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173

Molecular characterization of a region of DNA associated with mutations at the agouti locus in the mouse

(radiation mutagenesis/molecular analysis of mutations)

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ABSTRACT **Molecular characterization of a radiation-induced agouti (*a*)-locus mutation has resulted in the isolation of a segment of DNA that maps at or near the *a* locus on chromosome 2 in the mouse. This region of DNA is deleted in several radiation- or chemical-induced homozygous-lethal *a*-locus mutations and is associated with specific DNA structural alterations in two viable *a*-locus mutations. We propose that DNA probes from this region of chromosome 2 will be useful for ultimately characterizing the individual gene or genes associated with *a*-locus function.**

The agouti (*a*) locus in chromosome 2 of the mouse controls the production of two distinct types of pigment, phaemelanin (yellow) and eumelanin (black or brown, depending on alleles present at other loci), by the melanocytes within the hair follicle. Under control of the *a* locus, individual hairs may contain only eumelanin, only phaeomelanin, or a subapical band of phaeomelanin in an otherwise eumelanotic hair (agouti type). The *a* locus also regulates the distribution of phaeomelanotic, eumelanotic, or agouti hair over the surface of the animal; certain genotypes show a marked variation in dorso-ventral pigmentation, whereas others exhibit differential pigment patterns in various regions, including the perineum, pinnae, nipples, flanks, and tail. Interestingly, the regulatory signal produced by the *a* locus to control pigmentation does not originate within the melanocytes themselves but is transmitted from the follicular environment within which the melanocytes reside (1–5).

To date, 18 dominant and recessive alleles and pseudoalleles have been described for the *a* locus (6), and numerous other alleles, some associated with chromosome rearrangements, have been recovered in mutagenesis studies (refs. 7 and 8; L.B.R., unpublished). Genetic data can be interpreted as evidence for a complex, multiallelic gene that is regulated differently in each body region or as evidence for the existence of several closely linked genes that work in concert (1). A^y, lethal-yellow, described in 1905 (and the first lethal gene ever reported in a mammal) (9), has been shown to recombine with a number of *a*-locus alleles—namely, a^e (lethal nonagouti) (10), a^j (black-and-tan) (12), and a (nonagouti) (12)—the recombination frequency in each case being of the order of 0.1%, with A^y lying proximal to a^e (10, 12). One crossover product in each case (the presumed double wild type) was indistinguishable from A; the other product (presumed double mutant) has not been identified. The finding that among spontaneous *a*-locus mutations, certain ones (specifically, a to a^j and a to A^w) are common, whereas others are quite rare, also suggests that "*a*-locus alleles" may involve a complex of genetic sites, some of which, as is the case for A^y, are separable by recombination.

Although considerable effort has been expended in investigating the genetics and biology of the *a*-locus region, the structure and expression of the gene(s) remain unknown. The endogenous ecotropic murine leukemia virus *Emv-15* has been localized very close to A^y, and probes flanking *Emv-15* have been used for physical mapping experiments (12–19). However, the results of certain presumed spontaneous crossover events indicate that *Emv-15* does not lie between A^y and a (20).

We have earlier reported the derivation of a DNA probe associated with a radiation-induced *a*-locus mutation (7). Here we describe how we have used this DNA probe to detect genomic alterations associated with several mutations that express the a^j phenotype (a^j, jet-black, is probably equivalent to a^e, extreme nonagouti). The data we present support the hypothesis that the region represented by the probe is directly associated with the *a* locus and that cloned segments of DNA from this region will be useful for the ultimate characterization of the gene(s) associated with *a*-locus function.

MATERIALS AND METHODS

Animals. All mice were bred at our laboratory in stocks propagating induced mutations. Five of the mutations were induced at Oak Ridge (ref. 7; L.B.R., W. L. Russell, and P. A. Hunsicker, unpublished); one was induced at Harwell (21).

Southern Blotting. Ten micrograms of genomic adult liver or tail DNA was digested with restriction enzymes, electrophoresed through agarose gels, and blotted to GeneScreen (DuPont) with standard procedures (22, 23). Radiolabeled hybridization probes were prepared with the random-hexamer labeling technique (24).

Cloning of a 5.5-Kilobase (kb) Wild-Type Fragment from a Region Corresponding to the Distal Inversion Breakpoint of Is1Gso. A total of 250 μg of genomic adult liver DNA from an Is1Gso heterozygote (7) was digested to completion with *Eco*RI and size-fractionated on a 10–40% sucrose gradient (23). An EMBL4 phage library, prepared with fractions containing 5- to 6-kb fragments, was screened with standard procedures utilizing a 0.2-kb *Pvu* II fragment from the hatched region of the 7.5-kb *Eco*RI rearranged fragment corresponding to probe A in Fig. 1*A* (7, 22, 23). The 5.5-kb *Eco*RI fragment was then subcloned into the pGEM4 vector (Promega), restriction mapped, and partially sequenced as described (22, 23).

Cloning a Rearranged Fragment Associated with the a^{5MNU} Mutation. A total of 500 μg of genomic tail DNA from an a^{5MNU}/a^{5MNU} homozygote was partially digested with *Sau*3A and size-fractionated on a 10–40% sucrose gradient (23). An

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EMBL3 phage library was prepared with fractions containing 15- to 20-kb fragments and was screened by standard procedures (22, 23) utilizing a 1.0-kb *Eco*RI–*Xba* I fragment from the distal end of the 5.5-kb *Eco*RI fragment as a probe (see probe B, Fig. 1*A*). A rearranged 3.5-kb *Eco*RI fragment was subcloned into pGEM4 (Promega), restriction mapped, and partially sequenced as described (22, 23). Oligonucleotide primers were synthesized to facilitate the sequencing of the deletion breakpoint region.

Cloning a Large Region of DNA at or Near the Agouti Locus. Genomic spleen DNA from the strain 129/R1 was partially digested with *Sau*3A and size-fractionated on a 10–40% sucrose gradient (23). Fractions containing 35- to 45-kb fragments were ligated into the cosmid vector c2RB (25), packaged *in vitro*, and screened by standard procedures using a 0.2-kb *Pvu* II fragment from the hatched region of the 7.5-kb *Eco*RI rearranged fragment corresponding to probe A in Fig. 1*A*.

RESULTS

Identification of a DNA Probe Deleted in Several Radiation- or Chemical-Induced *a*-Locus Mutations. We previously described the genetic, cytogenetic, and molecular characterization of a radiation-induced mutation that simultaneously gave rise to new alleles at the limb deformity (*ld*) and *a* loci, two regions normally separated by 20 centimorgans on mouse chromosome 2 (7). This mutation, Is(17;In2)*ld*,a^{J}1Gso (abbreviated Is1Gso), contains an inversion of chromosome 2 that involves a segment lying between the limb deformity (*ld*) locus and a site that maps at or near the *a* locus (7). At the proximal end of the inversion, the breakpoint in the DNA caused a physical disruption and alteration in the expression of the gene giving rise to the formin family of mRNAs. The disruption of this gene appears to be associated with the limb-deformity defect in this and the ld^{Hd} mutant (7, 26). Since the extreme-nonagouti phenotype arose simultaneously with the limb-defect phenotype in Is1Gso, we reasoned that it was possible that the DNA breakpoint at the distal end of the inversion had caused a physical disruption and alteration in the expression of a gene directly associated with the nonagouti trait in this mutant.

To begin to test the possibility that the distal inversion breakpoint in Is1Gso is associated with a gene at the *a* locus, we utilized a DNA probe (probe A in Fig. 1*A*), consisting of a sequence adjacent to the proximal DNA breakpoint (hatched segment of the 7.5-kb fragment in Fig. 1*A*), to determine whether this region of the genome is altered in four radiation- or chemical-induced agouti mutations. We showed previously that the probe used in this experiment maps at or near the *a* locus (7). These four mutations, a^{Jl10}, a^{Jl41}, a^{Jl85}, and a^{9H}, all express the a^{J} (jet-black; probably equivalent to a^{e}, extreme nonagouti) phenotype and are lethal in the homozygous condition (Table 1). Viable compound heterozygotes were produced for this experiment by balancing each of these alleles with Is1Gso. One DNA breakpoint of Is1Gso is near the segment that was used as a probe, so the Is1Gso allele provided us with an *Eco*RI restriction fragment length variant for this region of DNA; the Is1Gso allele is associated with a 7.5-kb *Eco*RI fragment, whereas the wild-type allele appears as a 5.5-kb *Eco*RI fragment. Therefore, this restriction fragment length variant allowed us readily to determine whether the region identified by the probe was deleted in these agent-induced mutations.

Analysis of *Eco*RI-digested DNA from the above Is1Gso/a^{Jl} compound heterozygotes, as expected, revealed that each contained the 7.5-kb Is1Gso-specific fragment; none of the samples yielded a 5.5-kb fragment or any other detectable fragment associated with the agent-induced allele (Fig. 1*B*, lanes A–D). To rule out the unlikely possibility that the agent-induced allele in each of the four mutations appears as a 7.5-kb fragment that comigrates with the Is1Gso-specific fragment, we analyzed DNA from $+/a^{Jl}$ heterozygotes, which contain a 5.5-kb fragment associated with the wild-type chromosome (Fig. 1*B*, lanes H–L). No fragments of a length other than 5.5 kb were detectable. These data clearly indicate that all four of these *a*-locus mutations contain a deletion in the region corresponding to the distal inversion breakpoint in Is1Gso and support the hypothesis that this section of chromosome 2 contains at least one gene associated with the *a* locus.

Characterization of the Structure of the Distal Inversion Breakpoint in Is1Gso. The above data indicate that large radiation- and chemical-induced deletion mutations are useful for initially mapping DNA probes to the general vicinity of a particular gene on the genome. However, such mutations are often associated with the deletion of hundreds, thousands, or perhaps even millions of base pairs of DNA sequence and, therefore, may be deficient in multiple genes, not all of which are directly related to the phenotype of

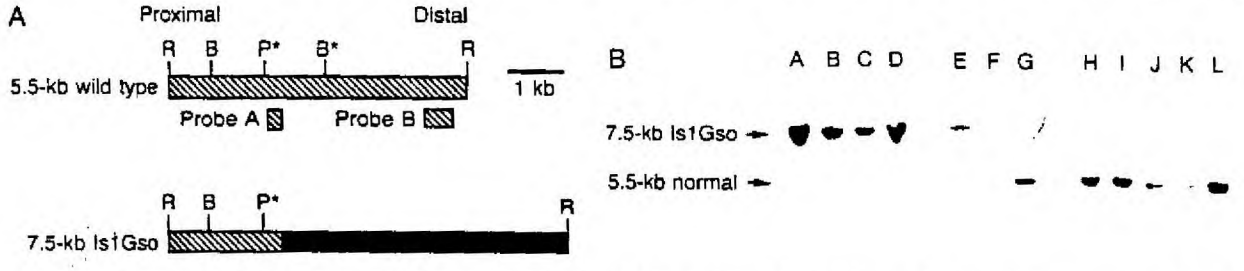

FIG. 1. (*A*) Structure of a 7.5-kb rearranged fragment from the Is1Gso radiation-induced mutation containing the proximal inversion breakpoint and the structure of a 5.5-kb wild-type fragment mapping at or near the *a* locus. The 7.5-kb fragment contains a portion of the *ld* locus (filled box) juxtaposed with a section of DNA (hatched) that we previously mapped to a region at or near the *a* locus (7). The 5.5-kb wild-type fragment was cloned utilizing a probe from the hatched region of the 7.5-kb fragment (region corresponding to probe A). Comparison of the two cloned fragments revealed that the inversion DNA breakpoint on the 7.5-kb fragment was ≈1.9 kb from the proximal end of the 5.5-kb fragment. B, *Bam*HI; P, *Pst* I; R, *Eco*RI. Probes A and B correspond to proximal and distal regions of the 5.5-kb fragment and are represented by hatched boxes. (*B*) Southern blot of *Eco*RI-digested genomic DNA from several radiation- or chemical-induced extreme-nonagouti mutations hybridized with probe A from the 5.5-kb fragment (see *A*). Individual mutations are described in Table 1. Lanes A and H, a^{Jl10}; lanes B and I, a^{Jl41}; lanes C and J, a^{Jl85}; lanes D and K, a^{9H}. The four individual radiation- or chemical-induced mutations were analyzed as compound heterozygotes balanced with Is1Gso (lanes A–D) or heterozygotes balanced with either a wild-type chromosome (lanes H–J) or the a^{16H} allele (21), which contains a normal-size 5.5-kb fragment in this region of DNA (lane K). (The a^{9H} mutation was balanced with a^{16H} in lane L to verify that the a^{16H} mutation contains a 5.5-kb fragment in this region.) Lanes E–G, homozygous, heterozygous, and wild-type DNA, respectively, from the Is1Gso mutant line. Hybridizing fragments corresponding to the Is1Gso-specific fragment, or to a wild-type-size 5.5-kb-specific fragment, are indicated.

Table 1. Radiation- and chemical-induced agouti-locus mutations that exhibit detectable structural alterations

Mutation (a^*)	Mutagen	Germ-cell type†	Phenotype		Structural alteration	Source or ref.
			a^*/a^*	a^*/a^{j}‡		
a^{j110}	X-rays	Oocyte	Lethal	Jet	Deletion	§
a^{j141}	Ethyl methanesulfonate	Spermatozoa	Lethal	Jet	Deletion	§
a^{j185}	γ Rays	Oocyte	Lethal	Jet	Deletion	§
a^{9H}¶	X-rays	Oocyte	Lethal	Jet	Deletion	21
Is1Gso	γ Rays	Spermatogonia	Jet	Jet	Inversion	7
a^{5MNU}	Methylnitrosourea	Spermatogonia	Jet	Jet	Deletion	‖

*Radiation- or chemical-induced mutant agouti allele.
†Specific germ-cell stage exposed to mutagen.
‡a^{j}, Jet black, the most recessive *a*-locus allele and probably equivalent to a^{e}, extreme nonagouti.
§L.B.R., unpublished results.
¶a^{9H} is the Harwell designation for a^{j} (11).
‖W. L. Russell and P. R. Hunsicker, unpublished results.

interest (27). Viable mutations, on the other hand, are more likely to be intragenic changes or smaller deletions that are considerably more useful for locating individual genes because any observed structural alteration is more likely to affect only the expression of the gene directly associated with the phenotype under consideration. For this reason, we subsequently focused our attention on the analysis of viable nonagouti mutations in an attempt to identify a gene associated with the function of the *a* locus.

Since the Is1Gso mutation is a viable allele at the *a* locus, we continued our analysis of this mutation, concentrating on the DNA structural alteration at the distal inversion DNA breakpoint. As described above, the initial hypothesis was that the distal inversion breakpoint itself caused a disruption of the expression of a gene directly associated with the function of the *a* locus. It was also possible that during the process of formation of the chromosomal structural alterations in Is1Gso, a modest-sized segment of DNA had been deleted from the distal breakpoint region on chromosome 2. (It was unlikely that a large deletion had occurred since the mutation is viable.) To begin to test this possibility, we cloned and characterized the wild-type 5.5-kb *Eco*RI fragment that hybridized with the probe used in the experiment described in Fig. 1. One end of this wild-type 5.5-kb fragment contains all of the 1.9 kb of sequence located adjacent to the proximal inversion breakpoint site in Is1Gso (corresponding to the hatched box at the end of the 7.5-kb rearranged fragment in Fig. 1*A*), whereas the other end of the fragment contains the remaining 3.6 kb of sequence. A probe prepared from the distal end of the remaining 3.6 kb of sequence (probe B, Fig. 1*A*) detected mutant allele-specific restriction fragment length variants in *Eco*RI- and *Pst* I-, but not *Bam*HI-, digested genomic DNA of wild-type, heterozygous, and homozygous animals from the Is1Gso line (Fig. 2). The Is1Gso restriction fragment length variant detected with this probe occurs at a position within a small interval between a *Pst* I and a *Bam*HI site on the 5.5-kb fragment (indicated with asterisks in Fig. 1*A*). Since this small interval occurs at approximately the same position as the structural alteration site detected on the 7.5-kb fragment—i.e., 1.9 kb from the proximal end of the 5.5-kb fragment—it appears that no significant deletion had occurred at the distal inversion breakpoint site of Is1Gso. In fact, in additional experiments to be reported elsewhere, we determined that only 29 base pairs (bp) of sequence were deleted from the distal inversion breakpoint site. No additional structural alterations were detected in this region of the mutant allele. This result supports our prediction that a gene associated with the function of the *a* locus has been disrupted by the breakage of the DNA at the distal inversion site of Is1Gso.

Characterization of a Structural Alteration in the a^{5MNU} Viable *a*-Locus Mutation. Major structural alterations like Is1Gso could exert position effects on genes that are not located at the actual DNA structural alteration site. Therefore, although we have demonstrated that no additional major structural alteration like a large deletion had occurred in the region near the distal DNA inversion breakpoint of Is1Gso, it was possible that the expression of the gene or genes at the *a* locus might be affected from a distance and that the *a* locus might not map within the immediate vicinity of the breakpoint region. For this reason, we examined other viable *a*-locus mutations with the DNA probes derived from the Is1Gso distal-breakpoint region.

One viable mutation, a^{5MNU}, arose in a specific-locus experiment (W. L. Russell, unpublished results; ref. 8) 6 weeks after methylnitrosourea exposure of differentiating (101 × C3H)F_1 spermatogonia (Table 1). DNA from an a^{5MNU}/a^{5MNU} homozygote, along with DNA from the C3H and 101 parental types, was digested with *Eco*RI, *Pst* I, and *Bam*HI. The samples were then hybridized with probe A or B, derived from either the proximal or distal end of the normal 5.5-kb fragment mapping at or near the *a* locus (Fig. 1*A*). Although the distal probe B hybridized to an identical *Bam*HI fragment in mutant and wild-type DNA, nonparental fragments were detected in the *Eco*RI- and *Pst* I-digested mutant samples (Fig. 3). Also, a deletion of the region of DNA corresponding to the proximal probe (probe A) had occurred in this mutant (Fig. 3). This result indicated that a DNA structural rearrangement, which included a deletion of the proximal end of the normal 5.5-kb *Eco*RI fragment, had occurred between the *Pst* I and distal *Bam*HI sites on the 5.5-kb fragment in a^{5MNU} (sites shown with asterisk in Fig. 1*A*).

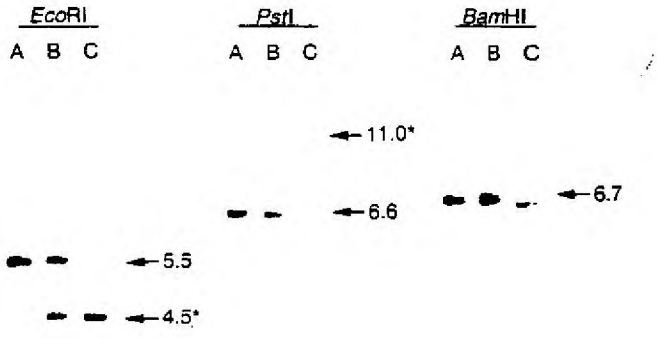

FIG. 2. Southern blot of wild-type (lanes A), heterozygous (lanes B), or homozygous (lanes C) adult genomic DNA from the Is1Gso mutant line. Each sample was digested with either *Eco*RI, *Pst* I, or *Bam*HI and then hybridized with probe B (Fig. 1*A*). Numbers refer to sizes of DNA fragments in kb; asterisks denote rearranged Is1Gso fragments.

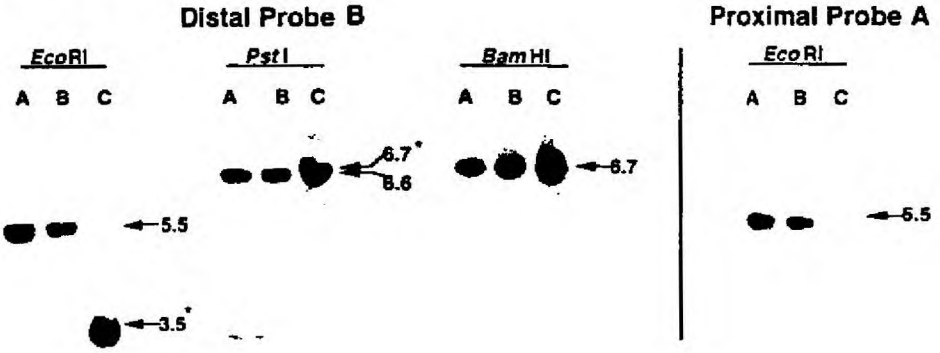

FIG. 3. Southern blot of C3H (lanes A), 101 (lanes B), or homozygous a^{5MNU} (lanes C) adult genomic DNA digested with *Eco*RI, *Pst* I, or *Bam*HI. Each sample was hybridized with either probe A or probe B from the proximal and distal ends of the cloned 5.5-kb normal fragment (Fig. 1*A*). Numbers refer to the sizes of the DNA fragments; asterisks denote rearranged a^{5MNU} fragments identified with the distal probe.

To characterize the nature of the structural alteration in the a^{5MNU} allele, the mutant 3.5-kb *Eco*RI fragment detected in the above analysis was cloned and compared with a large section of DNA derived from overlapping cosmid genomic clones from this region of the genome (Fig. 4*A*). DNA sequence analysis of the structurally altered region demonstrated that a small segment of DNA, estimated to be 2.8 kb in length, had been deleted from the a^{5MNU} mutant allele (Fig. 4*B*). No other major structural alterations were detected in the a^{5MNU} mutant allele within several kilobases of this deletion (S.J.B. and R.P.W., unpublished results). Interestingly, the distal deletion breakpoint site of the a^{5MNU} occurs only 129 bp away from the site of the distal inversion breakpoint on the Is1Gso mutation (Fig. 4*A*).

DISCUSSION

Characterization of the molecular structure of the *a* locus has been the focus of a considerable amount of research by many investigators over the past several years (12–20). We have utilized a DNA probe derived from a radiation-induced extreme-nonagouti mutation to characterize a region of DNA that has undergone structural changes in several *a*-locus mutations. Although several of these mutations are homozygous lethals and are deletions that may be very large, we did identify specific DNA sequence alterations in two viable mutations, Is1Gso and a^{5MNU}. We hypothesize that the sequence alterations in these viable mutations will likely define the position of at least one gene associated with *a*-locus function.

Interestingly, the Is1Gso and a^{5MNU} mutations contain a structural alteration at sites very close to each other in the DNA: the distal inversion breakpoint in Is1Gso is located within the 2.8 kb of DNA deleted in the a^{5MNU} allele, 129 bp from the distal a^{5MNU} deletion breakpoint. The fact that this region is deleted in several lethal mutations and has undergone a structural alteration at almost the same site in two viable mutations provides compelling evidence that this region contains at least a portion of the *a*-locus gene(s).

Molecular characterization of the agouti region should ultimately reveal whether the function of the *a* locus is associated with a single, large gene that undergoes complex regulation or whether there are multiple, linked genes that

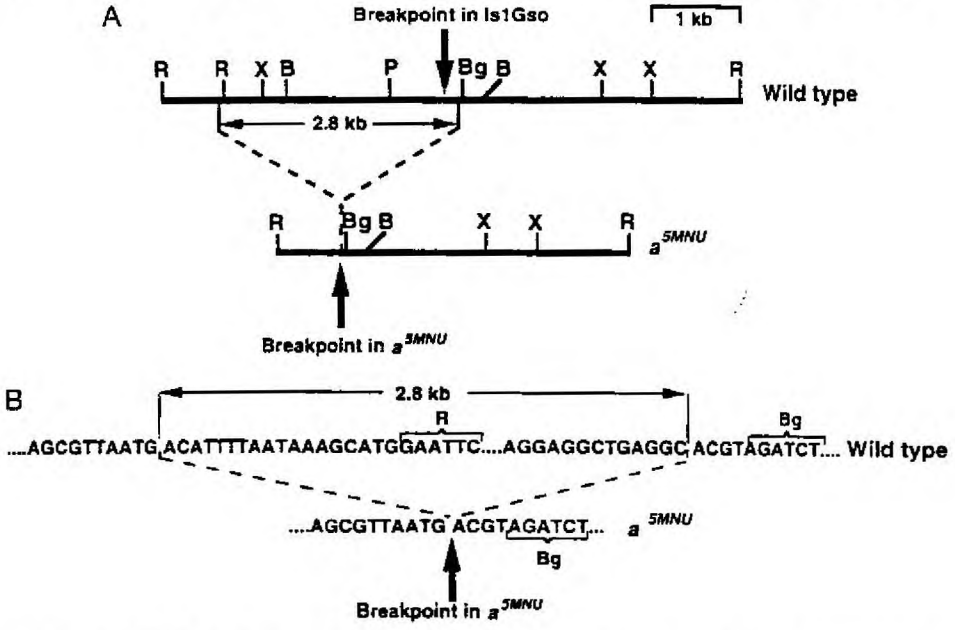

FIG. 4. (*A*) Structure of the 3.5-kb *Eco*RI fragment cloned from the a^{5MNU} mutation compared with the corresponding structure of a cloned segment of wild-type DNA. The presence of a 2.8-kb deletion in the 3.5-kb a^{5MNU} fragment is indicated with dashed lines. The position of the distal inversion breakpoint in Is1Gso is 129 bp from the distal deletion breakpoint in a^{5MNU} and is indicated with an arrow. B, *Bam*HI; P, *Pst* I; X, *Xba* I; R, *Eco*RI; Bg, *Bgl* II. (*B*) Nucleotide sequence analysis of the a^{5MNU} breakpoint region compared with the sequence of wild-type DNA. R, *Eco*RI; Bg, *Bgl* II.

undergo a coordinated pattern of expression. Additionally, cloning of cDNAs and the analysis of RNA transcripts arising from the gene(s) at the *a* locus, coupled with *in situ* hybridization techniques, may also aid in understanding the molecular basis for the complex biology associated with the differential production of phaeomelanosomes and eumelanosomes, which give rise to the banded agouti pattern of coat pigmentation.

The agouti region has also been of interest because some of the alleles or pseudoalleles are associated with an increased susceptibility to the development of various spontaneous and induced tumors (1, 28, 29), a stimulation in normal body growth (29), obesity (28–30), a diabetes-like syndrome (31), reduced fertility (32), and embryonic death (33–35). The lethality associated with A^y has been investigated in some detail; embryos homozygous for this mutation display characteristic abnormalities at the morula or blastocyst stage and die during implantation (33–35). Therefore, in addition to understanding the molecular basis for the agouti coat color, the characterization of the agouti region at the molecular level should also provide useful information for understanding the molecular basis of many or all of these additional phenotypes.

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