Molecular analysis of reverse mutations from nonagouti \((a)\) to black-and-tan \((a^t)\) and white-bellied agouti \((A^w)\) reveals alternative forms of agouti transcripts

Scott J. Bultman,\(^1,2\) Mitchell L. Klebig,\(^1\) Edward J. Michaud,\(^1\) Hope O. Sweet,\(^3\) Muriel T. Davisson,\(^3\) and Richard P. Woychik\(^1\)

\(^1\)Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077 USA; \(^2\)The University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee 37831-8077 USA; \(^3\)The Jackson Laboratory, Bar Harbor, Maine 04609 USA

The agouti gene regulates the differential production of eumelanin (black or brown) and phaeomelanin (yellow) pigment granules by melanocytes in the hair follicles of mice. The original nonagouti \((a)\) allele, which confers a predominantly black coat color, has been shown to revert to two other more dominant agouti alleles, black-and-tan \((a^t)\) and white-bellied agouti \((A^w)\), with an exceptionally high frequency. The \(a^t\) and \(A^w\) alleles confer phenotypes in which the pigmentation is not uniformly distributed over the dorsal and ventral surfaces of the animal; in both cases the ventral surface of the animal is markedly lighter than the dorsal surface due to an increase in phaeomelanin production. To understand the unusually high reversion rate of \(a\) to \(a^t\) or \(A^w\), and to decipher the molecular events associated with the different pigmentation patterns associated with these three agouti alleles, we have characterized \(a\), \(a^t\), and \(A^w\) at the molecular level. Here, we report that insertions of 11, 6, and 0.6 kb are present at precisely the same position in the first intron of the agouti gene in \(a\), \(a^t\), and \(A^w\), respectively. The \(a\) insertion consists of a 5.5-kb VL30 element that has incorporated 5.5 kb of additional sequence internally; this internal sequence is flanked by 526-bp direct repeats. The \(a^t\) allele contains only the VL30 element and a single, internal 526-bp repeat. The \(A^w\) allele has only a solo VL30 LTR. Based on the comparison of the structure of the \(a^t\) and \(A^w\) insertions, we propose that reverse mutations occur by excision of inserted sequences in \(a\) through homologous recombination, utilizing either the 526-bp direct repeats to generate \(a^t\) or the VL30 LTRs to generate \(A^w\). Moreover, the analysis of these three alleles has allowed us to identify additional exons of the agouti gene that give rise to alternatively processed forms of agouti mRNA. We demonstrate that the distinct insertions in \(a\), \(a^t\), and \(A^w\) cause pigmentation differences by selectively inactivating the expression of different forms of agouti transcripts.

[Key Words: agouti locus; \(a\), \(a^t\), \(A^w\); reverse mutations; VL30; homologous recombination]

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Black-and-tan is dominant to \(a\) but recessive to \(A^w\). It is dominant to \(A\) across the ventral surface of the animal, consistent with yellow being dominant to agouti, but is recessive to \(A\) across the dorsal surface of the animal, which is compatible with the observation that agouti is dominant to black. White-bellied agouti, which confers an agouti dorsal surface and a cream color over the ventral surface, is dominant to the \(A, a^l,\) and \(a\) alleles.

The \(a, a^l,\) and \(A^w\) alleles are also of interest because reverse mutations from \(a\) to \(a^l\) or \(A^w\) are more common than any other spontaneous agouti locus mutation (Dickie 1969). The incidence of \(4.7 \times 10^{-6}\) per generation \([3.29\) for \(a^l\) and \(1.41\) for \(A^w\)] represents one of the highest reverse mutation frequencies documented in the mouse (Schlager and Dickie 1967). This high reversion frequency implies that there may be some unusual structural features associated with the \(a\) mutation.

We and others previously cloned the agouti gene and determined that it contains four exons, the last three of which are coding (Bultman et al. 1992; Miller et al. 1993). The gene produces a 0.8-kb mRNA in \(A/A\) mice that encodes a novel 131-amino-acid protein that includes a putative signal peptide. The \(A\) protein has been hypothesized to be a secreted signaling molecule that functions as an antagonist to the \(\alpha\)-melanocyte-stimulating hormone receptor on the surface of melanocytes (Bultman et al. 1992; Jackson 1993). Because the structure and expression of the agouti gene are altered by the most dominant agouti locus mutation (lethal yellow, \(A^l\)), mutations in the middle of the dominance hierarchy (\(a\) and \(a^l\)), and the most recessive member of the agouti allelic series (extreme nonagouti, \(a^l\)), we proposed that there is only one gene associated with agouti locus function (Bultman et al. 1992).

Here, we report that the \(a, a^l,\) and \(A^w\) alleles are associated with DNA insertions at precisely the same position in the first intron of the agouti gene. A portion of each inserted fragment corresponds to a member of the VL30 family of retrovirus-like transposable elements in the mouse. We describe how homologous recombination events within the integrated sequences of the \(a\) allele are associated with the reversions from \(a\) to \(a^l\) or \(A^w\). Additionally, we have discovered that the agouti gene produces alternatively processed transcripts that are expressed in the \(a^l\) and \(A^w\) alleles but not in the \(A\) allele. We propose that the insertions in \(a, a^l,\) and \(A^w\) confer their unique coat-color phenotypes by selectively inactivating the expression of the different processed forms of agouti mRNA.

### Results

**Structural DNA rearrangements associated with the \(a, a^l,\) and \(A^w\) alleles**

As part of our recent molecular characterization of the agouti gene, we identified DNA insertions within a 700-bp interval in the first intron both in \(a\) and in an \(a^l\) mutation called \(a^l-2Gso\) [formerly referred to as SB B + T; Bultman et al. 1992]. This 700-bp region is defined by EcoRI and BglII restriction sites and is illustrated in Figure 1A. More recently, we determined that a new \(A^w\) mutation, called \(A^w-38f\), also contains a DNA insertion in this same 700-bp interval [see below]. To elucidate the molecular nature of the inserted sequences, overlapping DNA fragments spanning the insertions were cloned from the \(a, a^l-2Gso, \) and \(A^w-38f\) alleles. In all cases, the structure of the mutant fragments was compared with the corresponding cloned wild-type (\(A\)) region (Fig. 1).

These analyses demonstrated that insertions of \(-11, 6,\) and \(0.6\) kb are present in the first intron of the \(a, a^l-2Gso,\) and \(A^w-38f\) alleles, respectively [Fig. 1].

DNA sequence analysis of the cloned fragments revealed that the ends of the inserted DNA are identical in \(a, a^l-2Gso, \) and \(A^w-38f\) and correspond to the 5' and 3' regions of the long terminal repeat (LTR) of a retrovirus-like VL30 element (Figs. 1 and 2). Restriction mapping and DNA sequencing analyses of the inserted regions indicated that \(a\) and \(a^l\) each contain a complete 5.5-kb VL30 element, nearly identical to the element already reported [Adams et al. 1988; Hodgson et al. 1990] (Fig. 1). The VL30 sequences within both \(a\) and \(a^l-2Gso\) each contain an additional DNA segment that is unique to each allele [see below], whereas in \(A^w-38f\) only a single LTR is present [Fig. 1]. The VL30 sequences in all three alleles are integrated in the opposite transcriptional orientation relative to agouti (Figs. 1 and 2).

For both the \(a\) and \(a^l-2Gso\) mutations, sequence analyses indicated that the additional segment of DNA within the VL30 element interrupts the VL30 sequence between nucleotides 2473 and 2474 of the published sequence (Hodgson et al. 1990, Fig. 1). The size of this additional sequence was determined to be 5.5 kb for the \(a\) allele and 526 bp for the \(a^l-2Gso\) allele. The complete nucleotide sequence of the 526-bp insert within the VL30 element of \(a^l-2Gso\) was determined and compared with the sequence of selected regions from the \(a\) allele. This analysis revealed that the extra 5.5-kb insert within the VL30 sequence in \(a\) contains direct terminal repeats of 526 bp in length, each of which corresponds to the single 526-bp insert in the \(a^l-2Gso\) allele [Fig. 1]. Comparison of the sequence of this 526-bp direct repeat with GenBank indicated that it is 90% identical to a region between two T-cell receptor (TCR) \(\gamma\) variable region gene segments, V10.8A and V10.8B, which lie 3.5-kb apart in an opposite transcriptional orientation [Hayday et al. 1985; Garman et al. 1986] (Fig. 3). The functional significance of this homology is presently unclear because the 526-bp region does not correspond to a defined regulatory element or \(cis\)-acting sequence responsible for somatic recombination events that produce functional TCRs. Furthermore, hybridization of the 526-bp region to total mouse DNA revealed that it is repetitive in the mouse genome, suggesting that the 526-bp region is a member of an as yet uncharacterized family of DNA repetitive elements [data not shown]. The nature of the other sequences within the inserted 5.5-kb of DNA in the \(a\) allele is currently under investigation.

Characterization of the sequence flanking the inserted VL30 sequences for the \(a, a^l-2Gso,\) and \(A^w-38f\) alleles...
Figure 1. Identification of DNA insertions in the first intron of the agouti gene in the a, a*, and A" alleles. (A) Schematic representation of the genomic structure of the wild-type agouti gene with the 5' end enlarged immediately below. The four exons are depicted as solid boxes, and introns and flanking sequences are drawn as a solid, horizontal line. The insertion site in a, a*, and A" is indicated by a vertical arrow. The positions of two probes, 1.5 and 0.6, are indicated. (B) BamHI, (R) EcoRI, (BglII). (C) Schematic representation of the 5' end of the agouti gene in the a allele and a*. Insertions of 11 kb in a and 6 kb in a* are represented by rectangles. The wide portion of the rectangles represents VL30 sequence with crosshatched regions corresponding to the LTRs. Numerals refer to nucleotides within VL30 in the 5' → 3' orientation and are based on the published sequence (Hodgson et al. 1990). The narrow portion of the rectangles represents additional sequence integrated between positions 2473 and 2474 of VL30. This additional sequence is −5.5 kb in a and is bound by 526-bp direct repeats (shaded region with arrows above). All of this additional sequence is absent in the a* insertion, except for one of the 526-bp direct repeats (indicated by shaded box). (C) Schematic representation of the 5' end of the agouti gene in the a and A" alleles. All of the 11-kb insertion in a is absent in the A" allele, except for one VL30 LTR (indicated by the hatched box).

showed that the integrated DNA occurs at precisely the same position in each case (Fig. 2). Furthermore, a duplication of four nucleotides of host DNA at the integration site, and the fact that 2 bp are missing from the end of eachLTR, suggests that the integration of VL30 into the agouti gene occurred through the same mechanism that is normally associated with the integration of retroviral sequences [Dhar et al. 1980; Shimotohno et al. 1980; Van Buren et al. 1980; Varmus 1982]. This finding is in accord with VL30 integrations that have been characterized previously [Hodgson et al. 1983; Itin and Keshet 1983].

**Molecular analysis of several new a* and A" reverse mutations**

Based on the analysis of the a*2Gso and A"38I mutations, we hypothesize that each arose by excision of inserted DNA from the a allele through homologous recombination. In the case of a*2Gso, we predict that homologous recombination between the 526-bp direct repeats occurred to remove most of the additional 5.5 kb of inserted sequence, and in A"38I we predict that homologous recombination between the VL30 LTRs removed most of the 11 kb of inserted sequence present in the a allele (Fig. 1). If homologous recombination is playing a role in the reversion from a to a* or A", one would expect several independent a* and A" mutations that arise from a to exhibit the same structural alterations as those observed for a*2Gso and A"38I, respectively. To test this possibility, we analyzed DNA from 21 new mutant animals that arose in the C57BL/6J [a/a] colony at The Jackson Laboratory over the past 2 years. Eleven of these new
Figure 2. Nucleotide sequence of a region of DNA spanning the insertion site in \( a \), \( a' \), \( \alpha^{2-2Gco} \), and \( A^{w-38f} \). Wild-type agouti intron sequence is shown 5'→3' in uppercase letters. The sequences at the ends of the insertions in \( a \), \( a' \), and \( A^{w-38f} \) are identical and correspond to the LTRs of a VL30 provirus, as shown below in lowercase letters. The shaded box highlights a 4-bp duplication of agouti sequence at the VL30 integration site in \( a \), \( a' \), or \( A^{w-38f} \) but are absent in the repeat apposed with nucleotide 2473 of VL30 in \( a' \).

Figure 3. Nucleotide sequence comparison between the 526-bp direct terminal repeats in the \( a \) and \( a' \) insertions (shaded region of the narrow rectangles in \( a \) and \( a' \) in Fig. 1, top) and part of an intergenic regulatory region between two TCR \( \gamma \) variable gene segments (bottom). The sequence is presented in the same orientation as the arrows shown above the 526-bp repeats in Fig. 1B. The three capitalized nucleotides at the 5' end of the 526-bp repeat are present in \( a' \) and in the repeat apposed with nucleotide 2474 of VL30 in \( a' \) but are absent in the repeat apposed with nucleotide 2473 of VL30 in \( a \).

Table 1. New \( A^{w} \) and \( a' \) mutations analyzed by Southern analysis

| Remutation number | Strain of origin |
|-------------------|------------------|
| \( A^{w-34f} \)   | C57BL/6J         |
| \( A^{w-35f} \)   | C57BL/6J         |
| \( A^{w-36f} \)   | C57BL/6J         |
| \( A^{w-37f} \)   | C57BL/6J         |
| \( A^{w-38f} \)   | C57BL/6J         |
| \( A^{w-39f} \)   | C57BL/6J[N-Sp]   |
| \( A^{w-40f} \)   | C57BL/6J         |
| \( A^{w-41f} \)   | C57BL/Ks-\( dbm \) |
| \( A^{w-42f} \)   | C57BL/6J         |
| \( A^{w-43f} \)   | C57BL/6J         |
| \( a'^{46f} \)    | C57BL/6J         |
| \( a'^{47f} \)    | C57BL/Ks-\( dbm \) |
| \( a'^{48f} \)    | C57BL/6J         |
| \( a'^{49f} \)    | C57BL/6J         |
| \( a'^{50f} \)    | C57BL/6J         |
| \( a'^{51f} \)    | C57BL/6J         |
| \( a'^{52f} \)    | C57BL/6J         |
| \( a'^{53f} \)    | C57BL/6J         |
| \( a'^{54f} \)    | C57BL/6J         |
| \( a'^{55f} \)    | C57BL/6J         |
| \( a'^{56f} \)    | C57BL/6J × DBA/2J |
to the DNA insertions [see probe 1.5 in Fig. 1; data not shown]. Based on these data, we propose that all of the new reverse mutations contain the same DNA structural alterations that are present in the \(a^{2Gso}\) and \(A^{w-38f}\) mutations. Furthermore, because we analyzed each of the “original” mutants we were able to determine that the \(a^*\) reversions arose through intrachromosomal recombination. If the reversions had occurred through interchromosomal recombination, then we would have detected a size-altered restriction fragment associated with the reciprocal recombination product on the other chromosome, and we did not [data not shown]. Because of the relatively large size of the DNA segment that is excised from \(a\) in \(A^{w}\) revertants, we have been unable to identify a diagnostic restriction enzyme necessary to perform a similar analysis for \(A^{w}\).

**Additional forms of agouti transcripts**

As part of our recent molecular analysis of the lethal-yellow (\(A^y\)) mutation, we determined that \(A^y\) produces chimeric transcripts in which the first noncoding exon of agouti is replaced by the noncoding first exon of a closely linked gene called \(Raly\) [hnRNP associated with lethal yellow; Michaud et al. 1993]. During the course of this analysis, a number of \(A^y\) cDNA clones were isolated that contained differentially spliced segments, 111 and 46 bp in length, integrated between the \(Raly\) and agouti exons [Michaud et al. 1993]. These segments have been mapped to a region immediately 3’ to a deletion breakpoint associated with the \(A^y\) mutation, which positions them ~110 kb upstream from the first coding exon of agouti [Michaud et al. 1994]. We initially attributed these segments to cryptic splicing events associated with the \(A^y\) allele but could not rule out the possibility that the 111- and 46-bp segments actually represent additional 5’ exons of the agouti gene.

Here, we demonstrate that the 111- and 46-bp segments are differentially incorporated into agouti-specific transcripts that are structurally different from the agouti mRNA originally described [Bultman et al. 1992; Miller et al. 1993]. The RNAs that incorporate the 111- and/or 46-bp segments, here referred to as form II transcripts, were detected with a reverse transcriptase–polymerase chain reaction (RT–PCR) assay using an oligonucleotide primer from the 111-bp region [primer 1] coupled with a primer from the third agouti exon [primer 3; Fig. 6A]. These primers produced PCR products of 380 and 334 bp in size, depending on the presence or absence of the 46-bp exon in skin from the ventrum, but not the dorsum, of 4- and 5- day-old \(A^{w}/a\), \(a^y/a^y\), and \(A^{w}/A^{w}\) mice (Fig. 6B).

Both of the form II transcripts are also present in \(A^y/a\) neonatal skin (and in adult tissues, see Michaud et al. 1993), which was included as a positive control. In contrast, neither of the form II transcripts could be detected as ethidium-stained RT–PCR fragments in the ventrum or the dorsum of age-matched neonatal \(A^y/A^y\) or \(a/a\) mice (Fig. 6B), which explains why these forms of agouti mRNA were not detected originally [Bultman et al. 1992].

Additionally, a similar RT–PCR assay was utilized to detect the presence of agouti transcripts that incorporate the 5’ noncoding exon that we originally identified as the first agouti exon [Bultman et al. 1992]. This form of agouti mRNA, here referred to as the form I transcript, was detected with a primer from the originally defined noncoding first exon [primer 2] coupled with same third exon primer as above [primer 3]. With these primers, form I mRNA produced a single 315-bp PCR product of the expected size (Fig. 6A). Form I transcripts were detected in both the dorsum and the ventrum of 4- and 5- day-old \(A^y/A^y\), \(A^w/a\), and \(A^{w}/A^{w}\) mice, but not in the dorsum or the ventrum of age-matched \(a/a\) or \(a^y/a^y\) mice (Fig. 6C).

Overall, these RT–PCR analyses demonstrate that \(A/A\) mice produce form I transcripts in both their dorsal and ventral skin but do not produce form II transcripts. Conversely, \(a^y/a^y\) animals do not express form I transcripts and express form II transcripts but only in ventral skin and not in dorsal skin. Animals carrying the \(A^{w}\) allele [derived either from \(A^{w-42}\) or \(101\)] express both the form I and the ventral-specific form II transcripts, and \(a/a\) animals do not express detectable levels of either the form I or the form II transcripts (Table 2).

**Discussion**

**DNA insertions in \(a^y\), \(a^{2Gso}\), and \(A^{w-38f}\)**

As part of our recent molecular characterization of the agouti gene, we determined that the \(a\) and \(a^{2Gso}\) mutations each contain an extra fragment of DNA within an intervening sequence of the gene [Bultman et al. 1992]. More recently, we determined that a new \(A^{w}\) mutation, called \(A^{w-38f}\), also contains an extra fragment of DNA within this same region. Based on the analysis of genomic clones that span these inserted sequences, we now report that the insertions are 11, 6, and 0.6 kb in \(a\), \(a^{2Gso}\), and \(A^{w-38f}\), respectively, and that they are all integrated at precisely the same position. The 11-kb inser-
tion in $a$ includes an entire 5.5-kb VL30 element plus an internal 5.5 kb of additional sequence flanked by direct terminal repeats of 526 bp. The 6-kb insertion in $a^{vl30}$ also includes an entire VL30 element but contains only one 526-bp direct repeat internally. The 0.6-kb insertion in $A^{w-387}$ corresponds to a single, intact VL30 LTR.

VL30 elements are retrovirus-like structures, ~5.5 kb in size, that are dispersed throughout rodent genomes at ~100–200 copies (Besmer et al. 1979; Keshet et al. 1980; Keshet and Itin 1982). Although VL30 elements have not been shown to encode any polypeptides and are replication-defective, RNA copies of their genome are capable
of being copackaged by endogenous murine leukemia viruses. They can be retrotransposed when the pseudovirus infects new cells and the VL30 RNA undergoes reverse transcription and integrates into the host cell genome (Scolnick et al. 1979; Carter et al. 1986). Because retrovirus-like elements, particularly intracisternal A-particles, have been shown to affect the expression of numerous genes, even when integrated in the opposite transcriptional orientation (Kuff and Lueders 1988), we propose that the inserted VL30 elements in the a and a' alleles, brought about by homologous recombination between the VL30 LTRs. This model is supported by the expression data presented here. The fact that A/a' mice have a phenotype that is identical to that of A' [Dunn 1928; Silvers 1979] is consistent with the expression data presented here. A' produces both form I and form II transcripts, whereas A produces only the form I transcript and a' produces only form II transcripts. A'/a' mice would be expected to produce both forms of transcripts, and as a result exhibit a phenotype identical to that of A'.

From our analyses, form I and II agouti transcripts differ only in their S' noncoding regions and apparently encode the same protein. Additional analysis of the 111-bp segment has revealed that it may be comprised of two separate exons [Michaud et al. 1994], neither of which contains a termination codon [Michaud et al. 1993]. For this reason, we cannot exclude the possibility that the shorter form II mRNA that contains only the 111-bp segment actually has a 5' extension of the agouti open read-

Table 2. Dorsal (D) and ventral (V) distribution of form I and II transcripts in neonatal skin from mice with different agouti genotypes

| Genotype | Form I | Form II |
|----------|--------|---------|
| A/A      | D, V   | —       |
| A*/A     | D, V   | V       |
| a'/a'    | —      | V       |
| a/a      | —      | —       |

Multiple forms of agouti mRNA

We demonstrated previously that the agouti gene produces a 0.8-kb mRNA in neonatal skin and that the abundance of this mRNA correlates well with the presence of phaeomelanin (Bultman et al. 1992; Michaud et al. 1993). However, RT-PCR experiments presented here demonstrate clearly that instead of producing a single species of mRNA, the agouti gene produces a number of similarly sized transcripts that are different at their 5' ends. One class of transcript, referred to here as form I, consists of sequence derived from the first, second, third, and fourth agouti exons as described originally [Bultman et al. 1992; Miller et al. 1993]. This form I transcript is expressed in skin from both the ventrum and the dorsum of 4- and 5-day-old A/A, A*/a, and A*/A mice but neither in the ventrum nor the dorsum of age-matched a/a or a'/a' mice (Table 2). Furthermore, expression of the form I transcript is regulated during early postnatal development such that it is produced while melanocytes are actively depositing phaeomelanin into agouti hair follicles between days 3 and 7 (Galbraith 1964; Sakurai et al. 1975) but is absent both before and after this interval [data not shown]. A second class of transcripts detected by RT-PCR, referred to here as form II, consists of differentially incorporated 111- and 46-bp segments spliced to the agouti coding exons. Both of these transcripts are expressed in skin from the ventrum, but not the dorsum, of 4- and 5-day-old A*/a, a'/a', and A*/A mice. In contrast, form II transcripts are not detected in the ventrum or the dorsum of age-matched A/A or a/a mice [Table 2], this is the reason why these different forms of agouti mRNA were not identified originally in the analysis of the A allele, which was presumed to be wild type [Bultman et al. 1992]. Furthermore, the form II transcripts are expressed throughout the hair-growth process in the ventrum of A*/A mice (data not shown). Taken together, these findings provide compelling evidence that the form I transcript is associated with the production of the subapical band of phaeomelanin in agouti hair throughout the pelage and that the form II transcripts are responsible for the predominating yellow pigmentation over the ventral surface in the A' and a' alleles.
ing frame. Also, because the 111- and 46-bp segments each contain a functional splice acceptor at its 5' junction [Michaud et al. 1993, unpubl.], we predict that there must be at least one more exon upstream of the 111-bp segment that is normally at the 5' end of form II transcripts. These matters are currently under investigation.

Insertions in a and a' modulate the expression of form I and form II agouti transcripts

Comparison of the structure of the a, a', and A^w alleles suggests a model that explains how each insertion alters agouti gene expression. We propose that the VL30 element, along with the 5.5 kb of additional sequence, essentially shuts off agouti gene expression in the a mutation by negatively regulating the production of both form I and form II mRNA. This is presumably the case throughout the pelage, except for very restricted areas near the pinnae, mammae, and perineum, where pheomelanin deposition still occurs. It is likely that any agouti transcripts produced from the mammae in the animals with this mutation are not expressed. This is of particular interest because both A and A^w have traditionally been referred to as wild-type agouti alleles. The proposal that A^w is the true wild-type agouti allele is not only compatible with the molecular data presented here but is also consistent with reports that most populations of wild mice have a white-bellied agouti phenotype (Silvers 1979).

Materials and methods

Animals

All of the new a' and A^w mutants reported here arose spontaneously in the production colony at The Jackson Laboratory (see Table 1), except for a^-3G[CC] (formerly referred to as SB B+7T), which arose spontaneously at the Oak Ridge National Laboratory in a cross between strains SEC/E [a/a, b/b, c/b^/c^] and C57BL/E [a/a]. All animals were subsequently maintained at the Oak Ridge National Laboratory.

Cloning overlapping genomic DNA fragments that span the insertions in a, a', and A^w

A total of 250 µg of genomic liver DNA from wild-type A/A (C57BL/E), a/a (C57BL/E), or a'/a' [a'-5G[CC]] stock mice were digested with either BamHI or BglII, and were size fractionated on a 10-40% sucrose gradient (Sambrook et al. 1989). Fractions containing appropriately sized fragments (see below) were ligated into the EMBl3 vector, packaged in vitro and screened using standard procedures (Ausubel et al. 1988; Sambrook et al. 1989). Utilizing a fragment that includes the first agouti exon plus some 5'-flanking sequence and part of the first intron (probe 1.5, Fig. 1), BamHI fragments of 8, 16, and 11 kb were cloned from A/A, a/a, and a'/a' DNAs, respectively. Utilizing a 0.6-kb BamHI–PstI fragment in the first intron of agouti (probe 0.6, Fig. 1), BglII fragments of 2, 6, and 7 kb were cloned from A/A, a/a, and a'/a' DNAs, respectively. These fragments were subsequently subcloned into the pGEM (Promega) or pBluescript (Stratagene) vectors and purified as described [Ausubel et al. 1988; Sambrook et al. 1989]. For A^w, a total of 250 µg of genomic liver DNA was prepared from the original A^w-3G[CC] mutant, partially digested with Sau3A, and size fractionated on a 10–40% sucrose gradient (Sambrook et al. 1989). Fractions containing 15- to 20-kb fragments were ligated into the EMBl3 vector, packaged in vitro, and screened using standard procedures [Ausubel et al. 1988; Sambrook et al. 1989]. Clones that hybridized to probes 1.5 and 0.6 (see Fig. 1) were purified, and subfragments were subsequently isolated and subcloned into pGEM (Promega) or pBluescript (Stratagene) for further analysis.

DNA sequencing

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

Southern blot analysis

Genomic adult liver or tail DNA (10 µg) was digested with restriction enzymes, electrophoresed through 0.8% agarose
gels, and blotted to GeneScreen [DuPont] with standard procedures [Ausubel et al. 1988; Sambrook et al. 1989]. Radionucleotide hybridization probes were prepared with the random hexamer labeling technique [Feinberg and Vogelstein 1984], and posthybridization filter washing was conducted under high stringency conditions [0.2× SSC, 0.1% SDS, at 68°C].

RT–PCR analysis

Total RNA (10 µg) prepared by the guanidine isothiocyanate procedure [Ausubel et al. 1988], was reverse transcribed [Kawasaki 1990], ethanol precipitated, and resuspended in 20 µl of H2O. PCR was performed using 4 µl of the sample as described previously [Pieretti et al. 1991]. The sequences of the oligonucleotide primers in Fig. 6 are as follows: Primer 1, 5'-CAATTGCATCGAAGTACCA; primer 2, 5'-CACTCGTCCGCTCCTTGCTCCAGAAGT. The β-actin primers were 5'-ATGGGTCAGAAGGACTCTA (forward) and 5'-CAACATAGCACAGCTTCTCT (reverse). PCR products were electrophoresed through 3.0% agarose gels, transferred to GeneScreen Plus [DuPont], and hybridized to an agouti cDNA probe as described above.

Note added in proof

The sequence data described in this paper have been submitted to the GenBank data library under accession number L28700.

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