Differential expression of a new dominant agouti allele (A^{iapy}) is correlated with methylation state and is influenced by parental lineage

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The agouti gene normally confers the wild-type coat color of mice. Dominant mutations at the agouti locus result in a pleiotropic syndrome that is characterized by excessive amounts of yellow pigment in the coat, obesity, a non-insulin-dependent diabetic-like condition, and the propensity to form a variety of tumors. Here, we describe a new dominant mutation at the agouti locus in which an intracisternal A-particle (IAP) has integrated in an antisense orientation immediately 5' of the first coding exon of the gene. This mutation, which we have named A^{iapy} , results in the ectopic expression of the agouti gene through the utilization of a cryptic promoter within the IAP 5' long terminal repeat (LTR). The coat color of $A^{iapy}/-$ mice ranges from solid yellow to a pigment pattern that is similar to wild type (pseudoagouti), and the expressivity of this mutant phenotype varies with parental inheritance. Those offspring with a yellow coat ectopically express agouti mRNA at high levels and exhibit marked obesity, whereas pseudoagouti mice express agouti mRNA at a very low level and their weights do not differ from wild-type littermates. Data are presented to show that the differential expressivity of the A^{iapy} allele is correlated with the methylation status of the inserted IAP 5' LTR. These data further support the hypothesis that in dominant yellow mutations at the agouti locus, it is the ubiquitous expression of the wild-type agouti coding sequence that is responsible for the yellow coat color, obesity, diabetes, and tumorigenesis.

[Key Words: Agouti; spontaneous mutation; intracisternal A-particle yellow (A^{iapy}); CpG methylation; differential gene expression]

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The pelage of wild-type mice has a pigmentation pattern called agouti, in which individual hairs have a black tip, a subapical band of yellow, and a black base. The agouti locus in chromosome 2 regulates this alternating production of black (eumelanin) and yellow (phaeomelanin) pigment granules that are deposited in growing hairs. No fewer than 19 alleles have been identified at the agouti locus that result in varying amounts of phaeomelanin and eumelanin being distributed across the dorsal and ventral surfaces of the body. These agouti alleles form a complex dominance hierarchy in which alleles that lead to the production of phaeomelanin in one region of the body, for example the dorsum, are dominant to those alleles that produce eumelanin in the same body region (for review, see Silvers 1979).

Mice carrying agouti-locus alleles that are at the top of the dominance hierarchy can be readily identified by their predominantly yellow pelage. These alleles also

confer a number of dominant pleiotropic effects which, in addition to the excessive production of phaeomelanin in the coat, include obesity, an insulin-resistant diabetic condition, and the formation of a variety of tumors. Lethal yellow (A^y), the most dominant of the agouti-locus alleles, is characterized by mice with a solid yellow coat and a marked prevalence of the remaining pleiotropic effects. Homozygosity for A^y leads to preimplantation embryonic lethality, which may be attributable to the disruption of a second gene called *Raly* (hnRNP associated with lethal yellow) that is tightly linked to agouti (Michaud et al. 1993, 1994). The second most dominant allele in the agouti hierarchy is called viable yellow (A^{vy}), animals carrying this mutation may also have a solid yellow pelage and all of the other dominant pleiotropic effects of A^y , but, as the name implies, homozygotes are viable.

The A^{vy} allele is of special interest because the expres-

sivity of the phenotype can vary considerably from one individual carrying this mutation to another, even among siblings of a single litter. For example, $A^{vy}/-$ mice exhibit a broad spectrum of coat colors that range from solid yellow, to yellow with varying amounts of agouti mottling, all the way to a coat color that has been referred to as pseudoagouti, which is similar to wild-type agouti pigmentation. Interestingly, an increased amount of phaeomelanin in the coats of mice carrying the A^{vy} allele (i.e., the yellow and mottled phenotypes) is correlated with the expression of the other traits associated with this mutation, namely, obesity, diabetes, and tumorigenesis. Consistent with these observations, pseudoagouti mice remain lean and are not as likely to develop tumors as are their yellow siblings (for review, see Wolff et al. 1986; Wolff 1987). Additionally, the expressivity of the A^{vy} allele varies with parental inheritance. Female A^{vy}/a mice mated with nonagouti (a/a) males produce <1% of the wild-type-like pseudoagouti class of offspring, whereas A^{vy}/a males mated with a/a females produce anywhere from 10% to 34% pseudoagouti progeny, depending on the strain of the a/a female (Wolff 1971, 1978).

The agouti gene has been cloned and was shown to consist of three coding exons (Bultman et al. 1992; Miller et al. 1993) that are coupled with different 5'-noncoding exons to give rise to what we have referred to as form I or form II transcripts (Bultman et al. 1994). Both the form I and form II agouti transcripts are ~0.8 kb in size and are expressed in the skin during hair growth. The expression of the form I transcript appears to be associated with the banded agouti pigmentation on both the dorsum and the ventrum, whereas form II transcripts are produced mainly in ventral skin throughout the hair growth process, which correlates with the predominance of phaeomelanin on the ventral surface of mice carrying the white-bellied agouti (A^w) and black-and-tan (a^t) alleles (Bultman et al. 1994). The agouti mRNA has the potential to encode a 131-amino-acid protein with a consensus signal peptide (Bultman et al. 1992; Miller et al. 1993). This is consistent with the observation that the agouti protein acts in a non-cell autonomous manner from within the follicular environment of the dermis to signal the hair bulb melanocytes to synthesize phaeomelanin (for review, see Silvers 1979).

The A^y allele was recently characterized at the molecular level and was shown to result from a 170-kb deletion in sequences upstream of the agouti gene, including the entire coding region of an unrelated gene called *Raly* (Michaud et al. 1993, 1994). The net effect of this deletion is that the *Raly* promoter now directs the ubiquitous expression of agouti mRNA and protein in A^y heterozygotes. We have proposed that the deletion of *Raly* from the A^y allele causes the recessive embryonic lethality and that the ectopic overexpression of agouti in heterozygous A^y mice is responsible for the dominant pleiotropic effects associated with this allele (Bultman et al. 1992; Michaud et al. 1993, 1994). This paper describes a new dominant mutation at the agouti locus, intracisternal A-particle yellow (A^{iapy}), in which an IAP has in-

serted in an antisense orientation, 51 bp upstream of the first coding exon of agouti. Transcription of the A^{iapy} allele initiates from within the IAP, resulting in the ubiquitous ectopic overexpression of the wild-type agouti-coding sequence. The phenotype produced by A^{iapy} is remarkably similar to A^{vy} in that homozygotes are viable, and the coat color ranges from solid yellow, to varying amounts of yellow and pseudoagouti mottling, to pseudoagouti. Furthermore, yellow $A^{iapy}/-$ mice become severely obese, whereas their pseudoagouti siblings have normal weights. Importantly, we show that there is a positive correlation between increasing amounts of phaeomelanin in the coats of $A^{iapy}/-$ mice and increasing levels of the ectopically expressed agouti mRNA. Interestingly, the increased agouti expression is associated with decreased levels of methylation of the IAP 5' long terminal repeat (LTR).

Results

Genetic characterization of a new, spontaneous, dominant mutation at the agouti locus

The original spontaneous mutant was a female with a mottled yellow and pseudoagouti coat and was born on March 17, 1992 from the mating of a C3H (A/A) male to a C57BL/6J (a/a) female. The mutant founder female was backcrossed to her C3H father, resulting in four agouti offspring and five mutant offspring with coat colors ranging from solid yellow to yellow with moderate amounts of pseudoagouti mottling. Two of the agouti offspring from this backcross mating were sib mated, resulting in a litter of 10, 3 of which had nonagouti (a/a) black coats, indicating that one of the A alleles from the C3H father mutated to a new dominant allele (A^*). Therefore, the genotype of the original spontaneous mutant is A^*/a , which was subsequently verified by breeding this female to an a/a (C57BL/6N) male. Taken together, these and other breeding data suggest that A^* is an allele at the agouti locus that exhibits Mendelian inheritance and is dominant to both the A and a alleles.

The phenotype produced by A^* is essentially identical to A^{vy} in that homozygotes are viable (data not shown) and the coat color ranges from solid yellow, through varying degrees of yellow and pseudoagouti mottling, to pseudoagouti. Moreover, like A^{vy} , where the expressivity of the mutant phenotype is dependent on whether the allele is passed through the male or the female germ line (see introductory section), the A^* allele is also expressed in a differential manner depending on parental lineage. For example, the mating of A^*/A^* or A^*/a ($A^*/-$) females to a/a males produced 2 of 81 A^*/a progeny (2.5%) that were pseudoagouti. The mating of $A^*/-$ males to a/a females produced 43 of 106 A^*/a progeny (40.6%) that were pseudoagouti. Whereas the percentage of pseudoagouti offspring that arise from mutant male or female parents differs between A^* and A^{vy} , in all other respects the phenotypes of these two alleles appear to be identical. These data suggest that A^* may represent a new allele at the agouti locus that produces a phenotype like A^{vy} .

Genomic structure reveals an IAP insertion

To determine the molecular nature of the *A** mutation, genomic DNAs from the mutant founder and her parents were digested with a variety of enzymes, blotted, and hybridized with a ³²P-labeled, wild-type agouti cDNA probe. The cDNA probe detected restriction fragment length polymorphisms (RFLPs) only in the region near the first coding exon of the agouti gene in the *A** allele (data not shown). Subsequently, DNAs of the mutant founder and her parents were digested with *Bam*HI or *Eco*RI, blotted, and hybridized with a ³²P-labeled, 0.7-kb *Eco*RI fragment derived from wild-type DNA (probe A in Fig. 1B) that contains the first coding exon. Probe A detects the expected sized parental fragments, along with two RFLPs each in *Bam*HI- and *Eco*RI-digested DNAs that are specific to the mutant locus (Fig. 1A). These data, together with additional restriction mapping of the mutant locus (data not shown), indicate that a 5.2-kb fragment of DNA has inserted within the 0.7-kb *Eco*RI region that was used as a probe for these experiments (Fig. 1B). Additionally, it was determined by Southern blot analysis that this region of the agouti gene is not rearranged in the *A^{vy}* allele (data not shown), demonstrating that *A** does represent a new mutation of *A*.

To characterize the 5.2-kb fragment of DNA inserted into the mutant allele, probe A (Fig. 1B) was used to isolate a clone from an *A*/A** genomic λ library. Characterization and DNA sequence analysis of portions of the cloned region revealed that the inserted sequence in the mutant allele is an intact IAP of the Δ 1 type (Fig. 2). Complete sequence analysis of the IAP 5' LTR revealed that it contains all of the structural features associated with a functional LTR (Fig. 2B). Additionally, sequence analysis of the DNA flanking the inserted IAP revealed the presence of a 6-bp duplication of host genomic DNA precisely at the site of integration, suggesting that the IAP integrated through a mechanism common to retroviral-like elements in the mammalian genome (Heidmann and Heidmann 1991). Additional mapping demonstrated that the IAP inserted in an antisense orientation relative to the agouti gene, 51 bp upstream of the 5' end of the first coding exon of agouti (Fig. 2). Because the IAP insertion is not present in either parent and is the only rearrangement we have been able to detect in *A**, we have named this new agouti locus allele *A^{iapy}*. That the IAP insertion is responsible for the phenotype is apparent from the expression analyses presented below.

A^{iapy} expression

Previously, as an initial step in analyzing the molecular nature of the *A^{iapy}* mutation, we demonstrated that adult heterozygotes (*A^{iapy}/A*) with solid yellow coat colors ectopically overexpress wild-type-sized agouti mRNA in brain, testes, spleen, small intestine, kidney, and liver (see Fig. 8 in Michaud et al. 1993). Additionally, RNA was analyzed from the adult heart, lung, thymus, pancreas, fat, and muscle from *A^{iapy}/A^{iapy}* mice with solid yellow coats (Fig. 3). Collectively, these results

Figure 1. Southern blot analysis reveals that the new dominant yellow mutation at the agouti locus contains a 5.2-kb insertion immediately upstream of the first coding exon of the gene. (A) Genomic DNAs from the original spontaneous mutant (*A*/a*) and her parents [C3H (*A/A*) male and C57BL/6J (*a/a*) female] were digested with *Bam*HI or *Eco*RI, blotted, and hybridized with a ³²P-labeled fragment of DNA corresponding to the wild-type agouti 0.7-kb *Eco*RI fragment that contains the first coding exon (probe A in B). Because the founder (*A*/a*) arose from the mating of a single *A/A* male to two *a/a* females, and it was not known which of the two *a/a* females gave birth to the founder, DNAs from the male and both females were included in the analysis. Probe A detects the same sized parental DNA fragments for both the *A* and *a* alleles (data not shown; Bultman et al. 1992), so only a single parental DNA sample (*a/a* female) is shown. Probe A detects a 1.8-kb *Bam*HI fragment in parental DNA, and two RFLPs of 3.3 and 2.2 kb in the mutant DNA because the probe extends to both sides of the insertion. In *Eco*RI-digested DNA, probe A detects a 0.7-kb parental fragment and two RFLPs of 4.9 and 0.9 kb that are associated with the mutant allele. Sizes of DNA fragments detected are shown at left in kilobases. (B) Genomic restriction map of the new mutant agouti locus allele (*A**). The horizontal line represents wild-type agouti sequence. The solid boxes depict the four agouti exons associated with form I agouti mRNA (Bultman et al. 1994) and are numbered sequentially. In the mutant allele, a 5.2-kb fragment of DNA (open rectangle) has inserted immediately upstream of the first coding exon of the agouti gene (indicated by the ATG start codon in exon 2) in the paternal *A* allele. Probe A is indicated by a horizontal bar. The restriction map is drawn to scale, except for the exons that are enlarged for clarity. (B) *Bam*HI, (E) *Eco*RI.

Figure 2. The 5.2-kb fragment of DNA inserted in the agouti gene in the new dominant yellow mutation (A^{iapy}) is an IAP. (A) Genomic restriction map of the IAP inserted in A^{iapy} and the wild-type agouti sequence surrounding the site of integration. The IAP is shown as a rectangle, with the hatched regions representing the two LTRs and the open portion depicting the remainder of the proviral genome. The IAP inserted in an antisense orientation, 51 bp upstream of the first coding exon, and the insertion resulted in a 6-bp duplication of agouti sequence (underlined) at the site of integration. The estimated total size (5.2 kb) and the diagnostic internal 4-kb *HindIII* fragment suggest that this IAP is a ΔI element (Kuff and Lueders 1988). (B) *Bam*HI, (E) *Eco*RI, (H) *Hind*III. (C) Complete nucleotide sequence of the 5' LTR of the IAP inserted in A^{iapy} . The boundaries of the U3, repeat (R), and U5 regions are indicated above the sequence. In the U3 region, three enhancers are each underlined with a horizontal line, and the caat box sequence and tata box replacement sequence (gttgt) are shown in bold. Four CpG sites [three *Hha*I restriction enzyme recognition sites (gcgc) and one *Hpa*II site (ccgg)] are bracketed above and below the sequence. (C) Complete nucleotide sequence of the 3' LTR of the IAP inserted in A^{iapy} . The boundaries of the U3, repeat (R), and U5 regions are indicated above the sequence. Shown are the sense strands of both LTRs.

demonstrate that the expression of the agouti gene from the A^{iapy} allele is deregulated in a manner that results in the ectopic overexpression of a normal-sized agouti mRNA in a ubiquitous manner.

To determine whether the amount of yellow pigmentation in the coats of mice carrying the A^{iapy} allele correlates with the level of ectopic agouti mRNA expres-

Figure 3. Northern blot analysis demonstrates that A^{iapy} mRNA is ectopically overexpressed in a variety of adult tissues. A wild-type agouti cDNA clone was 32 P-labeled and hybridized to poly(A)⁺ RNA (~2.5 μ g/lane, except for pancreas and muscle, which are underloaded) from several adult A^{iapy}/A^{iapy} tissues and from 4-day-old A/A neonate skin, which served as a positive control.

sion, RNA from the liver and kidney of solid yellow, mottled, and pseudoagouti mice was evaluated by Northern blot analysis (Fig. 4). The data demonstrate that the level of ectopic agouti expression in the liver and kidney of A^{iapy}/a mice is directly correlated with the amount of pheomelanin in the coat; solid yellow mice express agouti ectopically at very high levels, pseudoagouti mice express agouti ectopically at barely detectable levels, and mottled mice express agouti at intermediate levels (Fig. 4). These results are consistent with the observation that yellow and mottled $A^{iapy}/-$ mice be-

Figure 4. Northern blot analysis of agouti locus expression in adult liver and kidney from A^{iapy}/a mice with either a solid yellow, moderately mottled (yellow plus pseudoagouti mix), or completely pseudoagouti coat color. The same wild-type agouti cDNA clone that was used in Fig. 3 was 32 P-labeled and hybridized to poly(A)⁺ RNA (~2.5 μ g/lane) from the kidney and liver of A^{iapy} mice exhibiting the two extremes (yellow and pseudoagouti) and an intermediate (mottled) in the full spectrum of coat color phenotypes. The filter was also hybridized with a chicken tubulin probe to control for the quantity and quality of RNA in each lane. With a longer exposure of this filter, a low level of agouti expression was detected in pseudoagouti liver but not in pseudoagouti kidney.

come markedly obese (up to 75 grams), whereas their pseudoagouti siblings have normal weights (~30 grams) (E.J. Michaud, unpubl.).

Transcription of the A^{iapy} mRNA initiates within the IAP

When IAPs transpose in the murine genome, they can up- or down-regulate the expression of other genes, depending on the orientation of the IAP and its position of integration relative to the target gene. These retroviral-like elements can act to enhance transcription from the endogenous promoter of a gene or can initiate transcription from within its LTRs (for review, see Kuff and Lueders 1988). To determine whether the ectopic transcription of agouti from the *A^{iapy}* allele initiates from the normal agouti promoters or from within the inserted IAP genome in an adult tissue that does not normally express the 0.8-kb agouti mRNA, we utilized a reverse transcriptase–polymerase chain reaction (RT–PCR) strategy (Fig. 5A). Total RNA was prepared from the thymus of an adult *A^{iapy}/A^{iapy}* mouse and was compared with RNA from 4-day-old dorsal skin of an *A/A* mouse, which pro-

duces only the normal form I agouti mRNA, and RNA from a sample of 5-day-old ventral skin of an *A^w/A* animal, which expresses both the normal form I and II agouti transcripts. These RNA samples were reverse transcribed and subjected to PCR with primers that would uniquely amplify the normal form I and form II transcripts, or transcripts that would arise from the antisense strand of the IAP 5' LTR (Fig. 5A). The PCR products were blotted and hybridized with a probe corresponding to the coding region of the agouti gene (Fig. 5B).

The cDNA probe detected PCR products of the expected size that were amplified from the form I and form II agouti transcripts in the skin RNAs from both the *A/A* and *A^w/A* controls (Fig. 5B). On the other hand, the primer combinations (II+IV and I+IV) that amplified these expected sized wild-type fragments failed to amplify any fragments in total RNA from the *A^{iapy}/A^{iapy}* mouse, clearly indicating that the ectopic expression in the mutant animals is not attributable to the activation of expression from the wild-type agouti promoters. However, the primer pair (III+IV) designed specifically to amplify transcripts arising from the IAP 5' LTR did amplify the expected sized *A^{iapy}* fragment (Fig. 5B). These data

Figure 5. RT–PCR analysis reveals that transcription of agouti from the *A^{iapy}* allele initiates from the inserted IAP genome. (A) RT–PCR strategy used to determine the location of initiation of agouti transcription from the *A^{iapy}* allele. Shown is the structure of the wild-type agouti gene as it was originally described (exons 1–4; Bultman et al. 1992) and the recently identified (Michaud et al. 1993; Bultman et al. 1994), additional 5′-noncoding exons A and B [the single box A actually represents two agouti exons (Michaud et al. 1994)]. Also shown is the IAP, its location of insertion into the agouti gene, and the 5′ → 3′ transcriptional orientation of agouti and the IAP. The wild-type agouti gene produces several different transcripts that each contain the three coding exons (2–4) of the gene but differ in their 5′-noncoding exons [form I transcripts contain exon 1, and form II transcripts contain exon A only or exons A and B (Bultman et al. 1994)]. Primers I or II were each used in conjunction with primer IV to detect the normal form II or form I agouti transcripts, respectively. Primer III was used in conjunction with primer IV to detect any transcripts initiating from the IAP 5′ LTR. (B) RT–PCR assay for determining the location of initiation of agouti transcription from the *A^{iapy}* allele. Total RNA (10 μg) from adult *A^{iapy}/A^{iapy}* thymus, 4-day-old *A/A* dorsal neonate skin (control for form I transcript), and 5-day-old *A^w/A* ventral neonate skin (control for form II transcripts) was reverse transcribed and subjected to PCR with different combinations of primers as indicated. The PCR products were electrophoresed through a 3% agarose gel, blotted, and hybridized with a ³²P-labeled agouti cDNA probe, which consists primarily of the three coding exons (2–4). Each lane is numbered below, and above is shown whether reverse transcriptase was (+) or was not (–) included in the RT reaction (control for contaminating genomic DNA), the genotype of the RNA sample, and the prim-

ers used in the PCR reaction. Oligonucleotide primers from the mouse β -actin gene were used as an internal control for each PCR reaction. A β -actin fragment of the expected size was observed in all five RT+ reactions (lanes 1,3,5,7,9) by ethidium bromide staining of the DNA in the agarose gel prior to transfer (data not shown). Primer combinations II and IV (lane 1), and I and IV (lane 7) amplified the expected sized fragments from form I and form II agouti transcripts, respectively. Two fragments are expected in lane 7 because exon B is alternately processed in ventral-specific transcripts of *A^w* mice (Bultman et al. 1994). Only primers III and IV amplified the expected sized fragment from the *A^{iapy}* RT template (lane 5). DNA molecular size standards are shown at left in base pairs.

indicate that ectopic transcription of agouti from the A^{iapy} allele in adult mice does not initiate from either of the agouti promoters that normally give rise to form I and form II transcripts in skin but, instead, initiates from within the inserted IAP 5' LTR.

The methylation state of the IAP 5' LTR is correlated with the level of A^{iapy} expression

It has been reported that IAP gene expression in mouse cells is regulated by CpG methylation of *HhaI* and *HpaII* restriction enzyme sites located in the 5' LTR and that increasing levels of methylation are correlated with decreasing levels of IAP expression (Morgan and Huang 1984; Feenstra et al. 1986; Falzon and Kuff 1991; Lamb et al. 1991). On the basis of this knowledge and the fact that transcription of agouti from the A^{iapy} allele is initiated from within the inserted IAP LTR, we considered the possibility that the range of coat color phenotypes and associated pleiotropic effects exhibited by $A^{iapy}/-$ mice may be a direct reflection of the differential methylation status of the individual IAP LTRs within these mice.

To test whether or not the methylation state of the integrated IAP 5' LTR correlates with agouti expression in $A^{iapy}/-$ mice, an experiment was designed to analyze directly the level of CpG methylation at the *HpaII* and *HhaI* restriction enzyme sites within this 5' LTR. For this purpose, genomic DNAs from solid yellow (A^{iapy}/A^{iapy}), pseudoagouti (A^{iapy}/a), and nonagouti control (a/a) mice were digested with *Bam*HI, *Bam*HI plus *Msp*I, *Bam*HI plus *Hpa*II, and *Bam*HI plus *Hha*I. These samples were blotted and hybridized with probe B, which lies immediately 3' of the inserted IAP element (Fig. 6A). The methylation-sensitive *Hpa*II and *Hha*I sites occur at defined positions within the 5' LTR (Fig. 2B). In *Bam*HI-digested DNAs, probe B detects a 1.7-kb fragment indicative of the balancer *a* allele used in these experiments, whereas a 3.3-kb fragment is detected for the A^{iapy} allele as a result of the insertion of the IAP immediately 5' of the second exon (Fig. 6B). For the *Bam*HI/*Msp*I-digested samples, as expected, probe B detects a 1.3-kb fragment for the A^{iapy} allele from both yellow and pseudoagouti mice because *Msp*I (a methylation insensitive isoschizomer of *Hpa*II) cuts the 3.3-kb *Bam*HI fragment to completion (Fig. 6B). When A^{iapy} DNA is digested with *Bam*HI and *Hpa*II, probe B detects only the 3.3-kb fragment in the pseudoagouti mouse, which indicates that the A^{iapy} allele is fully methylated at the *Hpa*II site within the IAP 5' LTR. However, in the DNA from the yellow A^{iapy} mouse, a substantial quantity of the 3.3-kb fragment is digested by *Hpa*II, which is indicated by the detection of both the 3.3- and 1.3-kb fragments. A similar result is seen with the *Bam*HI/*Hha*I-digested A^{iapy} DNAs (Fig. 6B). Taken together, these data demonstrate that several methylation-sensitive *Hpa*II and *Hha*I restriction enzyme sites in the regulatory region of the IAP 5' LTR in the A^{iapy} allele are almost completely methylated in pseudoagouti mice, but are hypomethylated in solid yellow mice.

Figure 6. Southern blot analysis reveals that the differential expression of agouti from the A^{iapy} allele is associated with the methylation state of the 5' LTR in the inserted IAP. (A) Genomic restriction maps of a portion of the A^{iapy} and *a* agouti alleles. The horizontal lines in each map correspond to agouti genomic sequence flanking exon 2, which is indicated by the solid box. The IAP proviral element present in the A^{iapy} allele is shown as a rectangle, with the hatched regions indicating the two LTRs and the open region depicting the remainder of the IAP genome. The IAP inserted in an antisense orientation relative to the transcriptional orientation of agouti, so that the 5' LTR is the one located closest to agouti exon 2 (see Fig. 5A). Shown above each map is the size (in kb) and position (extent of horizontal line) of the restriction fragments observed in the Southern blot in part B. The bracketed region below exon 2 corresponds to probe B used in B. (B) *Bam*HI, (E) *Eco*RI, (H) *Hha*I, (M) *Msp*I-*Hpa*II. There are three *Hha*I sites and one *Msp*I-*Hpa*II site present in the IAP 5' LTR (Fig. 2B), but only one of the *Hha*I sites is shown because of space considerations. (B) Southern blot analysis of the methylation state of the IAP 5' LTR in A^{iapy} mice with solid yellow vs. completely pseudoagouti coat colors. Genomic DNAs from mice with a solid yellow (Y, A^{iapy}/A^{iapy}), pseudoagouti (P, A^{iapy}/a), or nonagouti black (*a/a*) coat color were digested with *Bam*HI, *Bam*HI and *Msp*I, *Bam*HI and *Hpa*II, or *Bam*HI and *Hha*I, blotted, and hybridized with 32 P-labeled probe B. (The faintly detected 2.5-kb fragments seen only in *Bam*HI/*Hpa*II- and *Bam*HI/*Hha*I-digested DNAs of the mouse with the solid yellow coat probably result from the occasional cutting at additional *Hpa*II or *Hha*I sites located in the 3.3-kb fragment between the *Bam*HI and *Eco*RI sites in the IAP in cells where the *Hpa*II or *Hha*I sites located in the IAP 5' LTR are methylated and not cut). Sizes of DNA fragments detected are shown at left in kilobases.

Discussion

Dominant mutations at the agouti locus are each characterized by a number of pleiotropic effects that include the development of a yellow pelage, marked obesity, non-insulin-dependent diabetes, and a variety of spontaneous and induced tumors (for review, see Wolff et al. 1986; Wolff 1987). Our previous analysis of the *A^y* mutation (Bultman et al. 1992; Michaud et al. 1993, 1994) suggests that the development of these dominant pleiotropic effects is caused by the ubiquitous expression of agouti. In *A^y*, we determined that a 170-kb deletion that includes all of the coding exons of an upstream gene called *Raly* leads to the production of chimeric *Raly/agouti* transcripts. These chimeric transcripts retain the coding potential of the wild-type agouti gene and are ubiquitously expressed under the influence of the *Raly* promoter (Michaud et al. 1993, 1994). Here, we demonstrate that a new dominant yellow agouti mutation overexpresses agouti mRNA with a broad tissue distribution that resembles that observed for *A^y*. This new mutation, called *A^{iapy}*, arose from the insertion of an IAP proviral element 51 bp upstream of the first coding exon of the agouti gene. An RT-PCR analysis of the transcript expressed from this allele revealed that a cryptic promoter from within the IAP 5' LTR activates transcription of the downstream agouti coding exons and leads to the production of a wild-type-sized 0.8-kb mRNA. Although the transcript has a normal size, it contains a structural alteration at its 5' end that involves the replacement of the normal agouti noncoding sequences with a section of the IAP 5' LTR and the 50 bp of agouti intron sequence that occurs between the inserted IAP and the first coding exon of the agouti gene (determined on the basis of the size of the PCR product in a 3% agarose gel; Fig. 5). This structurally altered agouti mRNA nevertheless appears to retain the capacity to encode a wild-type agouti protein. Therefore, mice carrying either the *A^{iapy}* or *A^y* alleles each develop the dominant pleiotropic effects as a consequence of the ectopic overexpression of the agouti gene.

IAPs are defective retroviruses encoded by a large family of endogenous proviral elements that occur at ~1000 copies per haploid genome in *Mus musculus* (Lueders and Kuff 1980; Ono et al. 1980). Functionally, IAPs can transpose to novel places in the mouse genome and result in insertional mutations by activating or inactivating target gene expression (for review, see Kuff and Lueders 1988; Blatt et al. 1988; Kongsuwan et al. 1989; Brigle et al. 1992; Algate and McCubrey 1993). Particularly relevant to this study is the observation that some IAP LTRs are capable of acting in a bidirectional manner to activate target gene expression (Horowitz et al. 1984; Christy and Huang 1988). Therefore, the finding that an IAP element has transposed into the agouti gene in the *A^{iapy}* allele and that *A^{iapy}/–* mice express agouti mRNA under the influence of a cryptic promoter within the IAP LTR is consistent with these previous findings.

The new agouti allele described here, *A^{iapy}*, has a number of features in common with the *A^y* mutation. Ho-

mozygotes of both of these mutations are viable, which distinguishes them from the *A^y* mutation, and both develop the traits characteristic of dominant agouti alleles. Most notably, like *A^y*, the *A^{iapy}* allele shows a variable expressivity of its associated dominant traits. Specifically, the pelage of individual mice carrying the *A^{iapy}* or *A^y* mutation can range from entirely yellow, to varying amounts of yellow and pseudoagouti mottling, all the way to an entirely pseudoagouti pigmentation pattern, which is difficult to distinguish from the wild-type agouti coat color. Whereas *A^{iapy}* is phenotypically most similar to *A^y*, it is clear that it is a new allele of agouti and not a repeat mutation of *A^y* primarily because the *A^y* allele does not contain an IAP insertion at the same site as in *A^{iapy}*. However, on the basis of the overall similarity between *A^{iapy}* and *A^y* in their genetic and phenotypic characteristics, we propose that the agouti gene may be deregulated in a similar manner in both of these mutant alleles. It is possible that *A^y* may also be associated with the insertion of an IAP or perhaps another retroviral-like element. If *A^y* is the result of an IAP insertion, then the minor differences between the expressivity of the *A^{iapy}* and *A^y* mutations may result from different features of the regulatory regions of each IAP element and/or their different positions of integration into the agouti gene. Alternatively, the minor differences between the expression of these two mutations may simply be a consequence of their different genetic backgrounds.

The variability in the expression of *A^y* was previously attributed to metabolic characteristics of the maternal oviductal and uterine environment (Wolff 1978). Here, we show for the *A^{iapy}* mutation that increasing amounts of pheomelanin in the coats of these mice correlate well with increasing levels of ectopic agouti gene expression in the kidney and liver, which do not normally express agouti. This finding is particularly noteworthy because it suggests that the epigenetic factors resulting in variegated expression of agouti in the coat may also act on other, and possibly all, tissues to an equal extent within a given individual. Whereas the precise mechanism for the variegated expression is not entirely clear at the present time, we are predicting that methylation of the IAP 5' LTR plays a role in this process. Preliminary support for this prediction comes from our data, which showed that at least two CpG sites in the IAP 5' LTR of the *A^{iapy}* allele are hypomethylated in a solid yellow mouse, but hypermethylated in a pseudoagouti individual. Moreover, this predicted role of methylation in the expression of the *A^{iapy}* allele is in agreement with the observation that the expression of IAP proviral elements is inversely correlated with the methylation state of their 5' LTRs; increased levels of methylation are associated with decreased levels of expression (Feenstra et al. 1986). Recent evidence has also shown that transcription factors bind to domains in IAP LTRs that encompass CpG sites, and methylation of cytosine residues at these CpG sites can inhibit transcription factor binding and gene expression (Falzon and Kuff 1989, 1991; Lamb et al. 1991; Satyamoorthy et al. 1993). Given that the tran-

scription of agouti from the A^{iapy} allele appears to initiate from the 5' LTR of the inserted IAP, it seems quite likely that the wide range of agouti gene expression exhibited by $A^{iapy}/-$ mice may be attributable to the methylation state of the IAP regulatory region. Additional experiments are currently under way to investigate more fully the role of methylation in the differential expression of the A^{iapy} mutation.

On the basis of the fact that $A^{iapy}/-$ mice with mottled coats have regions of completely yellow hair adjacent to patches of fur that exhibit pseudoagouti-banded pigmentation, we predict that the methylation of the IAP regulatory region in each individual cell is an all or none phenomenon. This would suggest that in mice with mottled coats, the intermediate level of agouti expression detected by Northern analysis (Fig. 4) is attributable to mosaicism within the animal with respect to cells that do and do not ectopically express agouti. According to this proposal, those animals expressing the pseudoagouti phenotype would contain a fully methylated 5' LTR in virtually all of their cells and, hence, would express the agouti gene ectopically at extremely low levels. Moreover, the fact that the mice with the pseudoagouti phenotype develop a pigmentation pattern that is similar to the banded wild-type agouti coloration, as opposed to a solid black pelage, which is indicative of the inactivation of the agouti gene, suggests that the fully methylated LTR makes the IAP virtually invisible to the gene. Consequently, we predict that the form I agouti transcript will be expressed in a normal manner in the skin of pseudoagouti mice, which is consistent with the observation that some hairs of pseudoagouti mice appear identical to wild-type agouti hairs (Galbraith and Wolff 1974). However, because many aberrant banding pigmentation patterns are also present in the hairs of pseudoagouti mice (Galbraith and Wolff 1974), it is possible that very low levels of ectopic agouti expression can trigger a slightly altered pigmentation pattern, but not the obesity, diabetes, or tumors, which may require higher levels of constitutive agouti expression.

Interestingly, the variable level of expression of the dominant traits in mice carrying the A^{iapy} and A^{vy} alleles is influenced by parental inheritance. The expressivity of both alleles is higher when passed through the female germ line. This is particularly true for A^{vy} , where females produce <1% of the wild-type-like pseudoagouti class of offspring. In the A^{iapy} allele, there is also a marked effect of parental inheritance, but the expressivity of the mutant phenotype is lower than in A^{vy} , regardless of whether the mutation was passed through the female or the male germ line. This is evidenced by the fact that $A^{iapy}/-$ females produce 2.5% pseudoagouti offspring compared with <1% for A^{vy}/a females, and $A^{iapy}/-$ males have 40.6% pseudoagouti offspring compared with 10–34% for A^{vy}/a males. The fact that IAPs are hypermethylated during spermatogenesis but are hypomethylated in the developing oocyte (Sanford et al. 1987) leads us to speculate that the differential expression of the A^{iapy} phenotype (and possibly A^{vy}) associated with parental inheritance may result, in part, from dif-

ferences in DNA methylation of IAPs during oogenesis and spermatogenesis that persist after the time of extensive de novo methylation in the embryo (Sanford et al. 1987). Moreover, the fact that we observed a dramatically different degree of expressivity of the mutant phenotype on the basis of parental inheritance raises the question of whether mammalian imprinting mechanisms may be at least partly responsible for this differential expression of the A^{iapy} allele. If this proves to be the case, it is possible that the IAP 5' LTR is functioning as a fortuitous *cis*-regulatory imprinting element, which may be similar to the situation observed in several lines of transgenic mice where the transgene may be differentially methylated (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987) and expressed (Swain et al. 1987) depending on gamete of origin. Given this scenario, the A^{iapy} allele may prove to be particularly useful for studying imprinting because the coat color trait in individual mice would provide a visual indication of the degree to which the DNA was imprinted.

Materials and methods

Mice

The A^{iapy} allele arose from a spontaneous mutation of the A allele that occurred in a mating of a C3H (A/A) male to a C57BL/6J (a/a) female at The Jackson Laboratory (Bar Harbor, ME). The subsequent breeding and maintenance of this line was done at the Oak Ridge National Laboratory (Oak Ridge, TN). The A^{iapy} line is currently being maintained on a C57BL/6N background.

RNA analysis

Total cellular RNA from all tissues was extracted using the guanidine isothiocyanate procedure (Ausubel et al. 1988), enriched for poly(A)⁺ RNA using an oligo(dT)-cellulose column (Aviv and Leder 1972), electrophoresed through formaldehyde gels, and blotted to GeneScreen (DuPont) using standard procedures (Ausubel et al. 1988). Radiolabeled hybridization probes were prepared with the random hexamer labeling technique (Feinberg and Vogelstein 1984). Posthybridization filter washes were conducted at high stringency (0.2× SSC, 0.1% SDS, 68°C).

For RT-PCR analyses, 10 µg of total RNA was reverse transcribed (Kawasaki 1990), ethanol precipitated and resuspended in 20 µl of H₂O, and PCR analysis was performed with 2 µl of the sample as described previously (Pieretti et al. 1991). The sequences of the oligonucleotide primers used for the RT-PCR analysis in Figure 5 are as follows: I, 5'-gaactggcatcaaaagtacca-3'; II, 5'-caatgctcctgcctctgcca-3'; III, 5'-ccttggtcatgcgcagatt-3'; IV, 5'-ttccgcttctcggtctctt-3'.

Southern blot analysis

Genomic DNA (10 µg) was digested with restriction enzymes, electrophoresed through agarose gels, and blotted to GeneScreen (DuPont) using standard procedures (Ausubel et al. 1988; Sambrook et al. 1989). Probe preparation and posthybridization filter washes were performed as described above.

Isolation of genomic clones

Genomic spleen and kidney DNA from an A^{iapy} homozygote was partially digested with *Sau*3A and size fractionated on a

10–40% sucrose gradient (Sambrook et al. 1989). Fractions containing 18- to 23-kb fragments were ligated into the λ vector, EMBL3 (Stratagene), packaged in vitro, and screened with ³²P-labeled probe A (Fig. 1B) using standard procedures (Sambrook et al. 1989). Positive clones were purified, and portions were subcloned into pBluescript II (Stratagene).

DNA sequencing

Genomic clones were sequenced by the Sanger dideoxynucleotide method (Sanger et al. 1977) using T7 DNA polymerase (U.S. Biochemical) (Tabor and Richardson 1987). Analysis of DNA sequence was performed by use of the University of Wisconsin Genetics Computing Group sequence analysis programs (Devereux et al. 1984), and GenBank data base searches were conducted using the BLAST algorithm (Altschul et al. 1990).

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Note added in proof

Sequence data for IAP 5' and 3' LTR have been submitted to the GenBank data library under accession numbers L33247 and L33248, respectively.

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Differential expression of a new dominant agouti allele (Aiapy) is correlated with methylation state and is influenced by parental lineage.

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