B) Alignment of the cysteine-rich C-terminal sequence of human AGRP and mouse agouti proteins. Amino acids 87–132 of AGRP and 92–131 of agouti are shown. Conserved cysteines are highlighted in yellow, and the Mcr binding triplet, RFF, is shown in red. The pattern of disulfide bonding is indicated by brackets, with linkages 1–3 forming the “core” of the three-dimensional structure, as determined by Bolin et al. (1999), while bonds 4 and 5 appear to present and stabilize the RFF-containing loop. Asterisks below mouse agouti residues Cys110, Ser112, Cys115, and Arg118 correspond to the sites of mutations in alleles 20R, 14R, 19R, and 21–22R, respectively.

Visual phenotype analysis: In addition to agouti gene

notype group, with the exception of the severe hypomorphs. Overall, the C-terminal mutations were more devastating than the basic-region mutations, and the signal-peptide lesions were comparatively modest. Exceptions to these general rules are noted, however (a18R, a17R, a19R). Among the putative regulatory mutations, five alleles reflected lower mRNA levels in skin relative to the wild-type controls, although the absolute decrease in mRNA expression did not necessarily correlate with severity of the phenotype (e.g., a16R, a15R vs. a14R, a17R). On the basis of variation among the homozygous coding-region mutations and the wild-type controls, mild heterogeneity in genetic backgrounds accounts for no >20% fluctuation in mRNA levels. These data suggest that potentially significant differences in agouti transcriptional/translational control are likely to play a major role both temporally and spatially in determining how mutant phenotypes correlate with agouti mRNA expression patterns in some of the regulatory alleles. Further analysis using in situ hybridization of the skin and hair follicles and perhaps immunolocalization of the agouti protein should clarify the basis for many of the subtle hypomorphic phenotypes.
structure and expression patterns, other factors influence the appearance of agouti coat phenotypes. The expression level of wild-type agouti is determined not only by the degree of Mcr antagonism in vivo, but also by the dosage of partially functional agouti proteins. For example, mice homozygous for any given hypomorphic allele generally exhibited milder coat phenotypes (slightly more pheomelanogenic) than were apparent in mice heterozygous (a\(^d\)/a) for the same allele. Factors acting independently of agouti also influence agouti coat phenotypes. Chief among these is the effect of age on the spatial distribution of pheomelanin along the dorsal/ventral axis. These age-related changes are most striking between weaning and adulthood (3–12 weeks) and are particularly evident in mice carrying hypomorphic alleles. For example, those in the mild category appear nearly wild type as adults, but have a darker, umbrous dorsum at weaning age. Mice with more severe hypomorphic mutations (severe or umbrous-like as adults) typically exhibit nonagouti fur over the entire dorsum and agouti fur in the ventrum at weaning age. Reduced pheomelanin-banded hairs become apparent in adults on the sides and in the dorsum, depending on the phenotypic category. In contrast, amorphic mutations show black fur in both young and adult mice. For these reasons, we deliberately chose to analyze adult fur phenotypes (and primarily dorsal fur) rather than juvenile phenotypes, as the former were generally more stable and better represented each allele. Investigators in the past have consistently observed that pheomelanin is lost from dorsal fur prior to its loss from ventral fur as one progresses down the agouti dominance hierarchy (Silver 1979). Moreover, this effect is not entirely explained by the differential expression of ventral-specific agouti transcripts (Bultman et al. 1994; Vrieling et al. 1994), as equivalent expression of agouti variants in the dorsum and ventrum of transgenic mice consistently generates a more pheomelanic ventrum (Perry et al. 1995, 1996; Miltenberger et al. 1999). Our observation that the dorsal midline is most affected by pheomelanin loss resulting from a locus mutation further suggests that this is the region in which melanocytes are most susceptible to mild variations in agouti activity. One possibility is that hair-bulb melanocytes along the dorsal/ventral axis may express variable densities of Mcrs and/or the mahogany protein on their cell surfaces. The effective threshold concentration of agouti required by the melanocyte to switch from eumelanin to pheomelanin synthesis would be greater or more stringent in the dorsum than in the ventrum. Collectively these observations indicate that, in addition to the quality and quantity of agouti gene activity, independent and possibly dynamic factors within the hair follicle microenvironment contribute to agouti coat phenotypes.

Chemical analysis of melanins: The chemical method utilized for directly quantifying pheomelanin and eumelanin content of the fur allowed an objective interpretation of agouti loss of function in the various alleles. In general agreement with the visibly scored phenotypes, the amounts of the pheomelanin-specific degradation product AHP steadily declined over an ~10-fold range as one progresses down the allelic series analyzed here. However, because absolute AHP levels were not always in perfect agreement with the perceived severity of some agouti mutations, the visual phenotype may be a more sensitive discriminator of agouti activity in these cases than the chemical analysis. Seemingly random variation within a <2-fold range was observed for the eumelanin-derived degradation product PTCA in dorsal fur, indicating that, as anticipated, the primary effect of agouti mutation was at the level of pheomelanin, not eumelanin, synthesis. A surprising result from this analysis, however, is that the range of adult agouti phenotypes from wild type to jet black actually reflects only a minor change (2.3%) in the total melanin distribution. By converting dorsal fur AHP and PTCA values to pheomelanin and eumelanin content, respectively (Ito and Fujita 1985), dorsal midline fur of wild-type agouti mice contains 97.3% eumelanin and 2.7% pheomelanin. Moderate hypomorphic alleles, such as a\(^{11R}\), contain half as much pheomelanin or 1.4% (and 98.6% eumelanin), and visibly black fur, as in the amorphic allele a\(^{am}\), contains only 0.4% pheomelanin (and 99.6% eumelanin). The reflective qualities of pheomelanin granules and the manner in which individual hairs overlap along the coat probably augment these small differences in pheomelanin content such that even subtle changes in band width or pigment intensity are maximally exposed and readily detectable by the unaided eye.

Regulatory mutations: Using several levels of molecular analysis, we determined that 11 of the 25 agouti alleles (10 hypomorphs, 1 amorph) contained wild-type protein-coding sequence, suggesting that regulatory mutations somewhere in the locus interfere with proper and efficient temporal/spatial expression of the wild-type gene products in these mice. The precise genetic alterations remain unidentified, however, as very little is currently known about the elements that contribute to elaborated transcriptional control within the large agouti locus. Five of the noncoding hypomorphs that exhibited moderate (a\(^{1a}\), a\(^{1b}\), a\(^{2a}\)) to severe (a\(^{10b}\), a\(^{7b}\)) phenotypes expressed reduced levels of agouti mRNA in neonatal skin compared to wild-type controls. This finding probably accounts for their hypomorphic status, since a strong correlation has been established previously between the level of steady-state RNA synthesis in dominant agouti alleles and the amount of pheomelanin in the coat (Duhl et al. 1994; Michaud et al. 1994b; Yen et al. 1994; Hustad et al. 1995; Klebig et al. 1995; Zemel et al. 1995; Argeson et al. 1996; Miltenberger et al. 1999). Although, as discussed above, imperfect correlation was observed between the absolute level of agouti expression and the severity of phenotypes in some alleles, our data nonetheless suggest that approximately threefold or
greater decrease in agouti expression levels are sufficient to induce hypomorphic phenotypes in vivo. This finding probably reflects the delicate threshold of agouti activity required for effective Mcr antagonism.

Two hypomorphic alleles for which no DNA alterations were identified (a12R, moderate; a13R, umbrous-like) may represent a different class of regulatory mutation, as these mice expressed wild-type agouti message at normal to high levels in neonatal skin. Although the timing of agouti expression (day 4) was apparently normal in these mice, mutational alterations in spatial control elements could misdirect agouti expression to anomalous cell type(s) in the skin, leading to less optimal diffusion and localization of the wild-type agouti protein. Normally, the agouti protein is expressed by specialized cells in the dermal papillae (Millar et al. 1995) and has a limited diffusion distance in vivo, as suggested by skin transplantation (Silvers and Russell 1955) and transgenic experiments (Kucera et al. 1996). In situ hybridization or immunolocalization studies will be needed to determine if agouti is indeed mislocalized in the a12R and a13R alleles. Three other hypomorphs (a2R, mild; a5R, moderate; a6R, severe) and the amorphic allele a8R also exhibited no coding alterations and likewise may have acquired devastating lesions in critical regulatory element(s) that either reduce/eliminate agouti expression or sufficiently relocate its site of synthesis in vivo such that subthreshold levels of functional agouti protein reach hair-bulb melanocytes. As more data become available from mouse genome sequencing efforts, previously uncharacterized regions of the ≈125-kb agouti locus should become more amenable to mutagenesis screening, and the lesions in these 11 noncoding alleles more readily identifiable. In the meantime, these alleles will provide a rich mutant resource for future investigation into the poorly understood, yet complex, arena of agouti transcriptional control.

Signal peptide mutations: Two hypomorphic mutations (a8R, a9R) with moderate coat phenotypes were found to alter the coding potential of the agouti signal peptide. The a8R allele carries an in-frame deletion of 9 bp that eliminates Phe17-Cys26, the slightly more severe a9R allele contains a single missense mutation (T → A) that substitutes Asp for the conserved Val. Requirement for an intact signal peptide in vivo has been demonstrated previously (Hustad et al. 1995; Perry et al. 1996), in keeping with early seminal studies that indicated a paracrine role for agouti in pigmentation (Silvers and Russell 1955). Analyses by two neural networks, TargetP (Emanuelsson et al. 2000) and SignalP (Nielsen et al. 1997), provide clues to the cell biological defects caused by these mutations. Reduced hydrophobic-core length in the a8R signal peptide and the net negative charge at the a9R N terminus may interfere with targeting of the nascent preproteins to the endoplasmic reticulum (ER; TargetP score: wt 0.961, a8R 0.900, a9R 0.955). In addition, reduced efficiency of signal peptide processing at an alternative site (Ala22) in a9R and at the wild-type site (Ser66) in a6R may also play a role (SignalP Y score: wt 0.743, a6R 0.662, a9R 0.708). Although the functional impact of an altered N terminus (ΔHisLeuAla) in the mature a8R protein is not known, reduced ER targeting efficiency/fidelity of the a8R propeptide is probably sufficient to induce a loss-of-function phenotype in vivo. Potential ramifications include reduced secretion rate and delayed diffusion of the mature protein to target cells. Because the a8R allele is predicted to generate a wild-type mature protein, these analyses also suggest that reduced quantity, not quality, of mature secreted a8R protein is responsible for reduced agouti function in these mice.

Basic region mutations: All coding-region mutations were expressed at approximately wild-type levels, with the exception of the hypomorphic allele a10R. This allele contained a single base change at the splice-acceptor site in exon 4, prompting usage of an internal AG in the fourth exon that is apparently less efficient than the wild-type site. Abnormal splicing in a10R provides an in-frame message for translation and simultaneously deletes two byine residues (Lys66,Lys72) in the basic domain. Although the approximately threefold reduction in mRNA levels may contribute to the mutant phenotype in a10R, deletion of the two basic residues is probably of greater significance, since the regulatory mutations, such as a8R and a9R, exhibit greater reductions in mRNA levels yet display a less severe coat phenotype.

The central basic domain of agouti is highly conserved and important for activity in vivo, yet its precise biological role is poorly defined (Perry et al. 1996; Mittlerberger et al. 1999). More than one-half of the 29 aa in this domain are basic and often arranged in pairs, suggesting that endoproteolytic processing may generate smaller functional peptides in vivo. Western analysis of agouti in mouse tissues has since eliminated this early hypothesis (Ollmann and Barsh 1999), leaving alternative models, such as the following: (1) interaction with mahogany and/or mahoganoid (Dinulescu and Cone 2000; Gunn and Barsh 2000; He et al. 2001); (2) facilitation of intracellular trafficking/biogenesis of the mature agouti protein; (3) promoting diffusion to cellular targets following secretion; and/or (4) direct interaction with Mc1r (Virador et al. 2000). A common theme that emerged from characterizing the three point mutations (a2R, a12R, a13R) and one small deletion (a10R) that map to the agouti basic region is that net charge may be important for biological activity. In wild-type agouti, 16 basic (12 Lys, 4 Arg) and 2 acidic (Glu) residues generate a net +14 charge over the middle of the protein. Deletion of Lys72-Lys77 in a10R reduces the net positive charge to +12, generating a moderate dark-agouti phenotype. A single base change (G → A) in the mild allele, a6R, converts I of the 2 acidic residues (Glu66) to a basic aa (Lys), thereby increasing the net charge to +16. The second acidic aa (Glu72), which is absolutely conserved in all homologs, was also modified to Lys in
two more severe alleles, \(a^{12R} \) (CG \( \rightarrow \) AA) and \(a^{13R} \) (G \( \rightarrow \) A). Comparison of the phenotypes of \(a^{1R} \) (mild) vs. \(a^{12R} \) and \(a^{13R} \) (umbrous-like) suggests that the position of charged residues, rather than simply the net charge, influences agouti activity. Although the biological role of the basic domain remains unclear, the four mutations identified here could serve as useful tools for testing various hypotheses. Potential protein-protein interactions could be directly addressed in vivo by epistasis or double-mutant studies. In addition, although wild-type agouti is not processed beyond the signal peptide in vivo, the basic region mutations identified here could introduce new cleavage site(s) for proteolytic convertases, thereby yielding aberrantly processed forms of agouti in vivo.

**C-terminal mutations:** The final group of coding-region mutations maps to the C terminus of agouti, with most alleles being null except for \(a^{11R} \) and \(a^{15R} \). Spacing of the 10 cysteine residues in the C terminus is absolutely conserved in all agouti homologs, as are 9 out of the 10 cysteines in the C terminus of the agouti-related protein (AGRP, Figure 5B) that naturally antagonizes Mc3r and Mc4r in the central nervous system (Huszár et al. 1997; Shutter et al. 1997). This arrangement of cysteines bears resemblance to that of the disulfide-bonded \(\omega\)-conotoxins and \(\omega\)-agatoxins (Oliveira et al. 1994; Pallaghy et al. 1994), suggesting that agouti and AGRP fold into an “inhibitor cystine-knot” motif that preserves the conserved residues Arg116-Phe117-Phe118 (Figure 5B) that mediate direct interaction with Mcrs (Kiefer et al. 1998). Indeed, both mouse agouti (Willard et al. 1995) and AGRP (Bures et al. 1998) disulfide bond in the same pattern found in the \(\omega\)-agatoxins; however, agouti and AGRP both contain an additional disulfide linkage (disulfide 5 in Figure 5B). Based on NMR studies of a chemically synthesized and biologically active form of AGRP, three disulfide bridges (bonds 1–3 in Figure 5B corresponding to \(Cys_{92}-Cys_{107}, Cys_{96}-Cys_{113}, \) and \(Cys_{107}-Cys_{121}\)) in mouse agouti) build the core of the C-terminal domain structure (Bolin et al. 1999). These core linkages are located at the base of the structure to anchor the bottoms of loops that present Mcr-interacting motifs. The fourth disulfide linkage in AGRP, which is analogous to \(Cys_{115}-Cys_{122}\) in mouse agouti (disulfide 4 in Figure 5B), appears to stabilize the relatively rigid “active loop” that contains the \(Arg_{116}-Phe_{117}-Phe_{118}\) binding determinants. The fifth disulfide \(Cys_{119}-Cys_{121}\) (in mouse agouti; bond 5 in Figure 5B) is not present in the \(\omega\)-agatoxins, is highly reducible in vitro (Bures et al. 1998), suggesting that it may be more exposed on the exterior of the three-dimensional structure.

Using transgenic mice, Perry et al. (1996) determined that individual cysteines are indeed critical for the activity of mouse agouti in vivo. Individual cysteines were changed to serine, the mutant cDNAs were expressed under the control of the \(\beta\)-actin promoter, and founder mice were assayed for yellow-pigmented fur and maturity-onset obesity (Perry et al. 1996). Mutation of four of the five disulfide bonding pairs (\(Cys_{92}-Cys_{107}, Cys_{96}-Cys_{113}, Cys_{107}-Cys_{121}, \) and \(Cys_{115}-Cys_{122}\) bonds 1–4 in Figure 5B) completely abolished activity. In contrast, mutation at \(Cys_{910}\) or \(Cys_{311}\), which together form the fifth disulfide connection, resulted in partial loss of function, particularly a tendency toward black pigmentation in the dorsum and yellow pigmentation in the ventrum (Perry et al. 1996). Interestingly, this disulfide bond, which has no analog in the \(\omega\)-agatoxins, forms the most flexible peptide loop (Bolin et al. 1999) and was found to be more labile to reduction in vitro (Bures et al. 1998). Collectively, the in vitro and in vivo data suggest that the loop formed by \(Cys_{915}-Cys_{931}\) (disulfide 5 in Figure 5B) probably does not form the core structure of the agouti C terminus as originally suggested by Bolin et al. (1999), but instead serves a secondary role such as stabilizing or facilitating presentation of the \(Arg_{116}-Phe_{117}-Phe_{118}\)-containing loop.

In the present investigations, two alleles were found to disrupt C-terminal cysteine residues (\(a^{10R} \) \(Cys_{110} \rightarrow \) stop; \(a^{19R} \) \(Cys_{115} \rightarrow \) Ser); however, only the former was a true null. In \(a^{10R}\), a \(C \rightarrow A\) change at \(Cys_{110}\) introduces a stop codon, indicating no translation of the Mcr-binding determinants and elimination of three disulfide bonds, hence the null phenotype. The \(T \rightarrow A\) mutation in \(a^{19R}\), however, results in a nearly null phenotype in which residual agouti activity is detectable as pheomelanin around the mammae and perineum, but not on or behind the ears, thus placing this allele between nonagouti and true nulls in the agouti dominance hierarchy. The \(a^{16R}\) mutation prevents formation of the fourth disulfide pair \((Cys_{115}-Cys_{122})\) that in AGRP stabilizes the \(Arg_{116}-Phe_{117}-Phe_{118}\)-containing loop, but does not actually form the core of the C-terminal structure (Bolin et al. 1999). The nearly null phenotype of \(a^{10R}\) mice is consistent with these structural data. Furthermore, this phenotype suggests that the ventral hair follicle microenvironment around the mammae and perineum is extremely sensitive to minimal levels of agouti activity. Surprisingly, however, this allele is analogous to the \(Cys_{915}-Ser\) mutation studied by Perry et al. (1996) in which the transgenic mice exhibited no agouti activity whatsoever. Discrepancy between the phenotype of \(a^{10R}\) mice with the findings of Perry et al. (1996) may be explained by their use of transgenic founder animals that may not uniformly express the transgene in all cell types.

Two other null mutations were shown to affect the putative Mcr-binding determinant at \(Phe_{118}\) (\(a^{22R} \) \(Phe_{118} \rightarrow \) Ala121; \(a^{25R} \) \(Phe_{118} \rightarrow \) Ser). The \(a^{22R}\) mutation deletes the last aa in the triplet binding determinant for Mcrs, in addition to two other C-terminal aa, thus inducing a reading-frame shift that eliminates the disulfide bonding partners for three of the five disulfide connections. Because the \(a^{22R}\) allele is a single missense mutation at \(Phe_{118}\), it provides the first direct evidence that this aromatic residue is indeed critical for agouti activity.
in vivo. An alternative explanation for the eumelanin phenotype of $a^{25R}$, in particular, is that the point mutation of Phe$_{113}$ may convert agouti into a potent agonist of Mc1r (OLLMAN et al. 1998), rather than into a crippled antagonist. Although our genetic tests show no indication that $a^{28R}$ behaves differently from other jet-black agouti mutations analyzed here (i.e., $A\rightarrow a^{28R}$ looks identical to other null alleles balanced with $A$ or $A^*$), this hypothesis could be formally addressed by direct Mc1r binding studies with labeled mutant agouti protein in vivo.

Considering the complex structural constraints guiding the successful folding needed for building a functional agouti C terminus, it is not surprising that deletions that alter the entire coding potential of this region also induced amorphic phenotypes. In $a^{3R}$, a 3-bp deletion/2-bp insertion event at the end of exon 2 introduces six missense aa at positions 49–54, followed by premature termination just inside exon 3, thereby eliminating the entire basic and C-terminal domains. A 359-bp deletion was identified in $a^{70P}$, encompassing all of exon 3 and resulting in direct splicing of exon 2 to exon 4. A similar effect on the agouti mRNA has been described for the $a^{MN7R}$ allele that resulted from deletion of a larger genomic fragment (~5 kb) in this vicinity (Bültman et al. 1991).

Surprisingly, the mildest allele characterized in this study also carries a lesion within the agouti C terminus. A single base change at the stop codon (G → T) in $a^{6R}$ results in an additional 27 aa at the very C terminus of an otherwise normal agouti protein, producing apparently little impact on normal protein folding or on the interactions with Mc1r. This finding is consistent with the observation that the C-terminal end of AGRP exhibits very little structure in solution and is not involved in stabilizing or presenting the Arg$_{116}$Phe$_{117}$Phe$_{118}$-containing loop (Bolin et al. 1999). In contrast, the other hypomorphic mutation in the agouti C terminus ($a^{18R}$, T → C) converts Ser$_{112}$ → Pro and induces a more severe, umbrous-like phenotype. Due to the helix-disrupting or turn potential of prolines and the proximity of this mutation to the closely spaced, conserved cysteines (Figure 5B), this mutation may alter the pairing of one or more disulfide bonds. Furthermore, lower rates of agouti secretion could occur in $a^{18R}$ due to ER retention and retrograde translocation of improperly folded proteins to the proteasome (Wiertz et al. 1996).

Toxicology: In addition to new insights into the structure and function of agouti, analysis of these multiple $a$-locus alleles provides useful toxicological data for better understanding the molecular mechanisms by which the various mutagens impact the mammalian germline. Analysis of SLT mutations at seven loci in the mouse have shown that the germ-cell stage is the chief determinant of whether mutational lesions are multi or single locus (Russell 1991, 2002). The proportion of the latter type, which is generally compatible with homozygous viability, is considerably higher for mutations induced in spermatogonia than for those induced in post spermatogonial stages or oocytes. For the present investigations, only homozygous viable $a$-locus alleles were selected, and thus it is not surprising that 21 of the 23 (91%) that originated in mutagenized germ cells ($a^{671}$ and $a^{25R}$ were spontaneous) were induced in spermatogonia. The remaining 2 were induced by X rays, 1 in oocytes ($a^{25}$) and 1 in early spermatids ($a^{671}$).

Overall, differences were observed among the various treatment groups in the ratio of coding vs. regulatory mutations. Of eight mutations induced by chemicals in spermatogonia, six (86%) were coding-region alleles, and these were point mutations, not deletions. By contrast, fewer than one-half (6 out of 15 or 40%) of mutations induced by radiations in spermatogonia were coding-region alleles, with 3 of the 6 being deletions. Of the two mutations not induced in spermatogonial stages, one occurred in the protein-coding region. Other information derived from SLT-induced mutations (Russell 2002) indicated that mutational lesions induced in post spermatogonial stages are more extensive than those induced in spermatogonia and that, among mutations induced in spermatogonia, those induced by radiations are more extensive than those induced by chemicals. The proportion of $a$-locus alleles that are not in the coding region follows the same relative order. It might therefore be suggested that these alleles may turn out to include a relatively small percentage of single-base-pair changes.

Of the five mutations generated with N-ethyl-N-nitrosourea (ENU) in spermatogonial stem cells, only one was an uncharacterized regulatory mutation ($a^{18R}$), and four were single-base substitutions. The A/T → T/A transversions in $a^{671}$ and $a^{18R}$ and the A/T → G/C transition in $a^{671}$ typify ~80% of the ENU-induced mouse germ-cell mutations identified in other studies (Novoroske et al. 2000). The fourth ENU mutation ($a^{671}$), a G/C → A/T transversion, is a type that had been found rarely in the past. The N-methyl-N-nitrosourea (MNU)-induced allele, $a^{6}$, was shown to carry a G/C → A/T transition, as is characteristic of this methylaing agent in mouse germ cells (Vogel and Natarajan 1995; Favor 1999). Although the mechanism by which ethylene oxide induces mutations in the mammalian germline remains unclear (Dellarco et al. 1990), the A/T → T/A mutation identified in $a^{18R}$ is consistent with N-alkylation damage (Vogel and Natarajan 1995). Finally, synthetic fuel induced an unidentified regulatory mutation in $a^{25}$; however, this lesion could be a deletion like the previously characterized Bmp5$^{SIN62}$ allele that was also generated with synthetic fuel in SLTs (Mark et al. 1997).

Of the seven loci screened in SLTs, the $a$ locus is one of the two least mutable for either radiation or chemical treatment, representing only 0–7% of all mutants recovered (Russell 2002). Spontaneous mutations arising in mitotic stages of gametogenesis are also extremely
rare at the \( a \) locus. In fact, among the 66 such mutations reported for SLTs by three laboratories, none has been at the \( a \) locus (Russell and Russell 1996). On the other hand, among spontaneous mutations arising during the perigamic interval (Russell and Russell 1996; Russell 1999) over one-third involved the \( a \) locus. Two of these perigamic \( a \)-locus mutations were analyzed in the present study. One (\( a^{228} \)) was determined to be a single-base-pair change of a type \( (A/T \rightarrow G/C) \) that has been reported to be common after ENU mutagenesis (see above); the other (\( a^{48} \)) was found to be outside the coding regions, and the DNA alteration remains to be identified.

**Concluding remarks:** The extensive and diverse set of recessive, homozygous-viable, and intragenic mutations in the *agouti* gene generated in SLTs at ORNL has permitted us to perform detailed structure/function analysis of the agouti protein. As was previously shown for other SLT loci (Tyrlp, Sarangarajan et al. 2000; Tyr, Yokoyama et al. 1990 and Rinchik et al. 1993; Myo5a, Huang et al. 1998a,b; Bmp, Marker et al. 1997; and Ednrb, Shin et al. 1997), an allelic series of intragenic mutations that alter (but do not necessarily eliminate) a protein or its regulation is an invaluable tool in discovering the scope of *in vivo* biological functions of a gene. The community-wide effort to generate ENU-induced mutant alleles of genes throughout the genome, both by phenotype-driven and gene-driven approaches (Justice et al. 1999; Rinchik 2000; Brown and Balling 2001), underscores the usefulness of these tools and will provide important reagents for deciphering gene functions, interactions, and regulatory networks.

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