Expression and transgenic studies of the mouse *agouti* gene provide insight into the mechanisms by which mammalian coat color patterns are generated

Sarah E. Millar, Miles W. Miller, Mary E. Stevens and Gregory S. Barsh
Departments of Pediatrics and Genetics, and the Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305-5428, USA

**SUMMARY**

Expression of the *agouti* gene from two different promoters, one active at the midpoint of the hair cycle and the other specific for the ventrum, is responsible for generating a range of mammalian pigmentation patterns. We demonstrate that in postnatal mice transcripts from both promoters are confined to the dermal papilla of hair follicles, as predicted by classical transplantation experiments. Transcripts from the hair cycle promoter are detected in the embryonic whisker plate but not in other regions of the body before birth, whereas ventral-specific transcripts are detected in the ventral trunk of the embryo as well as ventral whisker plate. To investigate further the embryonic origins of adult pigmentation patterns, we carried out a detailed analysis of *agouti* expression in the embryo. The ventral-specific *agouti* isoform is first expressed at E10.5 in neural crest-derived ventral cells of the second branchial arch, in anterior regions of the forelimb buds and in a narrow stripe of ventral mesenchyme. By E14.5 a continuous layer of expression is observed in the upper cells of the dermis, including cells of the developing dermal papillae, and covering the entire ventral surface of the head and trunk and dorsal surfaces of the distal forelimb and hindlimb. This expression pattern reflects the domain of yellow coloration evident in adult animals and suggests that the *agouti* gene is regulated in part by factors responsible for establishing differences between the dorsal and ventral surfaces of the body during embryogenesis. To test the hypothesis that agouti is a paracrine signaling molecule that can influence pigment production by hair follicle melanocytes when expressed by either dermis or epidermis, as suggested by recombination and transplantation experiments, we created transgenic animals in which *agouti* is expressed in basal cells of the epidermis. These animals display stripes of yellow hairs corresponding to regions of epidermal *agouti* expression, confirming that *agouti* signals melanocytes to synthesize yellow pigment and providing direct evidence that it functions in a paracrine manner with a restricted radius of action.

Key words: *agouti*, coat color, dorsal-ventral patterns, mouse

**INTRODUCTION**

Molecular and cellular mechanisms that generate patterns in the mammalian coat are of interest in the study of both evolution and development, since many of the genes that affect pigmentation act early in embryogenesis and closely related individuals and species often exhibit remarkable diversity in their appearance. In the mouse, many patterns that vary according to their position along the anterior-posterior axis, such as transverse stripes, involve genes that affect melanocyte fate, migration or survival. In contrast, the most obvious pattern in rodents that varies according to regional expression of the *agouti* gene (reviewed in Silvers, 1979). *agouti* encodes a novel paracrine signaling molecule that causes hair follicle melanocytes to synthesize yellow instead of black pigment (Bultman et al., 1992; Miller et al., 1993). The agouti phenotype, a band of yellow pigment on an otherwise black hair, results from a short burst of *agouti* expression during the mid-portion of the hair growth cycle, indicating that the effects of *agouti* protein are short-lived and reversible. In animals that carry the wild-type white-bellied *agouti* (A<sup>w</sup>) allele nearly all dorsal hairs are banded, but ventral hairs are entirely yellow or cream-colored. This difference between ventrum and dorsum is the result of transcription from a second promoter that directs expression throughout the entire hair growth cycle of ventral but not dorsal hair follicles (Silvers and Russell, 1955; Bultman et al., 1994; Vrielings et al., 1994). In animals homozygous for the black-and-tan (a<sup>d</sup>) allele virtually all expression of *agouti* is controlled by this ventral promoter, resulting in black dorsal hairs and yellow ventral hairs (Vrielings et al., 1994). Ventral expression of *agouti* in A<sup>w</sup>a<sup>t</sup> and a<sup>d</sup>la<sup>d</sup> mice is likely to reflect a unique developmental fate acquired during embryogenesis and maintained after birth. While much progress has been made in identifying factors that determine anterior-posterior polarity in the vertebrate embryo, less is known about the mechanisms that influence dorsal-ventral patterning. Shortly after fertilization, dorsal-ventral differences in *Xenopus* embryos are thought to involve expression of the dorsalizing factor noggin and members of the Wnt gene family, particularly Xwnt-8 and Xwnt-11 (reviewed in Sive, 1993). Although
amphibian and mammalian embryos use different mechanisms to determine the orientation of the dorsal-ventral axis (Gerhart et al., 1989; Smith, 1980), the molecules involved in subsequent steps may be similar. Later in development, several genes have been shown to be expressed in a dorsal- or ventral-restricted fashion in the mouse limb bud and spinal cord, including members of the Wnt family, the TGF-β family member Bmp2, and the homeobox genes Dlx2 and Engrailed1 (Gavin et al., 1990; Parr et al., 1993; Lyons et al., 1990; Bulfone et al., 1993; Davis et al., 1991; Wurst et al., 1994). Recently it has been demonstrated that in the absence of Wnt7A, which is expressed in dorsal ectoderm of the limb, the limb mesoderm shows dorsal-to-ventral transformations of cell fate, indicating that Wnt7A provides a dorsalizing signal (Parr and McMahon, 1995). However, the molecular pathway by which Wnt7A establishes dorsal cell fate has not yet been elucidated.

The identification of a ventral-specific promoter for the agouti gene provides a means to investigate some of the mechanisms by which differences between the dorsal and ventral surfaces of the body are produced and maintained. Such differences include skin thickness and adiposity, the number and type of hair follicles and the timing of hair growth, in addition to variation of pigmentation. An important step towards understanding the mechanisms that lead to dorsal-ventral differences apparent in A+/A+ and a/a' mice is defining the timing and cell localization of agouti expression during development. Classical transplantation experiments in neonates performed by Silvers and Russell (1955) demonstrated that for each hair follicle, the agouti genotype of non-pigment cells determines the type of pigment synthesized within that follicle, but not in adjacent follicles. Later experiments in which dermis and epidermis were recombined from embryos between 13 and 17 days of gestation (B13-E17) suggested that for most, but not all, agouti alleles the genotype of the dermal cells rather than the epidermal cells determined the subsequent phenotype of hair follicles (Mayer and Fishbane, 1972; Poole, 1974, 1975; Poole and Silvers, 1976).

We demonstrate here that, in postnatal animals, expression from both hair-cycle and ventral-specific promoters of the agouti gene is confined to the dermal papilla. However, during embryogenesis, agouti expression is observed in a continuous layer of dermal cells covering the entire ventral surface, providing a unique marker for investigating factors that act during embryogenesis to produce differences between dorsal and ventral surfaces of the body.

To investigate further the hypothesized paracrine nature of agouti signaling and to determine whether agouti is capable of acting on hair follicle melanocytes when expressed in the epidermis, we placed the agouti cDNA under the control of a human keratin 14 promoter which directs expression in basal cells of the epidermis (Vassar et al., 1989). Transgenic mice carrying this construct had stripes of yellow hair in their coats corresponding to regions of epidermal agouti expression, confirming that agouti protein produced in the epidermis can direct the synthesis of yellow pigment in hair follicle melanocytes and providing direct evidence for a limited radius of agouti action. These results provide a molecular explanation for observations made by mouse geneticists several decades ago, suggest mechanisms by which pigmentation patterns arise during embryogenesis and shed light on the mechanism of action of agouti protein.

**MATERIALS AND METHODS**

**RT-PCR of embryonic RNA**

Mice homozygous for the A表示 allele (129/SvEv) were obtained from Taconic Farms and mice homozygous for the a表示 allele (MWT/Le) from The Jackson Laboratory. Males and females of each strain were mated naturally and embryo ages estimated from the time of appearance of a vaginal plug (E0.5). Pieces of skin from the mid-dorsum, mid-ventrum and whisker plate were dissected using fine forceps and iridectomy scissors. RNA was extracted and reverse transcription (RT) carried out using primer A1.AC (GAGGAATTCACCTGG-CACCTTCTTCATCGA) from exon 4 of the agouti coding region. RT reactions contained 2 pmol of primer, 5 μg RNA, 0.5 mM dNTPs, 0.01M DTT, 1 μl RNAsin (Promega, Madison, WI), 1 μl Superscript reverse transcriptase and 1× RT reaction buffer (Bethesda Research Labs, Gaithersburg, MD) in a volume of 50 μl and were incubated for 1 hour at 42°C. PCR was carried out using 1 μl of each RT reaction with 3’ primer O (GGATTTCTTGTCAGTGGCACG) from agouti exon 3, and 5’ primers V (AGTCTGATCGCCCCGACCT), X (CAAAACCTTGGCCCTGGG) and R (CAGGAATTCATGGAATGTCACCTGTGCGCTACT) from exons 1A, 1B and 2 respectively. 30 cycles of PCR were carried out in a volume of 50 μl using an annealing temperature of 45°C. 30 μl of each PCR run was run on a 2% agarose gel. Southern blotting was carried out using standard techniques (Sambrook et al., 1989). Blots were hybridized with a 32p-labeled oligonucleotide probe P (CGAGGTTATGAGGAT-TAATCGCCG) from exon 2 of the agouti coding region at 35°C in 50% formamide, and washed in 2× SSC, 0.1% SDS at room temperature.

**Preparation of RNA probes for in situ hybridization**

To construct an agouti probe suitable for in situ hybridization, the entire protein coding region plus 133 bp of 3’ untranslated sequence was PCR amplified from mouse testis cDNA and subcloned into pBluescript II (Stratagene, San Diego, CA). This plasmid was cut with BamHI and transcribed with T7 RNA polymerase to create antisense probes for agouti. The template plasmid was digested with BamHI and transcribed with T3 RNA polymerase to create a template for probe synthesis containing nucleotides 122-146 and 377-547 from the coding region and 3’ untranslated region of the agouti cDNA described in Miller et al. (1993). The template plasmid was digested with EcoRI and transcribed with SP6 polymerase to provide an antisense probe for Wnt5A. All probes were labeled with digoxigenin UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. 35S-UTP-labeled probes were synthesized by in vitro transcription, followed by alkaline hydrolysis.

**In situ hybridization to frozen sections**

Mid-dorsal and mid-ventral skin pieces were dissected from a/a’ (MWT/Le) mice from The Jackson Laboratory and A/A (C57BL/6J) mice from Simonsen at postnatal day 4. Skin pieces were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed in PBS, incubated overnight in 30% sucrose, transferred to Tissue-Tek OCT embedding compound (Miles Laboratories) for 1 hour and then embedded in OCT compound on dry ice. 8 μm cryostat sections were collected on slides coated with Vectorbond (Vector Laboratories) and allowed to dry for 30 minutes. Hybridization to digoxigenin-labeled probes was as described by Hebert et al. (1994) with the following modifications. Treatment with 10 μg/ml proteinase K was carried out for 6.5 minutes, hybridization was overnight at 60°C and washing in 0.2x SSC was for 1 hour at 60°C. The slides were blocked
in 0.1 M Tris, 0.15 M NaCl, pH 7.5 buffer containing 1% blocking reagent (Boehringer Mannheim), incubated with anti-digoxigenin antibody (Boehringer Mannheim) at 1:2000 dilution in the same buffer and stained for alkaline phosphatase activity. Sections were photographed using a Nikon Microphot-FXA microscope and Kodak Ektachrome 64T color slide film.

Whole-mount in situ hybridization

A<sup>w</sup>/A<sup>w</sup> and a<sup>fa</sup> embryos were obtained from naturally mated animals. Preparation of embryos and in situ hybridization were as described by Conlon and Rossant (1992) with modifications suggested by Parr et al. (1993) and the following additional modifications. Proteinase K treatments were carried out for 8 minutes for E8.5, 12 minutes for E9.5, 15 minutes for E10.5 and 20 minutes for E10.75 embryos; for embryos older than E8.5, prehybridization was carried out overnight, washing at 65°C in 50% formamide, 2× SSC, 0.1% CHAPS was carried out overnight and preblocking in 10% sheep serum was for 6 hours. Embryos were photographed using a Nikon SMZ-U microscope and Nikon FX-35DX camera with Kodak Ektachrome 64T color slide film.

In situ hybridization to paraffin-embedded sections

129/SvJ-A<sup>A</sup>/A<sup>A</sup> embryos were obtained from naturally mated animals. Fixation, embedding, sectioning of embryos, in situ hybridization and washing were as described by Frohman et al. (1990). Specimens were dehydrated in graded alcohols, air dried and dipped in Kodak NB2 emulsion. After 10 days exposure, the specimens were developed and counterstained with methyl green. Photography was carried out using a Nikon Microphot-FXA microscope and Kodak Ektachrome 64T color slide film.

Production and analysis of transgenic mice

A keratin-agouti-SV40 (KAS) transgene was made in pBluescript II (Stratagene, San Diego, CA) with a 2 kb EcoRI-BamHI fragment containing the human keratin 14 promoter ((Vassar and Fuchs, 1991), kindly provided by Dr Elaine Fuchs) ligated to a 525 bp PCR fragment containing nucleotides 204-729 of agouti cDNA and a 0.46 kb BamHI fragment that contained RNA processing signals derived from the SV40 small t intron and major polyadenylation site (Van Doren et al., 1984). Transgenic mice were produced by microinjection of C57BL/6J × FVB/N embryos and so were of genotype A/a; C/c. Expression of agouti in the skin of mice from transgenic lines was analyzed by in situ hybridization of digoxigenin-labeled antisense and sense agouti probes to 8 µm cryostat sections of dorsal skin from transgenic animals and non-transgenic littermates at postnatal day 5.

RESULTS

agouti transcripts from both hair-cycle and ventral-specific promoters are expressed in the dermal papillae of postnatal hair follicles

In newborn animals between P1 and P6, agouti mRNA isoforms are distinguished by the identity of their 5′ untranslated exons and their spatiotemporal pattern of expression (Vrieling et al., 1994; Bultman et al., 1994). Isoforms that contain exon 1A, referred to below as A-agouti, are expressed throughout the hair growth cycle but only in the ventrum; isoforms that contain exon 1B, referred to below as B-agouti, are expressed in both dorsum and ventrum but only during the mid-portion of the hair growth cycle (Fig. 2A). To determine the cell types in which these agouti isoforms are expressed in postnatal animals, in situ hybridization with a probe for the agouti-coding region was carried out using dorsal and ventral skin samples from postnatal day 4 animals of genotype A/A, in which only the B-isoform is expressed, and a<sup>fa</sup>, in which only the A-isoform is detected. In animals of both genotypes, agouti expression was observed in the dermal papillae of hair follicles, but not elsewhere in the skin (Fig. 1). Similar results were obtained with skin from animals of genotype A<sup>A</sup>/A<sup>a</sup>, in which both A- and B-agouti isoforms are expressed (data not shown). These results conform to the predictions of embryonic dermal-epidermal recombination experiments (Mayer and Fishbane, 1972; Poole, 1975; Poole and Silvers, 1976), which suggested that, in skin of genotypes A/A, a<sup>fa</sup> and A<sup>a</sup>/A<sup>a</sup>, agouti gene expression is controlled by cells of dermal origin. Previous results from our laboratory have indicated that agouti is expressed in suprabasal cells of the epidermis in plucked skin from A<sup>a</sup>/A<sup>a</sup> animals (Miller et al., 1993). The discrepancy between these two sets of data may be due to the fact that plucked skin was used in the earlier case, or may be caused by a difference in sensitivity of the two methods used.

Expression of agouti isoforms in A<sup>w</sup>/A<sup>w</sup> and a<sup>fa</sup> embryos

As a first step to determine the expression patterns of A- and B-agouti isoforms during embryogenesis, RNA was extracted from the mid-dorsum, mid-ventrum and whisker plate of black-and-tan (a<sup>fa</sup>) embryos at E13.5 and white-bellied agouti (A<sup>a</sup>/A<sup>a</sup>) embryos at E12.5, E13.5 and E14.5. After reverse transcription with a 3′ primer in exon 4, cDNA was PCR-amplified with primers from exons 2 and 3, which are shared among all agouti isoforms. agouti cDNAs were detected at each stage examined in the mid-ventrum and whisker plate, but not in the dorsum of a<sup>fa</sup> and A<sup>a</sup>/A<sup>a</sup> embryos (Fig. 2B, upper panel). PCR-amplification of the same cDNA samples with primers specific for the different isoforms indicated that the mid-ventral RNA samples contain only exon 1A, while the whisker plate RNA samples contain exons 1A and 1B (Fig. 2B, middle and lower panels). Embryonic expression of B-agouti in the whisker plate may be comparable to the postnatal expression of B-agouti observed in dorsal and ventral skin (Vrieling et al., 1994), since vibrissa follicles develop several days before hair follicles in the trunk and limbs.

agouti expression begins at E10.5 in the second branchial arch and forelimb buds

To determine the time and localization of earliest expression of agouti RNA in the embryo, whole-mount in situ hybridization with antisense and sense probes was carried out using a<sup>fa</sup> and A<sup>a</sup>/A<sup>a</sup> embryos at E8.5, E9.5, E10.5, E10.75 and E11. No expression was detected at E8.5 or E9.5 (data not shown). However at E10.5 hybridization to the antisense probe was observed in the second branchial arch, in the anterior ventral regions of the forelimb buds and in adjacent ventral mesenchyme. A small patch of expression was also seen on the anterior dorsal sides of the forelimb buds (Fig. 3C). At slightly later stages (E10.75 (Fig. 3A) and E11 (data not shown)), expression in the dorsal portion of the forelimb bud was no longer observed, but expression in the ventral region and adjacent mesenchyme of the trunk was more extensive. No hybridization to these areas was observed with the sense probe (Fig. 3B). Expression was not detected in the hindlimb buds of embryos at E10.5-E11. No differences were observed between the expression patterns of agouti in a<sup>fa</sup> and A<sup>a</sup>/A<sup>a</sup> embryos at these developmental stages.
The pattern of *agouti* RNA expression was compared with that of *Wnt5A*, a putative signaling molecule that has been suggested to be involved in regulating dorsal-ventral polarity of the limb bud (Parr et al., 1993). In both forelimb and hindlimb buds, *Wnt5A* is expressed at highest levels in mesenchymal tissue of the progress zone and in the overlying ventral but not dorsal ectoderm (Fig. 3D). *Wnt5A* expression can also be seen in the telencephalon and first branchial arch. The patterns of *agouti* and *Wnt5A* hybridization overlap in the ventral-lateral trunk but are clearly distinct in the limb buds and branchial arches suggesting that a direct involvement of *Wnt5A* in regulating early *agouti* expression is unlikely.

**As development proceeds the domain of *agouti* expression expands to include the upper dermal cells of the entire ventrum**

In order to examine the pattern of expression at later stages of embryogenesis when whole-mount in situ hybridization becomes impractical due to lack of probe penetration and to determine more precisely the cells in which *agouti* is expressed, tissue sections of paraffin-embedded *A/W*/*A*/ embryos were hybridized with 35S-labelled probes.

At E12.5, *agouti* expression is seen in ventral regions of the hindlimb and in the ventral neck and snout (Fig. 4A). Bright-field and dark-field photographs of the neck region taken at higher magnification show that the signal is quite diffuse and is in mesenchymal cells (Fig. 4C,D). One day later, at E13.5, expression in the ventral neck and snout regions has become confined to a narrow layer of cells in the developing dermis immediately underneath the epidermis (Fig. 5). Expression is also observed in dorsal regions of the front and hind paws in the dermis of the developing digits. By E14.5 expression in the dermis extends along the entire ventrum of the embryo and a short distance into the distal dorsal regions of the limbs, but is not seen in dorsal dermis of the trunk and head (Fig. 6 and data not shown), reflecting the pattern of pigmentation seen in the adult. Similar expression in the ventral dermis is observed at E15.5 (Fig. 7A,B).

![Fig. 1. Detection of *agouti* transcripts in A/A and a/a skin at postnatal day 4. (A) 8 μm cryostat section of mid-dorsal A/A skin hybridized with an antisense probe for *agouti*. Arrow indicates hybridization in the dermal papilla of a hair follicle. (B) Similar section to that shown in A hybridized with a sense probe for *agouti*. Bar, 25 μm. (C) Higher magnification view of a similar section to that shown in A hybridized with an antisense probe for *agouti*. (D) Adjacent section to that shown in C hybridized with a sense probe for *agouti*. Bar, 12.5 μm. (E) 8 μm cryostat section of mid-ventral a/a skin hybridized with an antisense probe for *agouti*. The arrow indicates hybridization to the dermal papilla of a hair follicle. (F) Adjacent section to that shown in E hybridized with a sense probe for *agouti*. Bar, 12.5 μm. d, dermis; e, epidermis; dp, dermal papilla.](image-url)
Careful examination of the whiskers of adult $a^t/a^t$ and $A^w/A^w$ animals reveals that the lower but not the upper vibrissae contain entirely yellow pigment. At E15.5 expression of $agouti$ is observed in the dermal component (dermal papilla) of developing lower vibrissa follicles as well as in the adjacent dermis (Fig. 7C,D). Taken together, these observations suggest that $agouti$ is capable of modifying vibrissa as well as coat hair color. Hair follicles of the trunk and limbs are much less well developed than the vibrissa follicles at this stage of embryogenesis; however, hybridization of $agouti$ was occasionally observed to developing dermal papillae in the limbs (data not shown).

Expression of $agouti$ in transgenic animals

The studies described above suggest that the major source of $agouti$ protein in postnatal hair follicles is the dermal papilla, a conclusion in accord with the results of dermal-epidermal recombination experiments performed with animals of genotypes $A/A$, $A^w/A^w$ and $a^t/a^t$ (Mayer and Fishbane, 1972;
Poole, 1974, 1975; Poole and Silvers, 1976). However, similar grafting experiments performed with animals in which agouti was expressed ubiquitously due to the $A_y$ mutation suggested that a functional agouti protein could also be produced by epidermal cells, since $A_y/a$ embryonic epidermis recombined with dermal cells of any agouti genotype gave rise to hairs that contained entirely yellow pigment (Poole, 1974, 1975). In all of these transplantation studies, yellow pigment was only observed close to the potential site of agouti expression, suggesting a limited radius of action regardless of the cell of origin.

To investigate further the nature of agouti signaling in vivo, a transgene (KAS) was created that contains the agouti cDNA fused to a human keratin 14 promoter which directs expression in basal cells of the epidermis (Vassar et al., 1989). The KAS transgene was injected into fertilized eggs of genotype $A^w/A^w; C/c$, which would normally give rise to animals with an agouti phenotype. From 15 weanlings, two female founder transgenic animals, KAS-14 and KAS-15, were obtained and were shown by Southern blot analysis of their genomic DNA to carry...
tandemly arrayed insertions of approximately 50 and approximately 20 copies of the transgene, respectively (data not shown). Both of the founder mice displayed stripes of yellow and agouti hair, unlike their non-transgenic littermates which were entirely agouti. When mated to a C57BL/6J-<math>^a</math> male, KAS-14 produced 19 progeny of which 10 were transgenic and displayed stripes of yellow and agouti hair or yellow and black hair (Fig. 8B). Further breeding studies confirmed that the genotype of animals containing yellow and agouti hair was <math>Tg.KAS; A/a</math>, and that the genotype of animals containing yellow and black hair was <math>Tg.KAS; a/a</math>. The KAS-15 founder produced 26 progeny after mating to a C57BL/6J-<math>^a</math> male, of which only 3 were transgenic, suggesting cellular mosaicism for transgene integration in the KAS-15 founder. However, the transgenic progeny of KAS-15, like those of KAS-14, displayed stripes of yellow and agouti or black hair (Fig. 8A). To determine the expression pattern of <math>agouti</math> transcripts in KAS transgenic lines, in situ hybridization with a probe for the <math>agouti</math> coding region was carried out on skin dissected at postnatal day 5 from regions of the dorsum of transgenic pups displaying yellow stripes. Skin from equivalent regions of non-transgenic littermates was used as a control. Patchy expression was observed in basal cells of the epidermis in regions of the skin that displayed yellow hairs (Fig. 9). Expression from the endogenous <math>agouti</math> gene in dermal papillae of both transgenic and control skin of genotypes <math>A/A</math> and <math>A/a</math> was at lower levels than seen at postnatal day 4 (data not shown). These results confirm the prediction of Poole (1974) that <math>agouti</math> expressed in the epidermis results in the production of yellow hairs. The occurrence of stripes of yellow pigmentation, corresponding to patches of basal epidermal cells expressing <math>agouti</math>, provide further evidence that the radius of agouti protein action is limited.

The striped phenotypes are most easily explained by position-effect variegation of the transgene, in which the yellow stripes represent clones of epidermal cells capable of expressing the transgene. Similar inherited mosaic patterns of expression have been described for a tyrosinase mini-gene, for a transgene containing tyrosinase coding sequences under the control of a metallothionein promoter and for a transgene with SV40 coding sequences controlled by a tyrosinase promoter (Tanaka and Takeuchi, 1992; Mintz and Bradl, 1991; Bradl et al., 1991).

Fig. 7. Expression of <math>agouti</math> in paraffin-embedded sectioned ventral skin and whisker plate of <math>A^vA^e</math> embryos at E15.5. (A) Bright-field view of sectioned ventral skin from an E15.5 embryo hybridized with antisense <math>agouti</math> probe. (B) Dark-field view of A showing hybridization of <math>agouti</math> to cells in the dermis immediately underlying the epidermis. (C) Bright-field view of sectioned ventral skin from the whisker plate of an embryo at E15.5 hybridized with antisense <math>agouti</math> probe. (D) Dark-field view of C showing hybridization to cells of the upper dermis and to the dermal component of developing whisker follicles. Similar signals were not detected in adjacent sections hybridized with a sense <math>agouti</math> probe (data not shown), indicating that the hybridization of antisense probe is specific and not related to cell density. d, dermis; dp, dermal papilla; e, epidermis; wf, whisker follicle. Bars, 25 μm.

Fig. 8. Phenotypes of KAS transgenic mice. Transgenic founders were mated to C57BL/6J-<math>a/a</math> animals. (A) Progeny of KAS-15. Alternating stripes of yellow and black hair are due to the genotype <math>Tg.KAS; a/a</math>. (B) Progeny of KAS-14. Alternating stripes of yellow and agouti hair are due to the genotype <math>Tg.KAS; A/a</math>.
ventral skin, the normal sites for postnatal expression of the agouti protein. The results presented here demonstrate that, in both dorsal and ventral skin, the normal sites for postnatal expression of the agouti protein.

**DISCUSSION**

The results presented here demonstrate that, in both dorsal and ventral skin, the normal sites for postnatal expression of the agouti protein are cells of the dermal papilla. Before birth, the hair cycle-specific isofrom, B-agouti, is expressed only in the whisker plate, but expression of the ventral-specific agouti isofrom, A-agouti, begins at 10.5 days of embryogenesis in the second branchial arch, forelimb buds and ventral mesenchyme, and, by E14.5, has expanded over the entire ventral trunk and the ventral and distal dorsal limbs. The early expression of A-agouti in the embryonic ventrum suggests that the gene may be regulated in part by factors responsible for establishing differences between the dorsal and ventral surfaces of the body.

The agouti protein antagonizes the action of alpha-melanocyte stimulating hormone (α-MSH), which has been shown to stimulate the proliferation of cultured melanocytes in conjunction with other growth factors (Halaban et al., 1993) and induces differentiation of epidermal melanoblasts into melanocytes when injected subcutaneously or added to skin in organ culture or to cultured melanoblasts (Hirobe, 1992). Thus, embryonic expression of agouti may inhibit the differentiation of melanoblasts migrating through the ventral dermis.

**Developmental origins of embryonic A-agouti expression**

The regulatory mechanisms that lead to agouti expression in the ventral trunk of late gestation and newborn animals may be influenced by differences in cell lineage since the dermis of ventral and lateral regions of the trunk arises from the outer layers of the somatic plate mesoderm (Christ et al., 1974a; Mauger, 1972), whereas dorsal trunk dermis is derived from dermatome (Mauger, 1972; Sengel, 1976). However, regional expression of agouti in limb bud dermis, which is solely derived from lateral plate mesoderm (Christ et al., 1979; Christ et al., 1974b), and cephalic dermis, which arises from the neural crest (Couly et al., 1992; reviewed in Noden, 1992), cannot be explained on the basis of cell lineage, suggesting that the factors regulating agouti expression are responding to environmental cues already present when dermal precursor cells migrate into the branchial arches and limb buds.

The early stage at which A-agouti is first expressed and the restriction of its expression to ventral regions of the embryo suggest that the factors regulating A-agouti transcription during embryogenesis may also be responsible for establishing and/or maintaining other differences between the dorsal and ventral surfaces of the body. Candidate paracrine signaling molecules that may be involved in regulating dorsal-ventral polarity in vertebrates include the TGF-β related growth factor BMP-2, which is expressed in the ventral ectoderm and apical ectodermal ridge of the limb bud (Lyons et al., 1990), and the putative paracrine signaling molecules Wnt5A and Wnt7A (Gavin et al., 1990; Parr et al., 1993). Wnt5A is expressed initially in the ventral ectoderm of the early limb bud and at later stages in the ventral apical ectoderm at the distal tip, at lower levels in the ventral distal mesenchyme and at lower levels still in the ventral proximal mesenchyme. As discussed in Results, the expression patterns of Wnt5A and agouti at E10.5 overlap in the trunk, but are distinctly different in the limb buds and branchial arches. Expression of Wnt7A is confined to the dorsal ectoderm of the limb (Parr et al., 1993) and mutation of the Wnt7A gene results in a dorsal-to-ventral alteration of cell fate in the limb mesenchyme (Parr and McMahon, 1995). It is possible that Wnt7A normally acts to repress transcription from the ventral promoter of agouti in the dorsal limb although, in distal regions of the limb, where Wnt7A has its most pronounced effects, expression of agouti is not strictly ventral-specific. Examination of the agouti pigmentation pattern in Wnt7A mutant mice on an A/a or A/w background will help to answer this question. The homeobox-containing gene Dlx2 is expressed in the ventral ectoderm of developing forelimb buds and members of the Hoxa gene cluster and Hoxd12 also show variation in their levels of expression along the dorsal-ventral axis of the limb bud (Bulfone et al., 1993; Dolle et al., 1989; Haack and Gruss,

**Fig. 9.** Detection of agouti transcripts in KAS transgenic skin. (A) 8 μm cryostat section of dorsal skin from a transgenic postnatal day 5 pup of line KAS-15 with genotype Tg.KAS; Aa hybridized with an antisense probe for agouti. Hybridization is observed in basal cells of the epidermis and is indicated by arrows. (B) Adjacent section to that shown in A hybridized with a sense probe for agouti. (C) 8 μm cryostat section of dorsal skin from a non-transgenic littermate of the pup whose skin is shown in A and B, with genotype A/a, hybridized with an antisense probe for agouti. d, dermis; e, epidermis. Bar, 12.5 μm.
1993). Another homeobox-containing gene, Engrailed1 (En1), is expressed in the ventral ectoderm of the limb buds and in adjacent ventral trunk epidermis, in ventrolateral regions of the spinal cord and dermatomes and, later in development, in ventral dermis of the trunk (Davis et al., 1991; Wurst et al., 1994). Analysis of the distribution of agouti transcripts in mice with a targeted disruption of En1 (Wurst et al., 1994) will aid in determining whether En1 plays a role in regulating the embryonic expression of agouti.

Since white-bellied phenotypes are common among mammals, factors regulating the agouti gene are likely to be conserved among mammalian species. Delineation of the cis-acting DNA elements involved in directing embryonic expression of agouti will provide a means to isolate and identify these factors, and to determine which, if any, of the genes mentioned above are involved in the regulation of ventral pigmentation patterns.

Expression of agouti in the epidermis of transgenic animals

Transgenic animals in which agouti is expressed from a keratin 14 promoter in basal calls of the epidermis display yellow hairs in regions of transgene expression, providing definitive proof that agouti expression directs melanocytes in hair follicles to produce yellow pigment, confirming the prediction of Poole (1974) that agouti expressed in the epidermis can influence pigment production by follicular melanocytes and providing a dramatic illustration of the limited radius of action of agouti protein. The pigment variegation observed in animals carrying the K14-agouti transgene is presumably due to position effects at the sites of integration of the transgenes and provides some insight into cell migration during epidermal development. Since yellow hairs are produced in these cases as a result of targeting agouti expression to the epidermis, the yellow stripes are likely to represent clones of epidermal cells capable of expressing the transgene and may therefore reflect a segmental pattern in the embryonic ectoderm. Regionalization of the ectoderm of the head into segmentally distributed stripes that correspond to the first, second, third and fourth branchial arches and mirror the distribution of the underlying neural crest cells has been demonstrated using chick-quail chimeras (Couly and LeDouarin, 1990). Similar fate mapping experiments have not been performed with trunk ectoderm. However, Mintz (1970) reported that mice chimeric for the epidermally expressed hair marker fuzzy (fz) showed narrow transverse bands with a mid-dorsal discontinuity, similar to those seen in the K14-agouti transgenic lines. The stripes observed in both fuzzy/wild-type chimeras and in the K14-agouti transgenic lines were narrower than those produced in transgenics displaying heritable coat color mosaicism due to variable expression of the tyrosinase gene, which have been interpreted as reflecting patterns of melanoblast migration in the embryo (Mintz and Bradl, 1991; Tanaka and Takeuchi, 1992).

CONCLUDING REMARKS

The agouti gene was first recognized by its effect on individual hairs, but it is now clear that a second function of the gene is to control dorsal-ventral differences in pigmentation. The developmental mechanisms that determine dorsoventral patterning of the coat are likely to be shared among vertebrates, and pigmentary systems that depend on α-MSH are present in both avians and reptiles. Therefore, it is possible that an ancestral agouti gene predates the origin of mammals. The melanogenesis inhibitory factor (MIF) produced by ventral but not dorsal frog skin described by Bagnara and colleagues (Bagnara et al., 1992; Fukuzawa and Bagnara, 1989) may represent a functional homolog of agouti.

Certain mammals, such as dogs, foxes and goats, exhibit coat color patterns with lateral stripes or patches of yellow or cream coloration that vary according to their position on the dorsal-ventral axis (Searle, 1968; Adalsteinsson et al., 1987, 1994). Although lateral stripes are not found in adult laboratory mice, expression of A-agouti in mouse embryos in the second branchial arch, the limb bud and the trunk may signify potential origins of such stripes in other mammals. For example, symmetrical markings that confer a reddish-yellow or pale coloration to the cheeks and lower jaw are observed in many strains of domestic goats (Adalsteinsson et al., 1994) and could arise from expression of an agouti homolog in the first and/or second branchial arch. Elucidation of the mechanisms that govern expression of A-agouti in mouse embryos may therefore shed light on the pathways by which complex pigmentation patterns are generated in other mammals.

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