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Differences in dorsal and ventral pigmentation result from regional expression of the mouse agouti gene

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ABSTRACT **The agouti coat color gene encodes a paracrine signaling molecule that controls the production of yellow and black pigment by melanocytes within hair follicles. Some agouti alleles affect the dorsum and ventrum independently, which has provided the basis for speculation that agouti gene action in different regions of the body is controlled by distinct genetic loci that are closely linked. Using a combination of cDNA cloning and RNA expression studies, we find that alternative isoforms of agouti mRNA contain different noncoding first exons located 100 kb apart, whose patterns of expression indicate independent control by regulatory elements that are either ventral specific or hair cycle specific. These results demonstrate that the apparent genetic complexity of the agouti locus is explained by the existence of multiple regulatory elements exerting control over a single coding sequence and provide a conceptual basis for understanding differences in dorsal and ventral hair coloration in many mammalian species. The ventral-specific agouti isoform represents an example of a transcript whose expression is restricted to ventral skin and provides an approach to investigate the mechanisms by which dorsal–ventral differences in gene expression are established and maintained.**

The external appearance of nearly all mammals is affected by morphological features that vary according to their location along the dorsal–ventral axis, including skin thickness, hair follicle type, and pigmentation. Although these characteristics are most evident in the adult animal, their cellular basis is established during embryogenesis, and little is known about mechanisms that underlie region-specific development of the skin, hair follicles, and pigment cells.

In mice and many other mammals, differences in dorsal and ventral pigmentation are caused by variation in action of the agouti gene (refs. 1 and 2; reviewed in ref. 3). Agouti gene expression induces melanocytes within hair follicles to switch from the synthesis of black pigment granules (eumelanosomes) to yellow pigment granules (phaeomelanosomes), which then become incorporated into a growing hair (2, 4). A series of elegant transplantation studies indicated that the gene product is produced normally by nonpigment cells and acts in a paracrine fashion, since it does not affect melanocytes in adjacent follicles (5–8). We (9) and others (10) have recently isolated the agouti gene, which appears to encode a secreted molecule produced within the hair follicle during the time of yellow pigment synthesis (9).

Three agouti alleles exemplify how agouti gene action can affect the dorsal and ventral parts of the body differently. In mice of agouti genotype A/A, yellow pigment is synthesized only during the midportion of the hair growth cycle; consequently, both dorsal and ventral hairs have a subapical band of yellow pigment on an otherwise black background. In contrast, the ventral surface of mice that carry the black-and-tan (a^t) or light-bellied agouti (A^W) alleles contains hairs in which yellow pigment is synthesized throughout the entire hair growth cycle, which leads to a ventrum that is entirely yellow, while their dorsal hairs are either black (a^t/a^t) or banded (A^W/A^W) (Fig. 1). Although more than 20 different agouti alleles have been described, most wild and inbred strains of mice carry A^W, A, or the nonagouti (a) allele, in which the only hairs that contain yellow pigment are located in specialized areas behind the ears or around the mammary glands and perineum.

Expression studies indicate that the level of agouti mRNA correlates with the synthesis of yellow pigment (9, 10), but it is not clear why agouti gene expression should be restricted to the ventrum of a^t/a^t mice or why agouti should be expressed differentially in the dorsum and ventrum of A^W/A^W mice. These characteristics, in combination with the observations that certain agouti alleles recombine with each other (11, 12) and that mutations of a to a^t or to A^W occur more frequently than expected (13), have led to speculation that agouti may be a complex locus in which distinct but closely linked genes are regulated independently in each part of the body (14, 15). An alternative "simple locus" hypothesis is that the apparent independence of dorsal and ventral pigmentation reflects differential competence of these areas to respond to the same level of agouti gene expression (ref. 16; reviewed in ref. 3).

Our recent molecular cloning studies suggested that a single copy of agouti coding sequences is present per haploid genome (9), which does not support the complex locus hypothesis. However, molecular heterogeneity among the 5′ ends of cDNA clones suggested that alternative isoforms of agouti mRNA might be produced with different 5′ untranslated sequences. Here we report that alternative isoforms of the agouti gene, which contain different sets of 5′ untranslated exons, are expressed in a region- and temporal-specific fashion. Based on these results, we present a general model for understanding differences in dorsal and ventral pigmentation.

MATERIALS AND METHODS

Mice carrying the A^W (129/SvJ), a^t(MWT/Le), and a (C57BL/6J) mutations were obtained from The Jackson Laboratory, and mice carrying the A (FVB/N) mutation were obtained from Taconic Farms. Isolation of agouti cDNA clones and analysis of their structure and expression were performed according to standard techniques (17) as described (9). Genomic clones were isolated from a bacteriophage P1 library (Genome Systems, St. Louis) with primer pairs described in the legend to Fig. 2.

RESULTS

Alternative 5′ Ends of the Agouti Gene Lie 100 kb Apart. In initial studies of agouti cDNA isolated from total skin RNA of

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Abbreviation: RT, reverse transcription.

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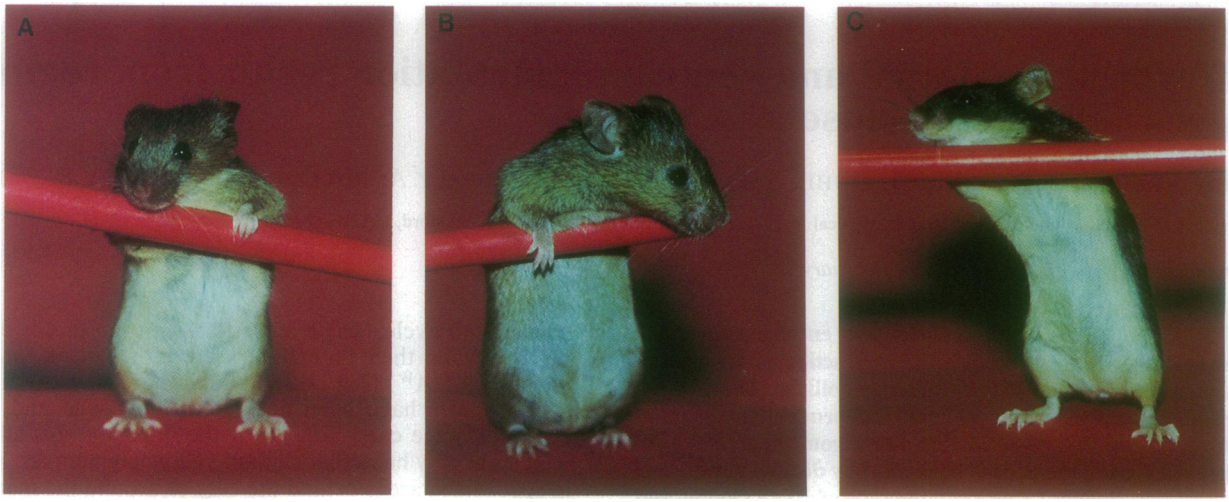

FIG. 1. Coat color phenotypes of light-bellied agouti (A^W/A^W), agouti (A/A), and black-and-tan (a^t/a^t) mice. (*A*) The agouti phenotype apparent in the head and dorsum of the A^W/A^W animal is caused by hairs that contain a subapical band of yellow pigment with black pigment at the tip and base. (*B*) These banded hairs cover the dorsum and ventrum of A/A animal. (*C*) In contrast, the a^t/a^t animal has dorsal hairs that are completely black. Hairs in the ventrum of the A^W/A^W and the a^t/a^t animal contain only yellow pigment. A sharp demarcation between dorsal and ventral colorations occurs in both animals.

5-day-old A/A mice, the majority of cDNA clones contained four exons, in which the predicted protein-coding sequences within exons 2, 3, and 4 were preceded by a 111-nt nontranslated first exon (9). A minor proportion of clones from these experiments contained a different 5′ end. To investigate the extent of this 5′ heterogeneity, additional cloning experiments were performed using ventral skin RNA from 1-day-old A^W/A^W mice. Among a total of 18 cDNA clones obtained from the two sources of RNA, four isoforms were apparent that differed only in their 5′ ends (Fig. 2*A*). Comparison of their sequences indicated that one isoform resulted from alternative splicing of a 46-nt exon (designated 1A′, Fig. 2); however, the other three isoforms appeared to each represent a different site of transcriptional initiation. None of the alternative 5′ ends

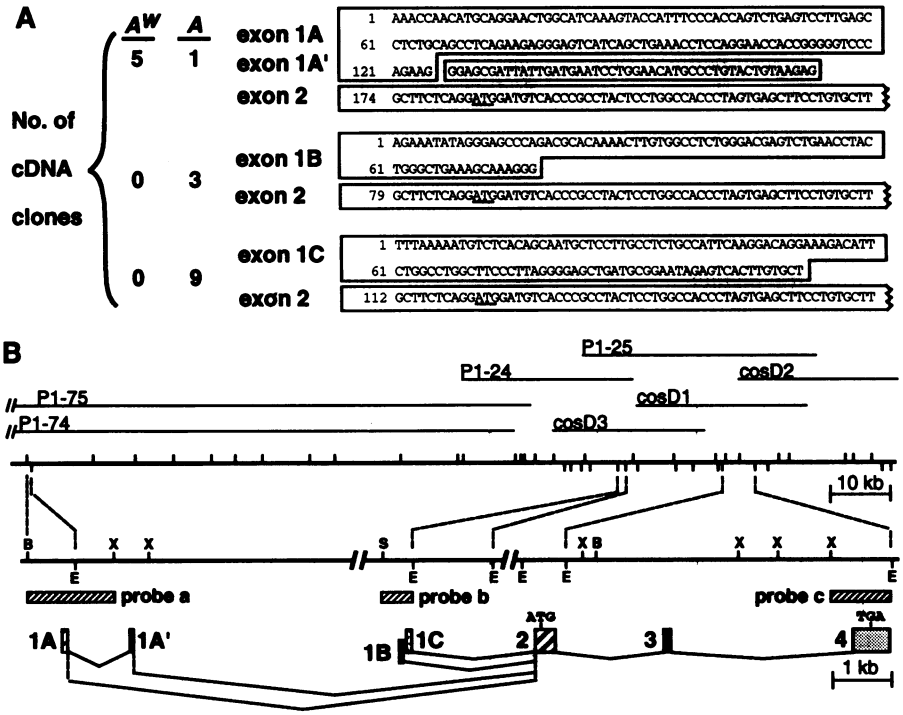

FIG. 2. Alternative promoters and splicing of the agouti gene. (*A*) Number, structure, and 5′ sequence of different agouti cDNA clones obtained from total skin RNA of 5-day-old A/A mice and ventral skin RNA of 1-day-old A^W/A^W mice. The cDNAs are divided into three groups, A, B, and C, based on the nature of their first exon. The sequences shown represent the longest clone obtained for each group. The location of exon boundaries was determined by comparison of the different cDNA clones to each other and to genomic clones. The genomic DNA sequence for all intron/exon boundaries conformed to expectations for vertebrate splice donor and splice acceptor sites (18). Except for a single A^W/A^W clone that contained an additional 46-nt exon after exon 1A, exons 1A, 1B, and 1C were always followed by exon 2 in which the probable translational initiation site is underlined. Exons 1A and 1A′ are also contained within one of several chimeric mRNAs that occur in the lethal yellow (A^y) allele (9, 19). (*B*) Locations of agouti exons on a 150-kb contig of genomic DNA. Isolation of the cosmid clones cosD1, cosD2, and cosD3 has been described (9). The bacteriophage P1 clones were isolated using the primer pairs 5′-CCTAGGTTTCTCTGTGTCCCC-3′ plus 5′-CAGAAGTCCCTGGTAGCTGC-3′ (P1-24 and P1-25) and 5′-AGTCTGAGTCCTTGAGCCTC-3′ plus 5′-TGGGACCCCCGGTGGTTC-3′ (P1-74 and P1-75). Restriction maps of the P1 clones were determined by partial digestion and indirect end labeling. Comparison of restriction maps allowed a 150-kb contig to be generated that included all the cosmids and P1-24, -25, -74, and -75. The locations of *Bam*HI and *Eco*RI restriction sites are indicated above and below the solid black line, respectively, or by "B" and "E" on the expanded map. Locations of *Eco*RI restriction sites were determined for all clones except P1-74 and P1-75. The *Bam*HI fragments that contained exons 1A, 1A′, 1B, and 1C were identified by hybridization of a radiolabeled oligonucleotide probe to P1 or cosmid DNA. These *Bam*HI fragments were then subcloned and partially sequenced, and the locations of additional restriction sites including *Stu* I (S) and *Xba* I (X) were determined. The exact position of each exon was confirmed by sequence analysis of genomic DNA. A second region in P1-74 and P1-75 that contains sequences similar but not identical to exon 1A is not shown. Probes used in subsequent hybridization experiments are designated as probe a, a 1.3-kb *Bam*HI–*Xba* I fragment that contains exon 1A; probe b, a 0.5-kb *Stu* I–*Eco*RI fragment that contains exons 1B and 1C; and probe c, a 1.0-kb *Xba* I–*Eco*RI fragment that contains exon 4.

contained an ATG predicted to function as a translational initiation site.

To determine the positions of the different 5′ ends relative to each other and to the protein-coding exons of the agouti gene, a 150-kb contig of cosmid and bacteriophage P1 genomic clones was generated, and the locations of each exon within the contig were established by Southern hybridization and DNA sequencing (Fig. 2*B*). The results indicated that two distinct sets of nontranslated first exons were located 100 kb apart. The furthest and most centromere-proximal group of exons, designated 1A or 1A1A′, was found in all of the cDNA clones isolated from ventral skin RNA of 1-day-old A^W/A^W mice and in 1 of 13 cDNA clones isolated from total skin RNA of 5-day-old A/A mice. The remaining 12 cDNA clones isolated from 5-day-old A/A mice began with one of two different first exons, designated 1B or 1C, that were separated by 40 nt of genomic DNA (Fig. 2*B*). These observations suggested that exons 1A and 1A′ might represent a previously unrecognized form of agouti RNA responsible for constitutive synthesis of yellow pigment in ventral skin in A^W/A^W mice.

Region- and Temporal-Specific Patterns of Expression Exhibited by Different Untranslated First Exons. To confirm that *agouti* isoforms were expressed differentially, we used genomic probes that contained either exon 1A (probe a) or exons 1B and 1C (probe b) to analyze dorsal and ventral A^W/A^W skin RNA from 1 to 8 days after birth by Northern hybridization (Fig. 2). In addition, we used a probe that contained exon 4 (probe c) as a measure of total agouti expression. This latter probe detects RNA levels that correlate with the time during hair growth when yellow pigment is synthesized, 1–7 days after birth in the ventrum and 3–6 days after birth in the dorsum (Fig. 3*A*). The probe that contains exon 1A detects similar levels of RNA in A^W/A^W ventral skin but detects no RNA in A^W/A^W dorsal skin. In contrast, the probe that contains exons 1B and 1C detects RNA in both dorsal and ventral A^W/A^W skin but only during the midportion of the hair growth cycle, which occurs 1–2 days later in the ventrum than in the dorsum (Fig. 3*A*).

To distinguish between exons 1A and 1A′, and between exons 1B and 1C, RNA from dorsal and ventral skin of neonatal A^W/A^W mice was subjected to reverse transcription (RT) and then enzymatically amplified using the polymerase chain reaction (RT-PCR) with oligonucleotide primers specific for the four different isoforms (Fig. 3*B*). The results were similar to those observed by Northern hybridization, confirming that expression of the different isoforms was specifically regulated, even at the level of sensitivity allowed by RT-PCR. In addition, no differences were observed for isoforms that contained exon 1A vs. exon 1A′ or for isoforms that contained exon 1B vs. 1C (Fig. 3*B*). Thus, region- and temporal-specific expression of the different isoforms is not controlled by alternative splicing but, instead, is likely to be controlled by mechanisms that affect transcriptional initiation of exons that lie 100 kb apart.

Expression of Agouti Isoforms in the *A* and a^t Alleles. To determine whether the observations made in A^W/A^W mice could be extended to other agouti alleles, we hybridized probes that contained the different sets of first exons with dorsal and ventral skin RNA from A/A and a^t/a^t mice (Fig. 4*A*). Samples from A^W/A^W mice were also included on the blot to allow a direct comparison between all three alleles. In a^t/a^t mice, the probe that contains exon 1A detected expression in the ventral but not the dorsal skin samples, identical

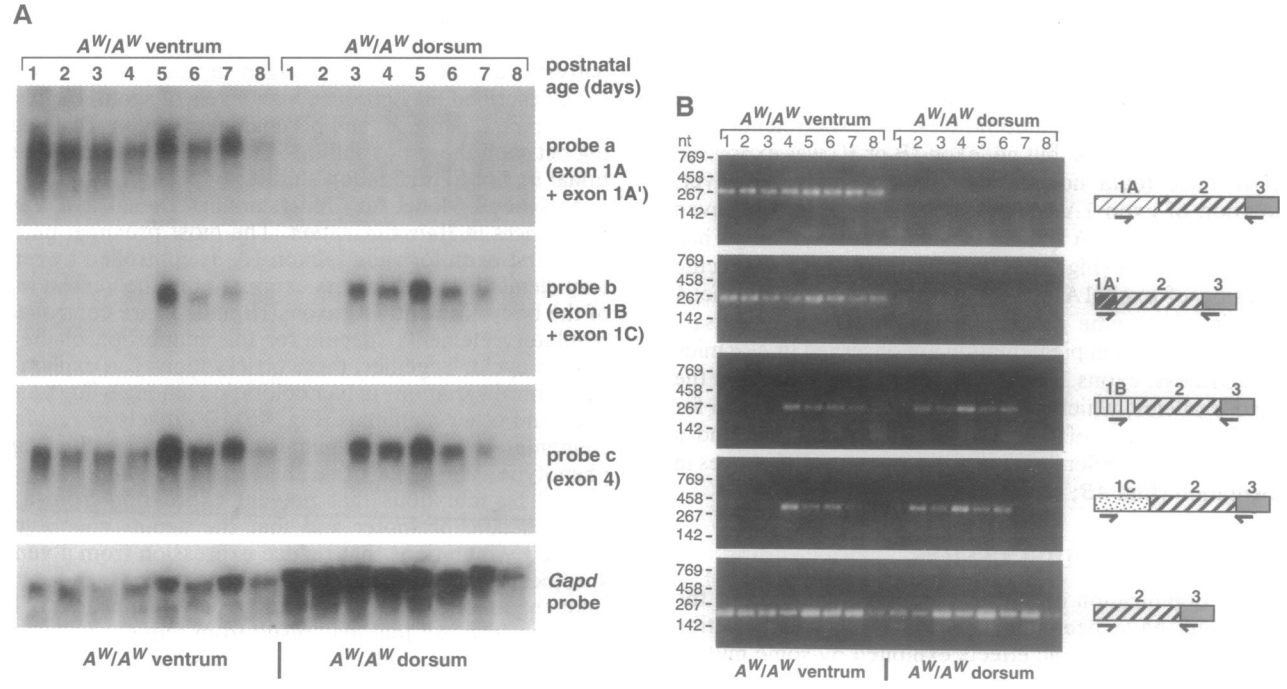

FIG. 3. Expression of different agouti exons in the ventrum and dorsum of neonatal A^W/A^W mice. (*A*) Total RNA was isolated from skin dissected from the ventrum and dorsum of 129/Sv-A^W/A^W mice at days 1–8 after birth. By using 30 μg per lane, the RNA was fractionated on formaldehyde/agarose gels, transferred to nylon membranes, and hybridized with probe a, b, or c and a glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) probe to control for different amounts and quality of the RNA loaded on the gels. Removal of residual probe after each hybridization was checked by autoradiography. (*B*) For RT-PCR experiments, 5 μg of RNA from each sample was subjected to RT using a primer from agouti exon 4, 5′-GAGGAATTCACCTTGCCACCTTCTTCATCGA-3′. One-twentieth of each reaction was then PCR-amplified using a primer located further 5′, 5′-GGATTTCTTGTTCAGTGCCACG-3′, paired with one of five sense primers as follows: exon 1A, 5′-AGTCTGAGTCCTTGAGCCTC-3′; exon 1A′, 5′-GATGAATCCTGGAACATGCC-3′; exon 1B, 5′-CAAAACTTGTGGCCTCTGGG-3′; exon 1C, 5′-CAGCAATGCTCCTTGCCTCT-3′; and exon 2, 5′-CAGGAATTCACCATGGATGTCACCCGCCTACTC-3′. The PCR was performed using parameters of 94°C for 45 sec, 45°C for 25 sec, and 72°C for 90 sec for 30 cycles of amplification. The identity of the amplified product was confirmed by direct sequencing and/or Southern hybridization using an internal radiolabeled oligonucleotide. An ethidium bromide-stained gel is shown in which the number of each lane refers to the age of the animal in postnatal days.

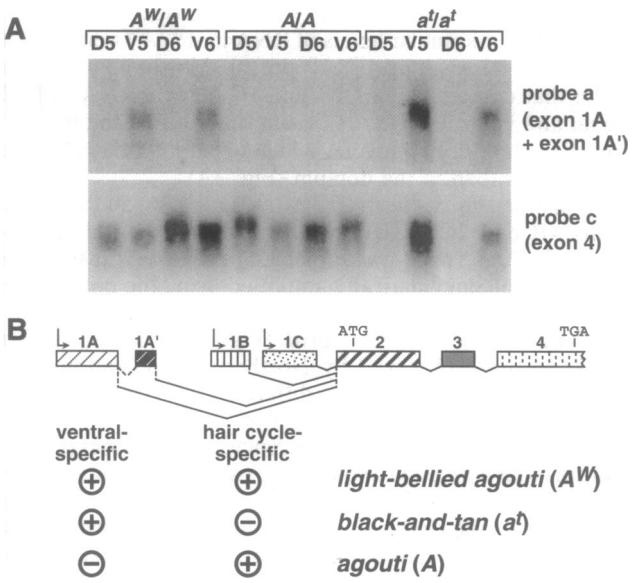

FIG. 4. Expression of agouti isoforms in the ventrum and dorsum of neonatal A^W/A^W, A/A, and a^t/a^t mice. (*A*) Northern hybridization studies were performed as described in the legend to Fig. 3*A*. The letter and number above each lane refer to the RNA source, dorsal (D) or ventral (V), and the postnatal age, respectively. Removal of residual probe after hybridization with probe a was checked by autoradiography before hybridization with probe c. (*B*) Diagram of the splicing pattern for agouti exons 1–3 and a summary of the results depicted in Figs. 3 and 4*A*. The lengths of the introns are not drawn to scale; ≈100 kb separates exon 1A' and exon 1B, exon 1B and exon 1C are separated by 40 bp, and ≈18 kb separates exon 1C and exon 2. Expression of the two different groups of exons, 1A1A' or 1B1C, characteristic for the alleles A^W, a^t, and *A* is indicated below the diagram.

to the pattern detected by a probe that contains agouti coding sequences (Fig. 4*A*). In addition, RT-PCR experiments demonstrated that exon 1A' but not exon 1B or 1C was expressed in a^t/a^t mice (data not shown). In A/A mice, however, expression of exon 1A was not detectable (Fig. 4*A*), even though 1 of 13 cDNA clones isolated from A/A mice had contained exon 1A (Fig. 2). It is possible that ventral-specific expression of exon 1A occurs at very low levels in the *A* allele, since on some genetic backgrounds, subtle dorsal–ventral differences in pigmentation are observed in A/a mice (3). Regardless, exons 1A and 1A' are ventral specific in the A^W and a^t alleles, which provides a unifying explanation for the molecular basis of the black-and-tan phenotype and a general model for understanding dorsal–ventral differences in pigmentation (Fig. 4*B*; see *Discussion*).

DISCUSSION

Genomic organization and transcriptional regulation of the agouti locus have been the subject of much speculation because of the pleiotropic effects exhibited by some mutant alleles and because different agouti alleles control gene action independently in specific parts of the body (reviewed in ref. 3). The latter observation is the basis for suggesting that the locus is genetically complex (reviewed in ref. 12)—i.e., a series of closely linked transcriptional units that may recombine with one another as pseudoalleles. The results presented here provide a molecular explanation for some of these observations, a model for understanding dorsal–ventral patterns of pigmentation in mice and in other mammals, and a tool with which to investigate the establishment and maintainence of differences in gene expression that depend on location along the dorsal–ventral axis.

Genetic Complexity of Agouti Regulatory, but Not Coding, Sequences. The initial hypothesis that agouti contained multiple closely linked genes, each regulated independently in different parts of the body (14, 15), was based on a model that did not take into account that alternative isoforms of a single gene can be produced by RNA splicing. In retrospect, the observations used to support the complex locus hypothesis are explained simply by the existence of alternative promoters regulating a single coding sequence. Thus, differences in the dorsum and ventrum of A^W/A^W and of a^t/a^t mice result from complexity of agouti regulatory regions and not from differential competence of these regions to respond to a common signal.

Because the different sets of untranslated first exons that we have identified are located 100 kb apart, recombination within this region could account for apparent examples of pseudoallelism. Intragenic recombination between A^y and *a* or between A^y and a^x predicts a genetic distance of 0.039–0.12 centimorgan (11, 12), which is roughly consistent with our observation that molecular lesions responsible for the A^y and a^x mutations are located 100–200 kb apart (20). Exons 1A and 1A', normally ventral-specific, are also found as internal components of some *agouti* isoforms expressed ubiquitously in the A^y allele (9, 10, 19). These A^y isoforms begin with the first exon of a gene named *Raly* (19) or *Merc* (33), which then becomes spliced abnormally to agouti exons 1A, 1A', or 2.

Null mutations of agouti such as nonagouti lethal (a^l) or extreme nonagouti (a^e) result in the complete absence of yellow pigment (21, 22). In contrast, the yellow pigment found in specialized areas of nonagouti a/a mice indicates that *a* is hypomorphic rather than amorphic, even though agouti RNA is not detectable in total skin RNA of these animals. It is possible that low levels of agouti are expressed in a generalized distribution in a/a mice and that follicular melanocytes in certain areas are especially sensitive. Alternatively, a regulatory mechanism in addition to those we have described may produce high levels of agouti expression in the pinna, perineal, and perimammary areas. Distinguishing between these possibilities should be feasible with additional *in situ* hybridization studies.

A General Model for Understanding Dorsal and Ventral Differences in Hair Coloration. The most proximal untranslated first exon for agouti, exon 1A, is controlled by regulatory elements that direct its expression to the ventral region of the body. In contrast, exons 1B and 1C are controlled by regulatory elements specific for the midportion of the hair growth cycle. Together, these observations can explain dorsal–ventral variations in hair coloration among wild strains of mice and other mammals (Fig. 4*B*). Both sets of regulatory elements are active in animals with a light-bellied agouti phenotype. We suggest that the black-and-tan phenotype is caused by mutations that prevent expression from a hair cycle-specific promoter and that the agouti phenotype is caused by mutations that reduce expression from a ventral-specific promoter. Thus, even though both *A* and A^W are frequent among wild strains of mice, *A* appears to be a mutation from A^W that may result from sequence alterations at or close to the ventral-specific promoter. Graded expression from the ventral-specific promoter may also account for phenotypes that are intermediate between agouti and light-bellied agouti, observed in many strains of wild mice (23). Finally, modifier genes such as Umbrous (*U*), which appear to have a greater effect on the dorsum than the ventrum, may act by reducing expression from the hair cycle-specific promoter (24).

This model makes several testable predictions. First, expression of all agouti isoforms is greatly reduced in the *a* allele, which is associated with an insertion between exon 1C and exon 2 (10). Because a reduction in the size of this insertion accompanies reversion of *a* to a^t, we suggest that

sequences within the insertion can influence expression from the ventral-specific promoter. Reversion of *a* to A^{W-J}, which also occurs frequently, may involve a similar mechanism in which expression from both the ventral-specific promoter and the hair cycle-specific promoter is derepressed. Second, the physical distance between exon 1A and exon 1B, 100 kb, is sufficiently large that recombination within this interval in an *A/a* animal should occasionally give rise to an A^W allele, assuming that the mutation from A^W to *A* resides close to exon 1A. Finally, the agouti gene is thought to exist in nearly all mammalian orders, with naturally occurring alleles accounting for a light-bellied agouti, agouti, or nonagouti phenotype (1). Besides rodents, the black-and-tan phenotype is present in certain species of rabbits, dogs, and deer; therefore, we suggest that agouti genes in these animals also contain multiple untranslated first exons that are regulated independently.

The Nature of Agouti Promoters. The agouti phenotype, a subapical band of yellow pigment on an otherwise black background, results from the expression of exons 1B and 1C, which are transcribed during the midphase of the primary hair growth cycle, between days 3 and 7 after birth. Because recapitulation of the agouti banding pattern is observed in actively growing adult hairs in response to plucking, it seems likely that regulatory elements associated with exons 1B and 1C respond to a set of coordinated signals implicated in the growth of hair matrix cells and melanogenesis. A similar pattern of expression has been described for genes thought to modulate cell proliferation and remodeling within the hair follicle (25–27).

In contrast, the ventral-specific expression pattern of exon 1A is, to our knowledge, unique. Although the ventral and dorsal embryonic dermis are morphologically indistinguishable, differential expression of agouti RNA indicates an underlying molecular and cellular heterogeneity established early in development. In limb bud mesenchyme, dorsal–ventral positional identity is thought to be acquired via paracrine signals transmitted by the overlying ectoderm (28), possibly via differential expression of *Wnt* gene family members (29). However, fate mapping experiments indicate that ventral and dorsal mesenchyme emerge from different parts of the primitive streak (30), and it is possible that ventral-specific agouti expression may reflect positional identity acquired through specific mesodermal cell lineages. These possibilities might be investigated using dermal/epidermal recombination experiments in conjunction with agouti RNA as a marker for ventral identity.

Evolutionary Conservation of Agouti Regulatory Elements? The presence of the characteristic agouti banding pattern in noneutherian mammals suggests the existence of a functional agouti gene early in mammalian evolution. With regard to other phyla, it is difficult to compare pigment patterns in skin appendages such as scales and feathers directly to mammalian hair. However, a common morphologic feature observed in many nonmammalian vertebrates is a sharp demarcation between the dorsal and ventral body surfaces. In the case of *Xenopus*, this difference appears to be controlled by a diffusible melanization inhibiting factor produced only by ventral cells (31, 32). Although the molecular relationship of *Xenopus* melanization inhibiting factor to mouse agouti remains to be determined, it is possible that the developmental pathway and associated regulatory factors that govern ventral-specific expression of agouti exon 1A are conserved among all vertebrates.

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