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Molecular Basis of the Pleiotropic Phenotype of Mice Carrying the *hypervariable yellow* (A^{hvy}) Mutation at the *agouti* Locus

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ABSTRACT

The murine *agouti* locus regulates a switch in pigment synthesis between eumelanin (black/brown pigment) and phaeomelanin (yellow/red pigment) by hair bulb melanocytes. We recently described a spontaneous mutation, *hypervariable yellow* (A^{hvy}) and demonstrated that A^{hvy} is responsible for the largest range of phenotypes yet identified at the *agouti* locus, producing mice that are obese with yellow coats to mice that are of normal weight with black coats. Here, we show that *agouti* expression is altered both temporally and spatially in A^{hvy} mutants. *Agouti* expression levels are positively correlated with the degree of yellow pigmentation in individual A^{hvy} mice, consistent with results from other dominant yellow *agouti* mutations. Sequencing of 5′ RACE and genomic PCR products revealed that A^{hvy} resulted from the integration of an intracisternal A particle (IAP) in an antisense orientation within the 5′ untranslated *agouti* exon 1C. This retrovirus-like element is responsible for deregulating *agouti* expression in A^{hvy} mice; *agouti* expression is correlated with the methylation state of CpG residues in the IAP long terminal repeat as well as in host genomic DNA. In addition, the data suggest that the variable phenotype of A^{hvy} offspring is influenced in part by the phenotype of their A^{hvy} female parent.

THE protein product of the murine *agouti* gene acts as a paracrine signaling molecule within the microenvironment of the hair follicle to control the decision between the production of eumelanin (black or brown pigment) and phaeomelanin (yellow or red pigment) by hair bulb melanocytes (reviewed in SILVERS 1979; SIRACUSA 1994). Expression of the *agouti* gene leads to the production of phaeomelanin, whereas lack of *agouti* expression results in eumelanin production. Because the *agouti* gene is normally expressed during the middle of the hair growth cycle, mice that are wild type at the *agouti* locus exhibit a subapical band of yellow pigment on an otherwise black hair, producing the overall classic agouti pattern. In addition to mice, this pattern is found in many mammals including cats, raccoons and sheep (reviewed in SEARLE 1968).

The *agouti* gene was cloned (BULTMAN *et al.* 1992; MILLER *et al.* 1993) and found to contain three coding exons (2, 3, and 4) and four alternatively spliced 5′ untranslated (UTR) exons (1A, 1B, 1C, and 1D) (BULTMAN *et al.* 1994; VRIELING *et al.* 1994). Exons 1A and 1B are located ~120 kb upstream of the first coding exon. Transcripts containing exons 1A and 1B are found only in the ventral skin of light-bellied mice. Exons 1C and 1D are hair cycle-specific exons that reside 18 kb upstream of exon 2. Transcripts containing exons 1C and 1D are found in both dorsal and ventral skin (BULTMAN *et al.* 1992, 1994; MILLER *et al.* 1993; VRIELING *et al.* 1994).

The *agouti* gene encodes a predicted 131-amino acid protein that contains a cysteine-rich region at the carboxyl-terminal end and a highly basic central region (BULTMAN *et al.* 1992; MILLER *et al.* 1993). The function of these regions is not known, but it has been postulated that the cysteine-rich carboxyl-terminal end of the agouti protein may have a role in the interaction with specific neurotransmitter receptors or ion channels (KLEBIG *et al.* 1995; MANNE *et al.* 1995). Consistent with this hypothesis is the recent work by ZEMEL and colleagues (1995) that implicates the agouti protein in the regulation of intracellular Ca^{2+} levels. The predicted agouti protein also contains an N-terminal consensus signal sequence but does not contain a transmembrane domain, suggesting that the agouti protein is secreted (BULTMAN *et al.* 1992; MILLER *et al.* 1993). Receptor-binding studies revealed that the agouti protein acts as an antagonist to the *α-melanocyte stimulating hormone receptor* (*α-MSHR*) (LU *et al.* 1994). These studies suggest that eumelanin is produced when *α-MSH* is bound to its native receptor, and conversely, phaeomelanin is produced when the agouti protein antagonizes the binding of the *α-MSHR* to its ligand (*α-MSH*).

The ease of identifying coat color variants has facilitated the identification of numerous phenotypically distinguishable mutations at the *agouti* locus in mice that exhibit a variety of coat colors ranging from pure yellow to pure black (reviewed in SIRACUSA 1994). The hierarchy of alleles dictated by the *agouti* locus indicate that

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the production of yellow pigment is dominant to the production of black pigment. In addition to controlling coat color, dominant yellow alleles at the *agouti* locus are associated with a number of pleiotropic effects including adult-onset obesity, noninsulin dependent diabetes, hyperinsulinemia and increased susceptibility to neoplasms (reviewed in YEN *et al.* 1994; SIRACUSA 1994).

Most mutations at the *agouti* locus produce mice of a single color. However, the alleles *agouti mottled* (a^m) (RUSSELL 1964), *agouti mottled-JAX* (a^{mj}) (HUSTAD *et al.* 1995), *viable yellow* (A^{vy}) (DICKIE 1962), and *intracisternal A-particle yellow* (A^{iapy}) (MICHAUD *et al.* 1994b) produce mice that exhibit variations in coat color. The a^m mutation resulted from radiation of an (C3H/Rl × 101/Rl) F_1 mouse, and therefore the mutated allele is either *agouti* (*A*) or *light-bellied agouti* (A^w) (RUSSELL 1964). The a^{mj} mutation arose spontaneously from a (C3H/HeJ × C57BL/6J) F_1 mouse and therefore the mutated allele is either *A* or *nonagouti* (*a*) (HUSTAD *et al.* 1995). Mice carrying the a^m and a^{mj} alleles can range in color from pseudoagouti to nonagouti (a^{mj} mice can also be completely black) and often exhibit mottling. Mice carrying the dominant A^{vy} and A^{iapy} alleles, which both arose spontaneously in the C3H/HeJ strain, can range in coat color from pseudoagouti to pure yellow and, like a^m and a^{mj}, often exhibit mottling. The *intermediate yellow* (A^{iy}) mutation also occurred spontaneously in the C3H/HeJ strain and results in another dominant allele that produces mice that are sooty yellow (DICKIE 1966). However, mice carrying the A^{iy} allele have not been reported to display mottled coats (DUHL *et al.* 1994b).

We recently identified a mutation at the *agouti* locus, named *hypervariable yellow* (A^{hvy}), that arose spontaneously in the C3H/HeJ strain (SIRACUSA *et al.* 1995). We demonstrated that A^{hvy} is responsible for the largest range of phenotypes yet identified at the *agouti* locus. Mice heterozygous or homozygous for A^{hvy} can be obese with yellow coats or of normal weight with almost pure black coats. In addition, most A^{hvy} mice are highly mottled, with yellow stripes or patches on an otherwise black or agouti background. Because of the extreme cellular heterogeneity exhibited by mice carrying the A^{hvy} mutation, we were interested in characterizing this allele at the molecular level. Here, we show that the A^{hvy} mutation results from the insertion of an intracisternal A-particle (IAP) within the UTR hair cycle-specific exon 1C. We show that methylation of CpG sites within this region is correlated with *agouti* expression. We postulate that an enhancer and/or cryptic promoter within the long terminal repeat (LTR) of the IAP controls *agouti* gene expression in A^{hvy} mice. A^{hvy} is the fourth example of an *agouti* allele that resulted from the insertion of an IAP that usurps control of *agouti* gene expression (reviewed in SIRACUSA 1994). Other mutations that resulted from IAP-element insertions include the dominant alleles A^{iapy}, A^{vy}, and A^{iy} (DUHL *et al.* 1994b; MICHAUD *et al.* 1994b). Although each of these insertions occurred in unique sites within the *agouti* gene, the *agouti* coding sequence was left intact, indicating that in A^{hvy}, as well as A^{iapy}, A^{vy} and A^{iy}, the phenotypes result from ectopic overexpression of the *agouti* gene due to regulatory mutations. Finally, we show that mostly yellow A^{hvy} female mice tend to produce offspring with greater amounts of phaeomelanin in their coats, suggesting that the maternal phenotype can influence the phenotype of resulting offspring.

MATERIALS AND METHODS

Mice: The spontaneous A^{hvy} mutation arose on the C3H/HeJ (C3H) strain at The Jackson Laboratory (Bar Harbor, ME) (SIRACUSA *et al.* 1995). The radiation-induced *extreme nonagouti* (a^e) mutation arose on the S strain (HOLLANDER and GOWEN 1956). The radiation-induced *nonagouti lethal* (a^l) mutation arose on a (C3H/HeH × 101/H)F_1 background (LYON *et al.* 1985) and was obtained from Harwell (MRC Radiobiology Unit, Chilton, UK). The A^{hvy}, a^e, and a^l mutations were maintained and propagated on a C57BL/6J (B6) background at the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and transferred to the Jefferson Cancer Center (Philadelphia, PA). Mutant progenitors were kindly provided by Dr. N. A. JENKINS and Dr. N. G. COPELAND (NCI-FCRDC, ABL-BRP, Frederick, MD). Most A^{hvy} mice used for these studies were at the N_{22}-N_{24} backcross generation. Mice of the C57BL/6J strain were purchased from The Jackson Laboratory.

Probes: The Clex1 probe was isolated from C3H *A/A* genomic DNA by PCR using primers specific to exons 1C and 1D. The primers were 1C2-5'TGGCCTCTGGGACGAGTCTGAACCTA3' and 1D2-5'AGCACAAGTGACTCTATTCC3'. The resulting 205-bp fragment was cloned into the TA cloning vector (Invitrogen, San Diego, CA). The Clex1 probe contains 55 bp of the 3' end of exon 1C, all of exon 1D, and the 40-bp intronic region that separates them. The E0.7 probe is a 0.7-kb *Eco*RI fragment that contains all of exon 2 and surrounding intron sequences. The H0.6 probe is a 0.6-kb *Hin*dIII fragment that contains all of exon 4 and sequence from the 5' intron. The E0.7 and H0.6 probes were isolated from a cosmid clone kindly provided by Dr. R. P. WOYCHIK (Oak Ridge National Laboratory, Oak Ridge, TN). The *agouti* cDNA probe used in Northern blot analysis, CRA, begins 10 bp 5' to the ATG start codon and ends 10 bp 5' to the TGA stop codon. CRA was isolated by PCR from a wild-type *agouti* cDNA construct kindly provided by Dr. M. P. ROSENBERG (Glaxo Pharmaceuticals, Morrisville, NC). The primers used to generate CRA were 2A-5'GCTTCTCAGGATGGATGTCAC3' and 4D-5'GTTGAGTACTCGACAGGTGC3'. The probes and primers listed above are shown in Figure 2.

Northern blot analysis: Total RNA was isolated from tissues as described with slight modifications (CHOMCZYNSKI and SACCHI 1987). Poly(A)$^+$ RNA was isolated from the skin of 4–6 day neonatal C3H/HeJ mice using oligo d(U) sephadex as described (MOORE and SHARP 1984). RNA was electrophoresed through 1.0 or 1.5% formaldehyde gels and transferred to Hybond N$^+$ (Amersham Corp., Arlington Heights, IL) or Zetabind (Cuno, Inc., Meriden, CT) membranes using standard procedures (SAMBROOK *et al.* 1989). Membranes were hybridized as described (CHURCH and GILBERT 1984) to random primed [α-^{32}P]dCTP labeled CRA or E0.7 probes.

Southern blot analysis: Genomic DNAs were isolated from mouse tails and tissues as described (SIRACUSA *et al.* 1987). For determination of genotype, mouse tail DNAs were digested with *Bam*HI, electrophoresed through 0.8% agarose

gels, transferred to Hybond N+ nylon membranes (Amersham Corp., Arlington, Heights, IL), and hybridized to the [α-^{32}P]dCTP-labeled Clex1 probe. Resulting fragment sizes were 14.3 kb for the *a* allele, 7.7 kb for the a^l allele, and 5.3 kb for the A^{hvy} allele. A^{hvy}/a^l mice were distinguished from A^{hvy}/a mice by the presence of a single 5.3-kb *Bam*HI restriction fragment for A^{hvy}/a^l mice compared to the presence of both 5.3- and 14.3-kb restriction fragments for A^{hvy}/a mice. For other Southern blot analyses, genomic DNAs were digested with restriction endonucleases (Boehringer Mannheim, Indianapolis, IN), transferred to nylon membranes, and hybridized to random primed [α-^{32}P]dCTP-labeled probes. Hybridization and washing conditions were as described (SIRACUSA *et al.* 1989).

Isolation of cDNA clones: cDNA clones were isolated using the 5'-Amplifinder RACE kit (Clontech, Palo Alto, CA). Poly (A)+ RNA was isolated from adult B6.C3H-A^{hvy}/A^{hvy} spleen using oligo d(U) sephadex as described (MOORE and SHARP 1984). The cDNA synthesis reaction was primed with an oligonucleotide from exon 3 (3B-$^{5'}$CTTGGAAGACCTCTTCCGC$^{3'}$) that corresponds to bp 207–225 of the *agouti* coding region (A in the ATG start codon is base #1). An oligonucleotide anchor was ligated to the 5' end of the cDNA. PCR fragments ranging in size from 300 to 350 bp were amplified using an oligonucleotide specific to the ligated anchor and an oligonucleotide (2B-$^{5'}$CCACGATAGAAACAGAGGAG$^{3'}$) that corresponds to bp 141–160 of the *agouti* coding region. PCR was performed using an initial denaturation at 94° for 4 min, followed by 35 cycles of 94° for 45 sec, 60° for 45 sec and 72° for 2 min with a final elongation step of 72° for 7 min. Resulting PCR fragments were cloned using the TA Cloning Kit (Invitrogen, San Diego, CA).

Long-range PCR: PCR fragments of 5.7 kb were amplified from 100 ng of B6.C3H-A^{hvy}/A^{hvy} genomic DNA as described (BARNES 1994) with modifications. The PCR protocol used was an initial denaturation at 94° for 4 min, followed by 25 cycles of 95° for 5 sec, 65° for 30 sec, and 68° for 7 min. DNA primers were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer (Foster City, CA). Oligonucleotide sequences were 1C4-$^{5'}$CATTAACTCTTTCTATAGAGTAACCTCAGTGC$^{3'}$ and 1D2L-$^{5'}$ACAGCACAAGTGACTCTATTCCGCATCAG$^{3'}$. Oligomer 1C4 is from the intron 5' to exon 1C, and oligomer 1D2L is from the 3' end of exon 1D.

Plasmid isolation and sequencing: Plasmid DNAs from the cDNA clones were isolated using the Qiagen Robotic Workstation (Qiagen Inc., Chatsworth, CA) at the Jefferson Cancer Center Nucleic Acid Facility (Philadelphia, PA). All clones were sequenced in both directions with oligonucleotides from the polylinker of the TA Cloning vector. DNA fragments corresponding to exons 2, 3, and 4 of the *agouti* coding region were obtained by PCR using primers specific to the flanking introns (J. MANNE, A. C. ARGESON, M. F. FERRARO and L. D. SIRACUSA, unpublished results). PCR products were purified with centricon-100 columns (Amicon, Inc., Beverly, MA) or with the QIAquick spin PCR purification kit (Qiagen Inc., Chatsworth, CA) to remove excess oligonucleotides and sequenced using primers specific to the surrounding introns. All sequencing was done with an Applied Biosystems Model 373 DNA Sequencer (Foster City, CA) at the JCC Nucleic Acid Facility.

RESULTS

Yellow A^{hvy} mice ectopically overexpress a 0.8-kb *agouti* message: We previously showed that A^{hvy} is a new allele at the *agouti* locus that produces mice ranging in color from yellow to nonagouti with most mice exhibiting mottling (SIRACUSA *et al.* 1995). Expression of the 0.8-kb wild-type *agouti* message is normally detected in 4–6 day neonatal skin (BULTMAN *et al.* 1992; MILLER *et al.* 1993). At this time, pigment synthesis by melanocytes switches from the production of eumelanin to the production of phaeomelanin, which results in the subapical yellow band that exemplifies the classic agouti pattern. Expression of the *agouti* gene in murine adult tissues has only been detected in testes (BULTMAN *et al.* 1992; MILLER *et al.* 1993) and in hair follicles during hair regeneration following plucking (MILLER *et al.* 1993). The different colored coat hairs exhibited by individual A^{hvy} mice, coupled with the ectopic overexpression of the *agouti* gene in obese yellow mice and a reduction in expression in nonagouti mice (reviewed in SIRACUSA 1994), led us to suspect that a regulatory mutation had occurred in the A^{hvy} chromosome. To test this hypothesis and to determine the size of the *agouti* transcript in A^{hvy} mice, we performed Northern blot analysis using poly (A)+ RNA from 4–6 day neonatal skin of control C3H *A/A* mice and total RNA from the liver and kidneys of a mostly yellow B6.C3H-A^{hvy}/A^{hvy} mouse (Figure 1). Total RNA from B6 A^y/a was included for comparison, since A^y produces the largest aberrant *agouti* transcript yet identified.

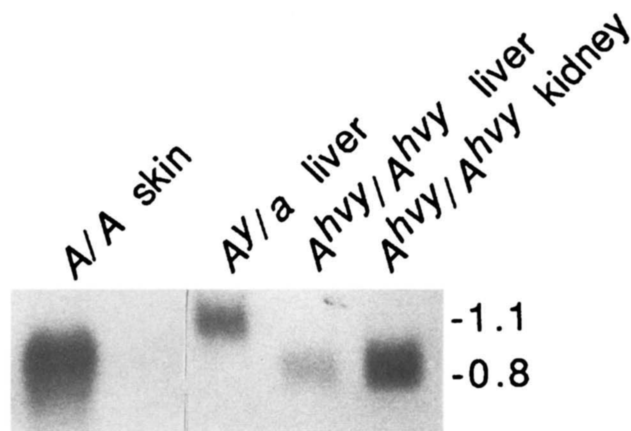

FIGURE 1.—The *agouti* transcript in A^{hvy} mice does not appear to vary in size from the wild-type transcript in *A* mice. Northern blot analysis of *agouti* RNAs hybridized to the [α-^{32}P]dCTP-labeled CRA probe from the *agouti* coding region. RNA samples from left to right are as follows: 7 μg poly (A)+ RNA from C3H *A/A* 4–6 day neonatal skin and 20 μg total RNA from B6 A^y/a adult liver, B6 A^{hvy}/A^{hvy} adult liver, and A^{hvy}/A^{hvy} adult kidney. A^{hvy}/A^{hvy} liver and kidney RNAs were isolated from the same mouse. Total RNA from the liver of an A^y/a mouse was used for size comparison, since A^y/a mice, unlike wild-type mice, ectopically overexpress a mutant 1.1-kb *agouti* message detectable at all developmental stages examined (BULTMAN *et al.* 1992; MILLER *et al.* 1993). Sizes of the *agouti* transcripts, in kb, are listed to the right.

The results indicate that both wild-type *A/A* and A^{hvy}/A^{hvy} mice express a 0.8-kb *agouti* transcript (Figure 1). This experiment also shows that *agouti* expression is deregulated in A^{hvy} mice. The 0.8-kb *agouti* transcript is not expressed in C3H *A/A* adult tissues (BULTMAN *et al.* 1992; MILLER *et al.* 1993) but is expressed in the liver

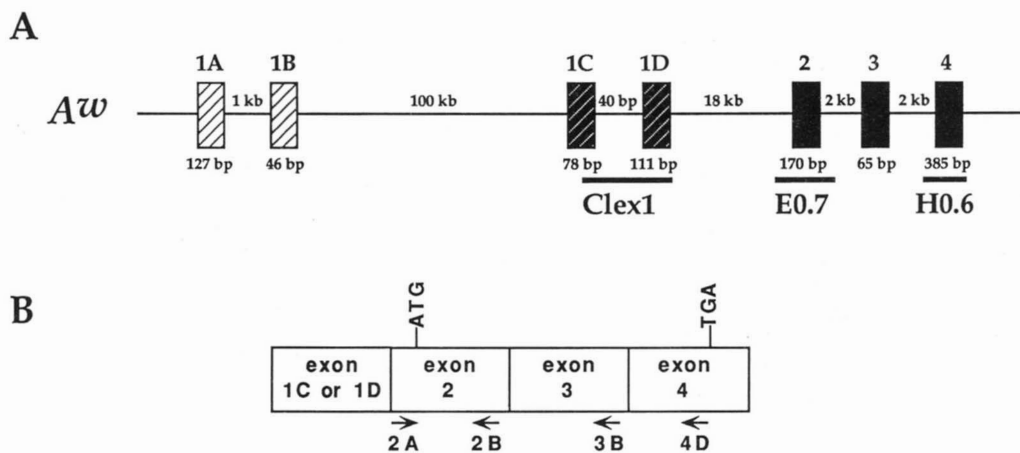

FIGURE 2.—Diagram of the *agouti* gene, probes used in Northern and Southern blots, and primers for PCR analyses. (A) The structure of the wild-type A^w allele is shown from proximal (left) to distal (right) (reviewed in SIRACUSA 1994). Noncoding ventrum-specific exons 1A and 1B are shown as white striped boxes and noncoding hair cycle-specific exons 1C and 1D are shown as black striped boxes. Coding exons 2, 3, and 4 are shown as ■. Numbers below the boxes indicate the length of exons, and numbers between the exons indicate the length of introns. The Clex1, E0.7, and H0.6 probes used for these studies are shown at the bottom (see MATERIALS AND METHODS). (B) Two different transcripts containing exons 1C or 1D are expressed during hair growth in *A/A* mice; an additional two alternatively spliced transcripts containing exon 1A or exon 1A and 1B are expressed continuously in the ventrum of A^w/A^w mice (not shown) but rarely in C3H *A/A* mice, the strain upon which A^{hvy} arose (BULTMAN *et al.* 1992, 1994; VRIELING *et al.* 1994; SIRACUSA *et al.* 1995). The 2A and 4D primers used to amplify the CRA probe, and the 2B and 3B primers used for the PCR step and cDNA synthesis step of 5′ RACE, respectively, are shown. The relative positions of the ATG start codon and the TGA stop codon are indicated.

and kidneys of adult A^{hvy} mice (Figure 1) and in other adult tissues (see below). The deregulation of *agouti* expression observed in A^{hvy} mice is consistent with the findings of ectopic overexpression of *agouti* detected in other dominant yellow *agouti* alleles such as A^y, A^{iapy}, A^{sy}, A^{vy}, and A^{iy} (BULTMAN *et al.* 1992; MICHAUD *et al.* 1993, 1994a,b; MILLER *et al.* 1993; DUHL *et al.* 1994b). These findings suggested that the mutation in the A^{hvy} allele affected a regulatory element such as a promoter and/or enhancer, or that a new promoter had usurped control of the *agouti* gene.

***Agouti* expression in A^{hvy} mice:** Previous studies have shown that the level of expression of the *agouti* gene in mice carrying various *agouti* alleles is positively correlated with the amount of yellow in their coats (BULTMAN *et al.* 1992; MICHAUD *et al.* 1994b; MILLER *et al.* 1993; DUHL *et al.* 1994b). To determine if this correlation was also true in A^{hvy} mice with identical genotypes (A^{hvy}/a) but exhibiting various coat color phenotypes, we performed Northern blot analysis of total RNA from A^{hvy}/a mice exhibiting a gradation of colors. We hypothesized that mostly yellow A^{hvy}/a mice would ectopically overexpress the *agouti* gene, but mostly black A^{hvy}/a mice would express *agouti* at much lower levels. Total RNA from the skin, liver, kidney, spleen, thymus, bone marrow, brain, and lung of adult mostly yellow and mostly black B6.C3H-A^{hvy}/a mice was screened with the E0.7 probe (see Figure 2). In mostly yellow A^{hvy}/a mice, we detected *agouti* expression in all adult tissues tested (Figures 1 and 3) (data not shown). Significantly lower levels or lack of expression of the *agouti* gene was observed in the tissues of black A^{hvy}/a mice. A representative expression panel of RNA from the adult kidneys of mostly yellow, intermediate, and mostly black A^{hvy}/a mice is shown in Figure 3. The results demonstrate that mostly yellow A^{hvy}/a mice ectopically overexpress the *agouti* transcript and that expression of the *agouti* gene is positively correlated with the amount of yellow in the coats of A^{hvy} mice. Accordingly, we previously showed that the amount of yellow in the coats of A^{hvy}/a^e and A^{hvy}/a mice is also positively correlated with the weight of the mice (SIRACUSA *et al.* 1995) (data not shown).

Southern blot analysis reveals a genomic alteration in the 5′ end of the *agouti* gene: The ectopic overexpression of the *agouti* gene observed in yellow A^{hvy} mice suggested that a mutation in a regulatory element is present in the A^{hvy} chromosome, similar to mutations in the *agouti* alleles A^y, A^{vy}, A^{iy}, A^{sy} and A^{iapy} that result in mostly yellow mice (MICHAUD *et al.* 1993, 1994a,b; MILLER *et al.* 1993; DUHL *et al.* 1994a,b). In support of this hypothesis, overexpression of the wild-type *agouti* cDNA in transgenic mice results in yellow mice displaying the same pleiotropic effects seen in spontaneous dominant yellow mutations (KLEBIG *et al.* 1995; PERRY *et al.* 1995; WILSON *et al.* 1995), whereas the coding region of the *agouti* gene remains unaffected. To date, only the *nonagouti-16H* (a^{16H}), *extreme nonagouti* (a^e), and *extreme nonagouti-5mnu* (a^{5mnu}) alleles are caused by mutations within the coding region of the *agouti* gene and result in mostly or completely black mice, respectively (BULTMAN *et al.* 1992; HUSTAD *et al.* 1995). Because of the yellow color of some A^{hvy} mice, we were not expecting a mutation within the *agouti* coding region. However, we screened for genomic DNA

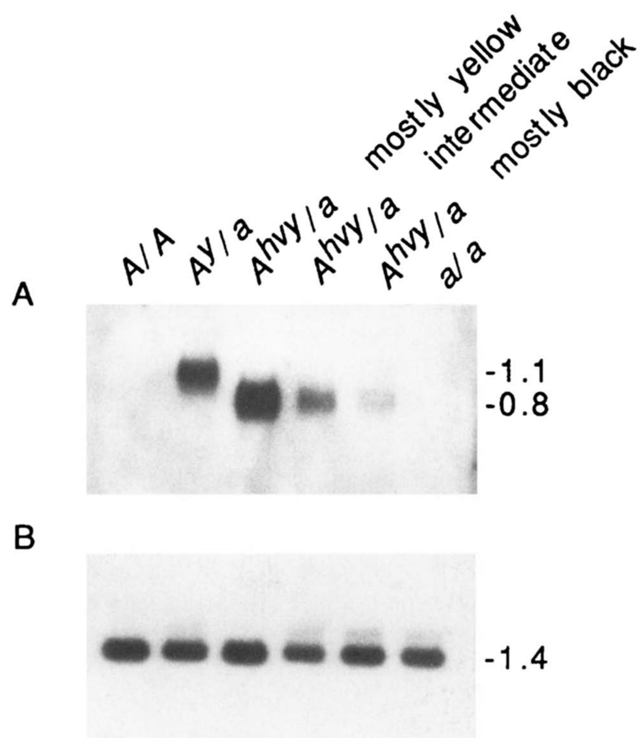

FIGURE 3.—Expression of the *agouti* gene is altered in A^{hvy} mice. (A) Ten micrograms of total RNA from the kidneys of C3H *A/A*, B6-A^y/a, three different colored A^{hvy}/a mice, (labeled mostly yellow, intermediate, and mostly black to describe the relative amount of yellow pigment in their coats) and B6 *a/a* was electrophoresed through a 1.5% formaldehyde gel and transferred to a nylon membrane. The resulting blot was hybridized to the [α-^{32}P]dCTP-labeled E0.7 probe (Figure 2). Sizes of the *agouti* transcripts, in kb, are listed to the right. (B) Hybridization of the same Northern blot with a *Gapd* probe serves as an indicator of RNA loading in each lane.

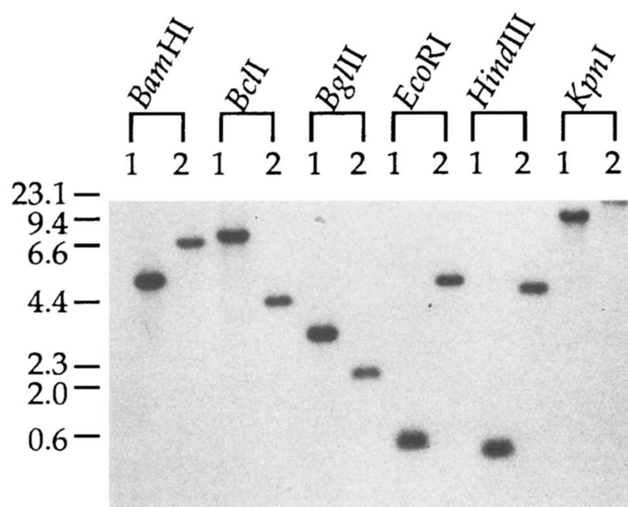

FIGURE 4.—Southern blot analysis of genomic DNA from A^{hvy}/A^{hvy} and *A/A* mice reveals an alteration in the 5′ end of the *agouti* gene. Genomic DNAs (7.5 μg) from B6.C3H-A^{hvy}/A^{hvy} and C3H *A/A* livers were digested with the restriction endonucleases listed at the top, electrophoresed through 0.8% agarose gels, transferred to nylon membranes and hybridized to the [α-^{32}P]dCTP Clex1 probe. Lanes marked 1 contain genomic DNA from B6.C3H-A^{hvy}/A^{hvy} mice and lanes marked 2 contain genomic DNA from C3H *A/A* mice. The sizes of *Hin*dIII-digested lambda DNA standards are listed on the left.

alterations spanning the entire coding region of the *agouti* gene as well as the UTR hair cycle-specific exons 1C and 1D using the probes Clex1, E0.7, and H0.6 (Figure 2). Transcripts containing the 5′ UTR ventral-specific exons 1A and 1B are rarely expressed in mice of the C3H *A/A* strain (VRIELING *et al.* 1994) on which A^{hvy} arose (SIRACUSA *et al.* 1995). We therefore omitted these regions from our analysis.

To identify a genomic alteration specific for the A^{hvy} mutation, A^{hvy}/A^{hvy} and *A/A* genomic DNAs were cleaved with restriction endonucleases to generate a Southern blot that was hybridized to the three probes listed above. The probes E0.7 and H0.6, which are specific to exons 2 and 4 of the *agouti* coding region respectively, did not detect polymorphisms between the DNA of A^{hvy}/A^{hvy} and *A/A* mice (data not shown). In contrast, the Clex1 probe, which contains the hair cycle-specific UTR exons 1C and 1D, detected polymorphisms with every restriction endonuclease tested (Figure 4). These results demonstrate that the alteration in A^{hvy} mice is within the genomic region surrounding exons 1C and 1D. PCR amplification and sequencing of *agouti* coding exons 2–4 using primers specific to the flanking introns confirmed that there were no mutations in these exons and that the splice donor and acceptor sites were intact in A^{hvy} mice. These findings supported the hypothesis of a regulatory mutation, rather than a structural mutation, in the A^{hvy} product.

A^{hvy} results from an insertion in the 5′ UTR exon 1C: To further characterize the *agouti* mRNA in A^{hvy} mice, 5′ RACE of A^{hvy}/A^{hvy} poly(A)$^+$ RNA was performed using oligonucleotide 3B for the RT and oligonucleotide 2B and an anchor primer for the PCR (Figure 2B) (see MATERIALS AND METHODS). Sequencing of several cDNA clones showed that the 5′ end of *agouti* exon 1C was replaced with novel sequences. The A^{hvy} cDNA did contain the last 55 bp from the 3′ end of exon 1C that were spliced perfectly to wild-type sequences from exon 2 (Figure 5). A GENBANK search revealed that the novel 5′-most sequences corresponded to the U3 region of an IAP LTR and suggested that an IAP had inserted within the UTR exon 1C in an antisense orientation (Figure 5). This finding also explains why the A^{hvy} transcript did not appear to vary in size from the wild-type *agouti* transcript (Figure 1), since in the mutant 80 bp from the IAP LTR replace the first 23 bp from the 5′ end of exon 1C or the entire 111 bp of exon 1D. No 5′ RACE clones containing exon 1D were found.

To confirm that the IAP had inserted directly into exon 1C, we performed long-range PCR of A^{hvy}/A^{hvy} genomic DNA using the oligonucleotides 1C4 and 1B that flank the probable IAP insertion site within exon 1C. The resulting fragment size was 5.7 kb instead of the

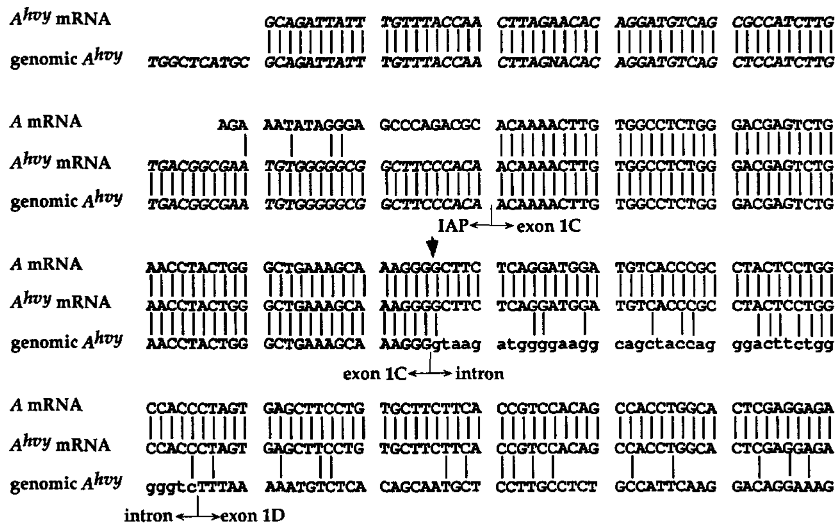

FIGURE 5.—Sequence comparisons of A^{hvy} and A mRNAs and the A^{hvy} chromosome. The 5′ portion of the hair cycle-specific A transcript containing exon 1C is shown on the top line; the dark arrowhead demarcates the boundary between exon 1C and exon 2. The longest sequence from 5′ RACE clones of A^{hvy} mRNA is show on the middle line. The genomic A^{hvy} sequence is shown on the bottom line with dual direction arrows showing the different junctions. Letters in italics represent IAP sequences, uppercase letters represent exon sequences and lowercase letters represent intron sequences. The diagram shows that in A^{hvy} mice, transcription starts within the U3 region of the IAP LTR and continues directly into the 3′ end of exon 1C. The 3′ end of exon 1C is perfectly spliced to exon 2 in both A and A^{hvy} transcripts.

335-bp fragment for control C3H *A/A* DNA (data not shown), demonstrating that a 5.4-kb insertion had occurred. Full-length IAP elements are 7.2 kb, but different types of IAPs are characterized by deletions (with or without substitutions) within the full-length IAP leading to a number of smaller classes of IAPs (reviewed in KUFF and LUEDERS 1988). The restriction map (data not shown) and size of the IAP are consistent with Type IΔ1 IAP elements (KUFF and LEUDERS 1988). Sequencing of the 5.7-kb genomic PCR product confirmed that both the genomic DNA and cDNA of A^{hvy} diverge from wild-type exon 1C sequences at the same location (Figure 5). The data demonstrate that a truncated IAP inserted directly into exon 1C in an antisense orientation, and that transcription of the *agouti* gene from the A^{hvy} chromosome initiates within the LTR of this IAP.

The coat color phenotype of A^{hvy}/a^l mice is correlated with the methylation status of CpG residues: We performed Southern blot analysis to determine if there were differences in the genomic DNA of A^{hvy} mice of different coat color phenotypes. To test the methylation status of only one homologue of the A^{hvy} chromosome in each mouse, we crossed B6-A^{hvy}/A^{hvy} mice to B6-a^l/a mice and selected for mice of the A^{hvy}/a^l genotype. Since the a^l allele is characterized by a deletion of the *agouti* gene (BULTMAN *et al.* 1991; A. C. ARGESON, M. A. FERRARO and L. D. SIRACUSA, unpublished results), the methylation status and expression of the *agouti* gene would be solely dependent upon the A^{hvy} chromosome in A^{hvy}/a^l mice.

Southern blot analysis of genomic DNA from different tissues of mostly yellow and mostly black A^{hvy}/a^l mice was performed (Figure 6). We were testing the methylation status of two *Msp*I/*Hpa*II sites that flank the Clex1 probe: one in the IAP LTR and one in the intron just 3′ to exon 1D. A 5.3-kb *Bam*HI restriction fragment that encompasses the junction including the IAP LTR and exon 1C was observed in all samples. *Bam*HI/*Msp*I double digestions produced a 0.7-kb restriction fragment in each sample, since neither of these two enzymes are sensitive to methylation differences. A^{hvy}/a^l genomic DNA was also cleaved with *Bam*HI and *Hpa*II, the methylation sensitive isoschizomer of *Msp*I. We expected a 2.6-kb *Bam*HI/*Hpa*II restriction fragment if only the 5′ *Hpa*II site was methylated and conversely, a 3.3-kb restriction fragment if only the 3′ *Hpa*II site was methylated. However, a prominent 5.3-kb restriction fragment was observed in genomic DNA from the black mouse but not the yellow mouse. An additional, less intense 0.7-kb restriction fragment was observed in genomic DNA from the black mouse. A prominent 0.7-kb restriction fragment was the only fragment detected in genomic DNA from the yellow mouse (Figure 6). The results indicate that the majority of A^{hvy} DNA is methylated at both sites in black A^{hvy}/a^l mice and that hypomethylation at both sites is correlated with expression of the *agouti* gene.

The coat color phenotype of A^{hvy} progeny is dependent on maternal phenotype: We performed several genetic crosses as a first step to investigate the factors responsible for the variable phenotypes exhibited by A^{hvy} mice. Mostly black (A^{hvy}/a) or mostly yellow (A^{hvy}/A^{hvy}) mice were mated with alternate sex black (a/a) mice. Offspring were classified into the following three categories: yellow, which includes mice that had ≥70% yellow fur; black, which includes mice that had ≥70% black fur; and intermediate, which includes mice that had mottled yellow and black fur that represented a roughly equal mixture of the two coat colors (Table 1).

The Chi-square statistic was used to evaluate differences between the proportions of A^{hvy} offspring within each coat color classification produced from the crosses listed in Table 1. Analysis of the proportions of offspring from crosses of yellow A^{hvy}/A^{hvy} *vs.* black A^{hvy}/a females to a/a males showed a significant difference ($\chi^2 = 10.43$, $P < 0.01$). Conversely, offspring distributions from crosses of black A^{hvy}/a females to a/a males showed no significant differences with offspring distributions from crosses of a/a females to black A^{hvy}/a males

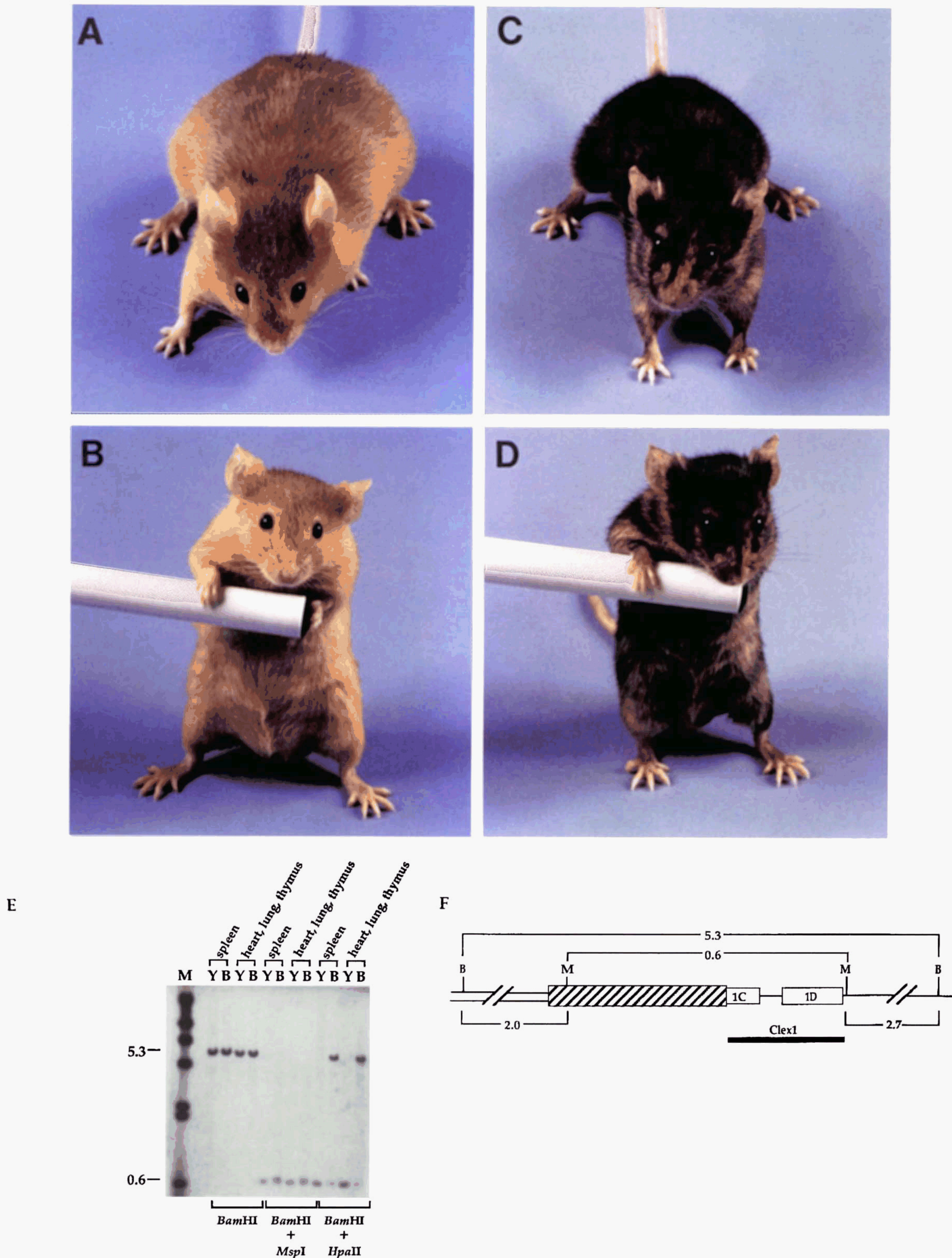

FIGURE 6.—The phenotype of A^{hvy} mice is correlated with the methylation state of the IAP LTRs. Dorsal (A) and ventral (B) views of a mostly yellow B6.C3H-A^{hvy}/a^{l} mouse used for comparative DNA methylation analysis. Dorsal (C) and ventral (D) views of a mostly black B6.C3H-A^{hvy}/a^{l} mouse used for comparative DNA methylation analysis. (E) Genomic DNAs (10 μg) from the mostly yellow (marked Y and shown in A and B above) and mostly black (marked B and shown in C and D above) mice were digested with the restriction endonucleases shown at the bottom, electrophoresed through 0.8% agarose gels, transferred to nylon membrane and hybridized to the [α-^{32}P]dCTP Clex1 probe. Sizes of the resulting fragments, in kb, are listed to the left. The specific tissues from which genomic DNA was extracted are listed above the lanes. *Hin*dIII-digested lambda DNA standards are shown in lane M. (F) Restriction map of the junction encompassing the IAP LTR and exon 1C. ▨, IAP LTR; □, exons 1C and 1D. The remaining IAP is to the left of the LTR. Sizes of restriction fragments (in kb) are listed above and below the A^{hvy} map. The cleavage sites of the restriction endonucleases *Bam*HI (B) and *Msp*I/*Hpa*II (M) are shown at the top. The thick black line below the map represents the Clex1 probe used in Southern blot analysis.

TABLE 1

Percentage of A^{hvy} progeny of different coat color phenotypes resulting from backcrosses of A^{hvy} mice to B6 a/a mice

Cross[a]	Color of A^{hvy} parent	A^{hvy}/a Yellow[b]	A^{hvy}/a Intermediate	A^{hvy}/a Black	a/a
B6 a/a × B6.C3H-A^{hvy}/A^{hvy}	Yellow	31 (44.9)	14 (20.3)	24 (34.8)	0
B6.C3H-A^{hvy}/A^{hvy} × B6 a/a	Yellow	26 (89.7)	2 (6.9)	1 (3.4)	0
B6 a/a × B6.C3H-A^{hvy}/a	Nonagouti	14 (58.3)	0 (0)	10 (41.7)	25
B6.C3H-A^{hvy}/a × B6 a/a	Nonagouti	10 (50.0)	3 (15.0)	7 (35.0)	20

[a] Offspring from the last two crosses were tail-clipped to determine genotype. Only A^{hvy}/a mice were classified into the three phenotypic categories.
[b] For description of coat color classifications see RESULTS. Values in parentheses are percentages.

($\chi^2 = 3.67$, $P > 0.1$). However, a highly significant difference was found between offspring proportions from crosses of yellow A^{hvy}/A^{hvy} females to a/a males *vs.* a/a females to yellow A^{hvy}/A^{hvy} males ($\chi^2 = 17.04$, $P < 0.005$). Finally, differences in the proportions of offspring from a/a females crossed to black A^{hvy}/a or yellow A^{hvy}/A^{hvy} males were not significant ($\chi^2 = 5.74$, $0.1 > P > 0.05$). These results show that yellow A^{hvy}/A^{hvy} mothers produce offspring with more yellow pigment in their coats than black A^{hvy}/a or a/a mothers. In addition, offspring that inherited A^{hvy} from a yellow mother were more likely to be yellow than offspring that inherited A^{hvy} from a yellow or black father.

DISCUSSION

The results presented here identify the molecular basis of the A^{hvy} mutation at the *agouti* locus in the mouse. A Type IΔ1 IAP is inserted in an antisense orientation within the 5′ UTR exon 1C in the A^{hvy} chromosome. The A^{hvy} mRNA originates from the LTR of the truncated IAP; U3 LTR sequences are directly adjacent to sequences from the 3′ end of exon 1C, followed by the wild-type sequence of *agouti* coding exons 2, 3, and 4. Although normal agouti protein is made, the protein is ectopically overexpressed in obese yellow A^{hvy} mice and is not expressed or is expressed at low levels in lean black A^{hvy} mice. Thus, aberrant *agouti* gene expression due to IAP LTR activity is the cause of A^{hvy} phenotypes.

IAPs are endogenous retrovirus-like particles widely dispersed (~2000 copies per haploid genome) throughout the mouse genome that are capable of transposition, thus leading to insertional mutagenesis (reviewed in KUFF and LUEDERS 1988). IAP elements are flanked by LTRs that include a 218-bp U3 regulatory region that contains at least five binding sites for transcription factors (FALZON and KUFF 1988) as well as the retroviral promoter (LUEDERS *et al.* 1984). Based on the results of 5′ RACE experiments, an enhancer element and/or cryptic promoter within the LTR of the IAP appears to control *agouti* gene expression in A^{hvy} mice.

During murine development, abundant IAP gene expression has been detected from the four to eight cell stage to the mid- to late-blastocyst stage (YOTSUYANAGI and SZOLLOSI 1984). High levels of expression have also been found in many mouse tumors (reviewed in KUFF and LUEDERS 1988). Although IAP expression in later stage embryos and most adult cells (excluding thymus) is typically low, tissue-specific expression found in adult tissues is believed to be controlled by the binding of transcription factors and the methylation state of the LTRs (reviewed in KUFF and LUEDERS 1988). In most nontransformed cells, the LTRs of IAPs are highly methylated, which presumably results in low levels of gene expression (MIETZ and KUFF 1990). In contrast, IAP LTRs of many transformed cells are hypomethylated, correlating with high levels of IAP gene expression (FEENSTRA *et al.* 1986). A correlation between methylation status of the IAP 5′ LTR and *agouti* gene expression was found in the variably expressed *agouti* allele A^{iapy} (MICHAUD *et al.* 1994b). Preliminary evidence has shown at least two hypomethylated CpG sites in the IAP 5′ LTR of yellow A^{iapy} mice that are highly methylated in pseudoagouti-colored mice of the same genotype (MICHAUD *et al.* 1994b). Our data show that the hypomethylation of at least two CpG sites, one in the IAP LTR and the other in host genomic DNA, is correlated with *agouti* gene expression in A^{hvy} mice.

To date, A^{hvy} is one of six dominant mutations (A^y, A^{hvy}, A^{vy}, A^{sy}, A^{iy}, and A^{iapy}) identified at the *agouti* locus that result in the ectopic overexpression of the *agouti* gene product leading to greater phaeomelanin and less eumelanin in coat hairs. In each of these alleles, the *agouti* coding region remains unaltered whereas transcript overexpression is evident and caused by insertions or deletions that place the *agouti* gene under the control of heterologous promoters (see below). In the *lethal yellow* (A^y) mouse, overexpression results from a large (120–170 kb) genomic deletion that removes the coding region of the upstream *Raly* gene, leaving only a single 5′ noncoding exon (BULTMAN *et al.* 1992; MICHAUD *et al.* 1993; MILLER *et al.* 1993). Although each of the 5′ UTR ventrum-specific and hair cycle-specific exons of the *agouti* gene remain unaltered, deletion of the *Raly* sequences places the stronger *Raly* promoter in close proximity of the *agouti* gene, allowing the *Raly*

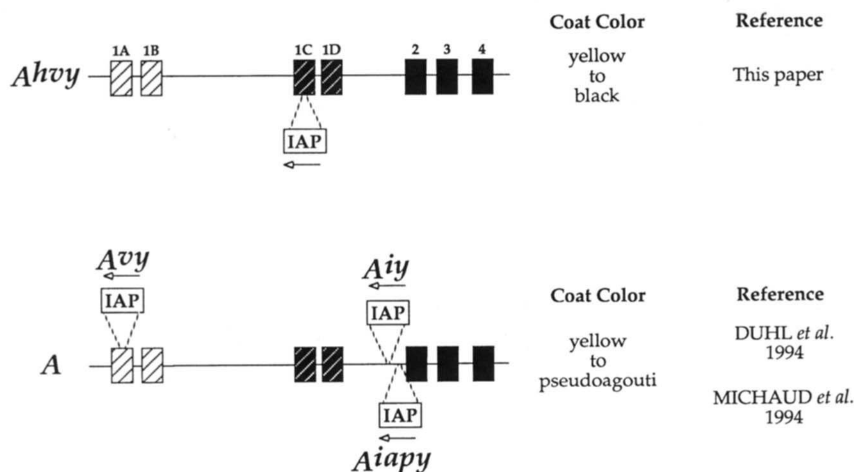

FIGURE 7.—Genomic structure of the A^{hvy} mutation compared to other *agouti* alleles. The A^{hvy} allele is shown at the top, with coding exons represented as ■, and noncoding exons represented as striped boxes. The clear boxes represent the IAPs. The dashed line indicates the site of inserted sequences in each mutant allele. Arrows depict the direction of transcription of the IAP element. Below the A^{hvy} allele, three other *agouti* alleles that arose on the C3H/HeJ strain as a result of IAP insertions (A^{vy}, A^{iy} and A^{iapy}) are shown. In each case, Type IΔ1 IAP elements have inserted in an antisense orientation. The columns to the right list the range of coat colors that can result from each mutation and references describing the molecular basis of each mutation.

promoter to usurp control of *agouti* expression (BULTMAN *et al.* 1992; MILLER *et al.* 1993; VRIELING *et al.* 1994). Each of the other five dominant *agouti* alleles that produce completely or mostly yellow mice have also resulted from genomic insertions (Figure 7). A^{hvy}, A^{vy}, A^{iapy}, and A^{iy} contain insertions of IAP elements in antisense orientations within distinct regions of the *agouti* gene that control *agouti* expression in mice carrying these alleles (DUHL *et al.* 1994b; MICHAUD *et al.* 1994b; SIRACUSA *et al.* 1995). In addition, the A^{sy} mutation presumably places the *agouti* gene under the control of yet another promoter, but unlike the alleles mentioned above, A^{sy} results from the insertion of novel sequences 10–15 kb upstream of exon 2 (DUHL *et al.* 1994b).

A number of *agouti* alleles produce offspring that exhibit variable coat color phenotypes. Mice carrying the a^{m} and a^{mj} mutations can range in color from nonagouti to pseudoagouti (RUSSELL 1964; HUSTAD *et al.* 1995), whereas mice carrying the A^{vy}, A^{iy}, and A^{iapy} alleles can range from pseudoagouti to pure yellow or sooty yellow (DICKIE 1962; MICHAUD *et al.* 1994b; J. R. KERSHNER, A. C. ARGESON and L. D. SIRACUSA, unpublished results). Mice carrying any of these variable alleles can exhibit mottling or striping. In contrast, A^{hvy} is the only mutation at the *agouti* locus that can produce mice ranging in color from almost pure black to pure yellow with a similar broad range in their overall weights (SIRACUSA *et al.* 1995). A^{vy}, A^{iy}, A^{iapy} and A^{hvy} are similar in that they are caused by IAP insertions in an antisense orientation and produce variable phenotypes. However, unlike A^{hvy}, the A^{vy}, A^{iy}, and A^{iapy} mutations cannot produce nonagouti-colored (black) mice. Differences in the methylation state of the IAP LTRs in each allele could account for the variable expression (MICHAUD *et al.* 1994b) but not the lack of nonagouti colored mice in A^{vy}, A^{iy} and A^{iapy}. This lack is probably due to the position of the IAP insertion in each allele (Figure 7). The A^{vy}, A^{iy}, and A^{iapy} mutations are caused by IAPs inserting into either the 5′ UTR exon 1A or into the intron just upstream of coding exon 2 (DUHL *et al.* 1994b; MICHAUD *et al.* 1994b). Thus, these insertions do not disrupt hair cycle-specific promoters. In A^{hvy}, the insertion within exon 1C can essentially block *agouti* expression from both hair cycle-specific promoters, resulting in black mice. Yellow A^{hvy}/A^{hvy} and A^{hvy}/a mice result from the constitutive expression of *agouti* dictated by the IAP insertion. Furthermore, several lines of evidence indicate that A^{hvy} cannot result in pseudoagouti-colored offspring. First, crosses of [C3H $A/A \times$ B6.C3H-A^{hvy}/A^{hvy}] F_1 females to B6 a/a males revealed no agouti-colored progeny carrying the A^{hvy} allele in >95 mice examined. Second, intercrosses of A^{hvy}/A^{hvy} and A^{hvy}/a mice, backcrosses of A^{hvy}/A^{hvy} and $A^{hvy}/a \times a/a$ mice and testcrosses of A^{hvy}/A^{hvy} and $A^{hvy}/a \times a^{e}/a^{e}$ mice revealed no agouti-colored progeny carrying the A^{hvy} allele in >500 mice examined (A. C. ARGESON and L. D. SIRACUSA, unpublished results). Thus, the absence of agouti-colored A^{hvy}/A^{hvy} and A^{hvy}/a mice makes this mutation unique in the *agouti* series and appears due to the position of the IAP insertion in A^{hvy} relative to the hair cycle-specific promoters. Since individual A^{hvy} mice can have both black and yellow coat hairs, A^{hvy} is both a dominant gain of function and a loss of function mutation.

The four Type IΔ1 IAP insertions found in the A^{vy}, A^{iy}, A^{iapy} and A^{hvy} mutations lead to the question of why the *agouti* gene appears susceptible to insertion of these retroviral-like elements. One hypothesis is that the *agouti* gene sequence contains a target or integration hotspot for IAP elements. This idea assumes that *agouti* is expressed before separation or differentiation of germinal and somatic lineages so that its chromatin is in a conformation where *agouti* sequences are preferentially selected as targets. In fact, preliminary evidence suggests that the *agouti* gene is expressed in preimplantation mouse embryos (J. MANNE, J. ROTHSTEIN and L. D. SIRACUSA, unpublished results) concordant with high levels of IAP expression. A second hypothesis is that IAP insertions are occurring randomly throughout the genome but are detected and propagated more often in genes that produce a dominant visible phenotype and less obvious in genes that, once inactivated, lead to embryonic lethality. Yellow or agouti coat color is an

example of a readily observable phenotype. This second hypothesis may be true, since IAP insertions within the *agouti* gene have produced dominant alleles in which the original mutant mosaic contains readily observable stripes or patches of yellow or agouti-colored hairs on an otherwise agouti or nonagouti background (DICKIE 1962, 1966; MICHAUD *et al.* 1994b; SIRACUSA *et al.* 1995). Finally, it is intriguing that all four spontaneous mutations that occurred in C3H/HeJ contain Type IΔ1 IAPs in an antisense orientation. This orientation may favor direct expression of adjacent genes leading to dominant visible phenotypes.

The factors responsible for the variation in phenotype of A^{hvy} offspring could in part be the result of genomic imprinting, since larger numbers of yellow A^{hvy} offspring result from yellow A^{hvy} mothers than from yellow A^{hvy} fathers (Table 1). Previous studies had shown that in reciprocal crosses of mottled yellow A^{vy}/a and nonagouti a/a mice, crosses of yellow mothers resulted in significantly fewer A^{vy}/a pseudoagouti progeny than crosses of yellow A^{vy}/a fathers (WOLFF 1978). An even greater maternal effect was observed for the a^{m} allele in reciprocal crosses of mottled a^{m}/a and a/a mice (WOLFF 1978), although the etiology of the a^{m} mutation remains unknown. The expression of the A^{iapy} allele is also dependent on parental inheritance; higher expressivity of A^{iapy} is observed when passed through the female germ line (MICHAUD *et al.* 1994b). Although these sex-specific effects could be due to genomic imprinting, our data also suggest that differences between the maternal phenotype of yellow *vs.* black A^{hvy} mothers can influence the spectrum of phenotypes of A^{hvy} offspring. This finding is consistent with a report that demonstrates a difference in *agouti* phenotypes depending on the parental coat color of female A^{vy}/a mice (WOLFF 1978).

The proportion of pseudoagouti A^{vy}/a mice also differed depending on the strain of the female parent (WOLFF 1978), and preliminary results for A^{hvy} suggest similar effects (A. C. ARGESON and L. D. SIRACUSA, unpublished results). These strain-specific differences suggest the existence of modifier loci that could influence the phenotype of mutant progeny. The factors that regulate these processes may include host genes that interact with IAP LTRs (FALZON and KUFF 1988), genes involved in establishing imprints, or genes that regulate DNA methylation (reviewed in SURANI 1994). Identification and characterization of the factors that influence the phenotype of A^{hvy} offspring would provide insight into the molecular mechanisms responsible for cellular heterogeneity.

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