# Physical and Genetic Characterization of a 75-Kilobase Deletion Associated with $a^l$, a Recessive Lethal Allele at the Mouse *agouti* Locus

**Gregory S. Barsh and Charles J. Epstein**

*Department of Pediatrics and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143*

Manuscript received July 27, 1988
Accepted for publication December 22, 1988

ABSTRACT

The *agouti* locus (*A*) of the mouse determines the timing and type of pigment deposition in the growing hair bulb, and several alleles at this locus are lethal when homozygous. Apparent instances of intragenic recombination and complementation between different recessive lethal alleles have suggested that the locus has a complex structure. We have begun to investigate the molecular basis of agouti gene action and recessive lethality by using a series of genetically linked DNA probes and pulsed field gel electrophoresis to detect structural alterations in radiation-induced agouti mutations. Hybridization probes from the *Src* and *Emv-15* loci do not reveal molecular alterations in DNA corresponding to the $a^e$, $a^x$, and $a^l$ alleles, but a probe from the parotid secretory protein gene (*Psp*) detects a 75-kilobase (kb) deletion in DNA containing the non-agouti lethal allele ($a^l$). The deletion is defined by a 75-kb reduction in the size of *Bss*HII, *Not*I, *Nru*I and *Sac*II high molecular weight restriction fragments detected with the *Psp* probe and is located between 25 kb and 575 kb from *Psp* coding sequences. Because the genetic distance between *A* and *Emv-15* is much less than *A* and *Psp*, there may be a preferred site of recombination close to *Psp*, or suppression of recombination between *A* and *Emv-15*. The $a^l$ deletion has allowed us to determine the genotype of mice heterozygous for different recessive lethal alleles. We find that three different recessive lethal complementation groups are present at the agouti locus, two of which are contained within the $a^l$ deletion.

THE mouse *agouti* locus controls the developmental timing and type of pigment granule synthesis in follicular melanocytes (SEARLE 1968; SILVERS 1979). In mice of the genotype *A/A*, a back and forth switch between eumelanin and phaeomelanin production results in the characteristic agouti phenotype of black dorsal hairs with a subapical yellow band. There are more than 20 alleles at the agouti locus, and several have additional phenotypic effects such as obesity (HESTON and VLAHAKIS 1961), variability of expression (WOLFF and PITOT 1973; WOLFF, ROBERTS and GALBRAITH 1986), and/or embryonic death in the homozygous state (ROBERTSON 1942; PAPAIOANNOU and MARDON 1983; LYON, FISHER and GLENISTER 1985). Despite recent efforts to produce a high-resolution genetic map surrounding the agouti locus (SIRACUSA *et al.* 1987a, b), it is not known whether these additional effects, in particular—homozygous lethality—result from altered expression of the coat color genes or of other closely linked genes.

Four recessive lethal alleles, $A^y$ (lethal yellow), $a^x$ (lethal non-agouti), $a^{16H}$ (non-agouti-16H), and $a^l$ (non-agouti lethal) have been well characterized from a phenotypic, genetic and embryologic perspective (EATON and GREEN 1963; CALARCO and PEDERSEN 1976; PAPAIOANNOU and MARDON 1983; LYON, FISHER and GLENISTER 1985). A fifth recessive lethal allele, $a^{jl}$ (jet lethal), has not yet been fully investigated (SIRACUSA *et al.* 1987b). The combinations $A^y/a^x$, $A^y/a^{16H}$, and $a^{16H}/a^l$ are fully viable, but the combination $A^y/a^l$ is lethal.

With respect to coat color, alleles associated with phaeomelanin production tend to be dominant over those associated with eumelanin production, and mice homozygous for the "null" alleles $a^e$ (extreme non-agouti) or $a^{4H}$ (non-agouti-4H) are entirely black (assuming the + allele is carried at the *b* locus). Mice carrying the $A^y$ allele opposite to $a^e$ or $a^{4H}$ are entirely yellow, those carrying the $a^l$ allele are entirely black, and those carrying the $a^x$ or $a^{16H}$ alleles have an intermediate or umbrous phenotype—black dorsal and pinna hairs with dark agouti flanks and ventral hairs (RUSSELL, MCDANIEL and WOODIEL 1963; LYON, FISHER and GLENISTER 1985).

The agouti locus is located on mouse chromosome 2, closely linked to the morphologic markers *bp* (brachypod) and *kr* (kreisler). Like mutations at the *c* (albino) and *d* (dilute) loci (DISTECHE and ADLER 1984; RINCHIK *et al.* 1986), the pleiotropic effects of some radiation-induced agouti alleles may be due to large deletions that affect multiple transcriptional units. We have attempted to define the physical position of the agouti locus by using a series of genetically linked DNA probes and pulsed field gel electrophoresis to detect structural rearrangements in the radiation-induced agouti mutations $a^e$, $a^x$ and $a^l$. The endogenous

ecotropic murine leukemia virus, *Emv-15*, maps within 0.55 cM (95% confidence limits of 0.01–3.4 cM) of the $A^y$ allele (COPELAND, JENKINS and LEE 1983; SIRACUSA *et al.* 1987b) and probes from the *Emv-15* insertion site have been previously used to study the agouti locus (LOVETT *et al.* 1987; SIRACUSA *et al.* 1987b). Additional DNA probes in this region include the parotid secretory protein (*Psp*) and cellular Rous sarcoma (*Src*) genes (OWERBACH and HJORTH 1980; BLATT *et al.* 1984). We now report that a probe from the *Psp* gene detects an approximately 75 kilobase (kb) deletion in DNA containing the $a^l$ allele. In combination with a recently identified restriction fragment length polymorphism (RFLP) at the *Emv-15* locus (LOVETT *et al.* 1987), this deletion has allowed us to test further the complementation relationships between different homozygous lethal agouti alleles. We find that the $a^l$ allele contains two distinct recessive lethal complementation groups, as defined by the $a^x$ and $A^y$ alleles, and that the $a^{16H}$ allele represents a third complementation group.

## MATERIALS AND METHODS

**Mouse strains and mutations:** The strains, C57BL/6J-$A^{w\text{-}J}$ and AEJ/GnLe-$a^e/A^{w\text{-}J}$, of agouti genotypes $A^{w\text{-}J}/A^{w\text{-}J}$ and $a^e/A^{w\text{-}J}$, respectively, were obtained from The Jackson Laboratory, Bar Harbor, Maine. The black offspring of AEJ/GnLe-$a^e/A^{w\text{-}J}$ intercrosses were used as a source of homozgous $a^e/a^e$ animals. Mice carrying the $a^x$ allele were provided by L. B. RUSSELL and have been repeatedly backcrossed to C57BL/6J-*a*/*a* mice in our laboratory. Mice carrying the $a^{16H}$ and $a^l$ alleles were provided by M. LYON and have been propagated in our laboratory as a balanced lethal stock.

**Pulsed field gel electrophoresis:** DNA was prepared from a single cell suspension of homogenized spleen tissue after embedding the cells in blocks of low melting temperature agarose at a concentration of $5 \times 10^6$ cells/ml (VAN OMMEN and VERKERK 1986). After equilibration with the appropriate digestion buffer, 40 μl blocks of agarose were incubated for 8 hr with 5–10 units of the indicated restriction enzymes (conditions according to the manufacturer, New England Biolabs). Digestion was terminated by washing the blocks in a solution of 50 mM EDTA, 50 mM Tris (pH 8), after which the blocks were melted at 70° and gently pipetted into the wells of a 1% agarose gel.

For field inversion gels, electrophoresis was performed at 240 V (7 V/cm) in half-strength Tris-borate-EDTA buffer (45 mM Tris, pH 8, 45 mM boric acid, 1 mM EDTA) and 0.5 μg/ml ethidium bromide, with recirculation of buffer through a heat exchanger to maintain the gel temperature at 13° (CARLE, FRANK and OLSON 1986). The electric field was periodically inverted by two computer-controlled relays according to a program that increased the forward interval exponentially from 10 to 60 sec during a 4-h cycle, such that half of the increase in forward switching time had been achieved by 40% through the cycle. The reverse interval was maintained at one third of the forward interval, the field was interrupted between each inversion by a period equal to 2% of the forward interval, and total electrophoresis time was 16 hr.

For orthogonal pulsed field gels, fractionation was performed on a commercial apparatus (LKB Pulsaphor) at 330 V (10 V/cm) and 10° for 40 hr. A switching time of 60 sec was used for fragments between 200 and 900 kb, and a switching time of 100 sec was used for fragments up to 1500 kb.

**DNA blotting, hybridization and probes:** Limited acid hydrolysis, capillary transfer, and hybridization in the presence of 10% dextran sulfate to probes radiolabeled by random priming were all performed according to standard procedures.

DNA probes from the *Emv-15* insertion site were either a 0.5-kb *Pst*I or a 1.1-kb *Eco*RI fragment from the 5′ or 3′ flanking region of the *Emv-15* insertion site, respectively (LOVETT *et al.* 1987). The DNA probe from the *Src* gene, kindly provided by NANCY QUINTRELL, was a 1.5-kb *Xba*I-*Bam*HI fragment located immediately 3′ to coding sequences. The DNA probe from the *Psp* gene, kindly provided by PHILIP SHAW, was a 2.1-kb *Bam*HI fragment isolated from the bacteriophage λ psp I (SHAW and SCHIBLER 1986) containing 5′ flanking and coding regions.

## RESULTS

**High molecular weight restriction fragments in mice carrying radiation-induced agouti mutations:** Factors that suggest a particular agouti mutation might represent a large deletion are failure to observe reversion or instances of similar spontaneous mutations, phenotypic consequences beyond effects on coat color (*e.g.*, recessive lethality), and a history of induction by X-irradiation. Reversions of the radiation-induced alleles $a^e$, $a^x$ and $a^l$, have never been observed, and both $a^x$ and $a^l$ are lethal when homozygous. We hypothesized that a deletion in one of these alleles would alter the mobility of high molecular weight restriction fragments fractionated by pulsed field electrophoresis and that such fragments (which would presumably contain the agouti locus) might be detected by one or more of the genetically-linked DNA markers, *Emv-15*, *Psp* and *Src*.

To search for deletions of the agouti locus, we initially focused on *Emv-15* because it is very closely associated with the $A^y$ allele (SIRACUSA *et al.* 1987b). DNA from $A^{w\text{-}J}/A^{w\text{-}J}$ (white-bellied agouti), $a^e/a^e$, $a^x/a$, and $a^{16H}/a^l$ mice was digested with a series of rare-cutting restriction enzymes, fractionated by field inversion gel electrophoresis, and transferred to a nylon filter. Multiple restriction fragments between 150 kb and 1000 kb were detected by the *Emv-15* insertion site probe, but no structural rearrangements were apparent in any of the DNA samples. We then expanded our hybridization experiments to include probes from the *Psp* and *Src* genes. In mice of the agouti genotype $A^{w\text{-}J}/A^{w\text{-}J}$ the *Psp* probe detects fragments of 675, 1000, 850, 950, 900, and 200 kb, produced by the restriction enzymes *Bss*HII, *Mlu*I, *Not*I, *Nru*I, *Sal*I, and *Sfi*I, respectively (Figure 1). Fragments of 675 kb and 575 kb were generated by *Sst*II, probably due to incomplete digestion as a consequence of partial methylation (BIRD 1987). DNA from $a^e/a^e$ and $a^x/a$ mice produces a pattern identical

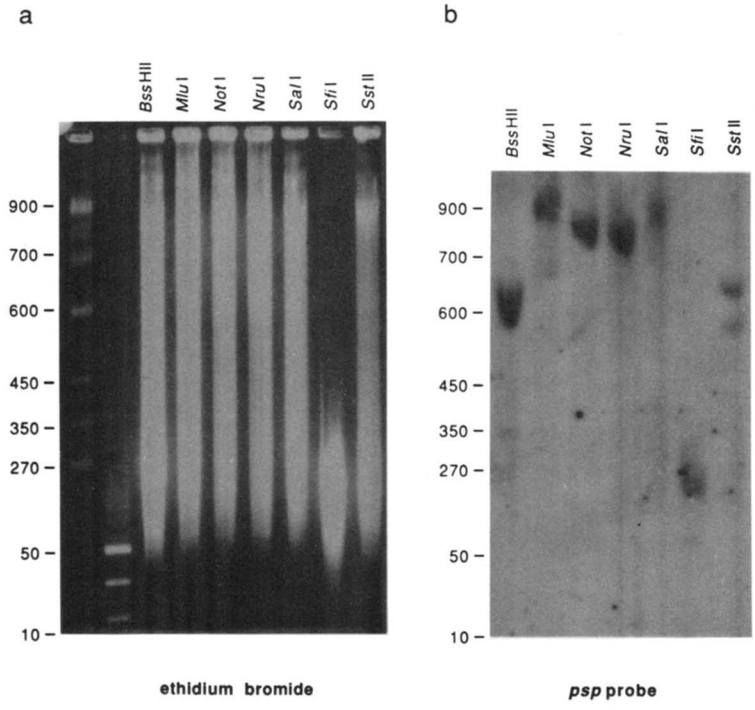


FIGURE 1.—Field inversion gel electrophoresis and blot hybridization of $A^{w\text{-}J}/A^{w\text{-}J}$ DNA with a *Psp* probe. (a) Ethidium bromide-stained pattern of high molecular weight DNA from spleen cells of a C57BL/6J-$A^{w\text{-}J}$ mouse digested with the indicated "rare-cutting" restriction enzymes. The two left-hand lanes contain yeast chromosomes from the strain AB 1380 (BURKE, CARLE and OLSON 1987) or *Hin*dIII-digested λ DNA used to estimate molecular weights of the mouse genomic fragments. (b) Hybridization pattern to genomic *Psp* sequences. See text for discussion.

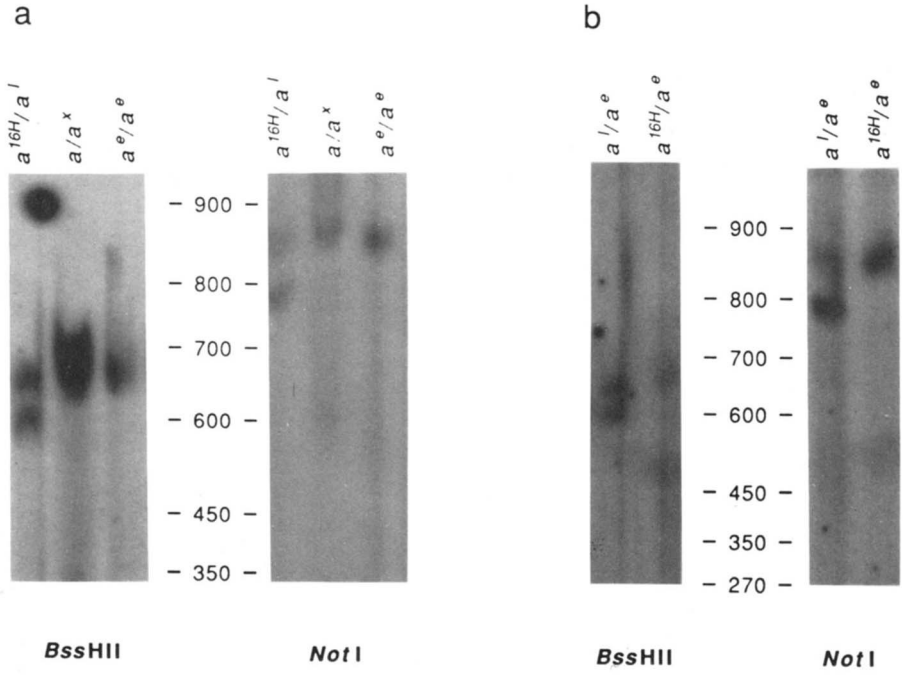


FIGURE 2.—Blot hybridization of a *Psp* probe to DNA from mice carrying radiation-induced agouti mutations. (a) Lanes 1, 2 and 3 in each panel correspond to DNA from $a^{16H}/a^{l}$, $a^{x}/a$, and AEJ/Gn $a^{e}/a^{e}$ mice, respectively. Samples in the left panel were digested with *Bss*HII, and those in the right panel were digested with *Not*I. (b) Lanes 1 and 2 in each panel contain DNA from $a^{l}/a^{e}$ and $a^{16H}/a^{e}$ mice, respectively. These animals were identified on the basis of their phenotypic similarity to $a^{l}/a^{4H}$ and $a^{16H}/a^{4H}$ mice (LYON, FISHER and GLENISTER 1985); the $a^{e}$ allele is genetically and phenotypically similar to the $a^{4H}$ allele but of independent origin. The samples in the left panel were digested with *Bss*HII, and those in the right panel were digested with *Not*I. Estimation of fragment sizes was based on AB 1380 chromosomes as in Figure 1.

to that of DNA from $A^{w\text{-}J}/A^{w\text{-}J}$ mice. By contrast, DNA from $a^{16H}/a^{l}$ mice produces 675- and 600-kb *Bss*HII fragments and 850- and 775-kb *Not*I fragments (Figure 2a). For both enzymes, the upper band is identical to that seen in DNA from all other samples examined to date and thus represents the wild type, whereas the lower band represents an apparent agouti mutation.

**The $a^{l}$ allele appears to contain a 75-kb deletion:** To determine whether the altered *Bss*HII and *Not*I restriction fragments corresponded to $a^{l}$ or $a^{16H}$, we followed their segregation pattern through successive generations. In a cross of $a^{16H}/a^{l} \times a^{e}/a^{e}$, 10 of 18 progeny had black pinna and dorsal hairs, and umbrous or dark agouti flanks and ventral hairs; these animals were therefore identified as $a^{16H}/a^{e}$ (LYON, FISHER and GLENISTER 1985). The remaining eight progeny were entirely black and were therefore identified as $a^{l}/a^{e}$. Comparison of DNA from $a^{16H}/a^{e}$ and $a^{l}/a^{e}$ mice shows that the altered *Bss*HII and *Not*I restriction fragments segregate with the $a^{l}$ allele (Figure 2b).

Field inversion electrophoresis can produce a nonmonotonic relationship between mobility and molecular weight under some conditions (CARLE, FRANK

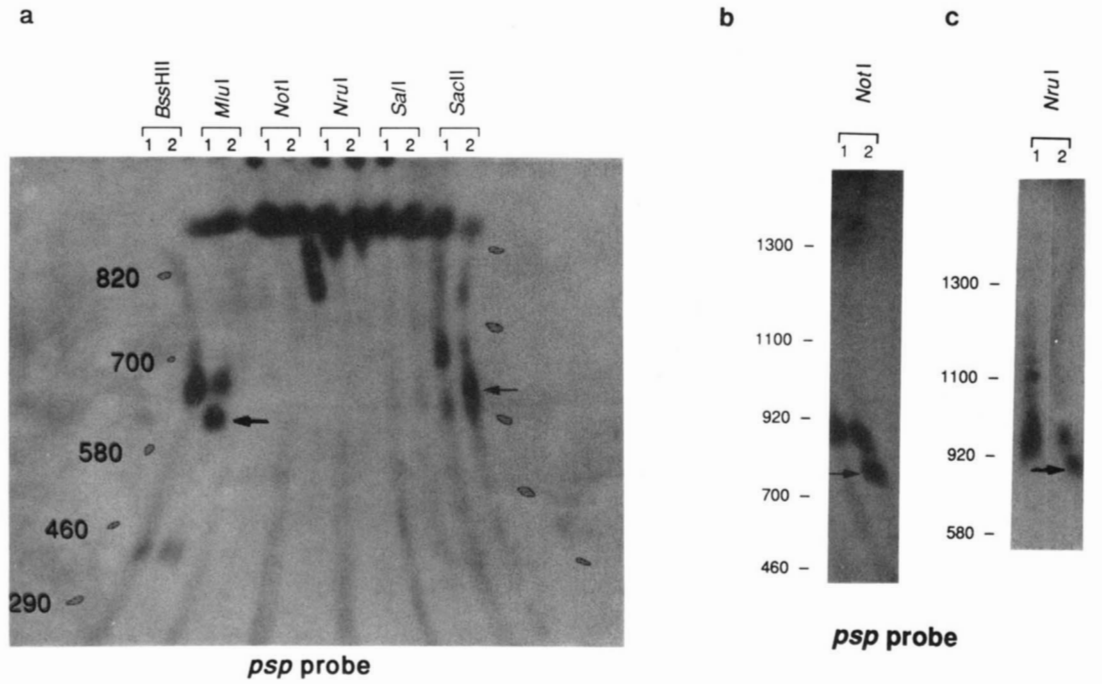


FIGURE 3.—Orthogonal pulsed field electrophoresis and blot hybridization of DNA from $a^{16H}/a^e$ and $a^l/a^e$ mice to the *Psp* probe. Spleen cell DNA was prepared and digested with the indicated enzymes as described in MATERIALS AND METHODS. For each enzyme, lanes 1 and 2 contain DNA from $a^{16H}/a^e$ and $a^l/a^e$ mice, respectively. For the enzymes *Bss*HII, *Not*I, *Nru*I, and *Sac*II, DNA from $a^l/a^e$ mice produces a fragment 75 kb shorter (arrows) than DNA from $a^{16H}/a^e$ mice. The position and size in kb of yeast chromosomes from the strain HY-1 (BURKE, CARLE and OLSON 1987) are indicated on each side of the autoradiograph. (a) Fractionation at a pulse time of 60 sec. For *Bss*HII and *Sac*II, lower molecular weight fragments of 400-kb and 575-kb respectively, are identical between lanes 1 and 2. In the case of *Sac*II, this "normal" 575-kb fragment migrates very close to the "deleted" 600-kb fragment. (b) Fractionation at a pulse time of 100 seconds after digestion with *Not*I. (c) Fractionation at a pulse time of 100 sec after digestion with *Nru*I.

and OLSON 1986). To ensure that the $a^l$-associated restriction fragments of increased mobility truly represented a decline in molecular weight, we compared the hybridization pattern of DNA from $a^e/a^e$ to $a^l/a^e$ mice after fractionation by orthogonal field alternating electrophoresis. High molecular weight DNA was digested with *Bss*HII, *Mlu*I, *Not*I, *Nru*I, *Sal*I or *Sac*II, and fractionated with a 60-sec pulse time, which separates fragments between 200 kb and 900 kb. Hybridization to the *Psp* probe detected $a^l$-specific 600-kb *Bss*HII and 600-kb *Sac*II fragments, each shorter than their $a^e$-specific counterpart by 75 kb (Figure 3a). Under these conditions, the 850-kb *Not*I fragment is not separated from the bulk of higher molecular weight fragments, and the distance between the allelic 775-kb and 850-kb *Not*I fragments is greater than the distance between the allelic 600-kb and 675-kb *Bss*HII fragments because orthogonal pulsed field electrophoresis produces a region of compression in the middle third of the gel and a region of expansion in the upper third of the gel (VOLLRATH and DAVIS 1987). However, fractionation at a pulse time of 100 sec resolved the 775-kb and 850-kb *Not*I bands from the bulk of higher molecular weight DNA, and also revealed a 950-kb and 875-kb *Nru*I doublet (Figure 3b). Hybridization of the same filters to the *Emv-15* insertion site probe and the *Src* probe resulted in identical patterns of DNA from $a^e/a^e$ compared to $a^l/a^e$ mice (data not shown), excluding the possibility that increased mobility of DNA corresponding to the $a^l$ allele is a general phenomenon and showing that the altered *Bss*HII, *Not*I, *Nru*I and *Sac*II restriction fragments are only detected with the *Psp* probe.

Although it is theoretically possible that the $a^l$-specific restriction fragments result from polymorphic point mutations, we have failed to observe similar fragments in more than fifteen other agouti alleles and inbred strains, including C57BL/6J-$A^y$/a, $A^{vy}/a$ (viable yellow), $A^{iy}/a$ (intermediate yellow), $A^{sy}/a$ (sienna yellow), $A^{w\text{-}J}/A^{w\text{-}J}$, $A^i/a$ (intermediate agouti), $a^t/a^t$, YBR/Ki-$A^y/a$, C3H/HeJ, and BALB/cJ mice. Instead, because the $a^l$ mutation was produced by X-irradiation and because the sizes of the $a^l$-specific fragments generated by *Bss*HII, *Not*I, *Nru*I and *Sac*II are all reduced by the same amount, the molecular lesion most likely responsible for the altered restriction fragments in $a^l$ DNA is a deletion of approximately 75 kb. The deletion does not appear to contain a cluster of rare-cutting restriction sites because all of the rare-cutting restriction enzymes we have tested on $a^l$ DNA generate either a normal fragment or one that is shortened by 75 kb. Furthermore, the maxi-

mum distance between *Psp* and the deletion is determined by the smallest fragments that contain the deletion: 675-kb *Bss*HII and 675-kb *Sac*II fragments. If sites for these enzymes were immediately adjacent to *Psp*, the proximal deletion endpoint could lie no farther away from the *Psp* gene than 600 kb. We have attempted to further define this limit by searching for rare-cutting restriction sites close to *Psp* in two cosmids recovered from chromosome walking experiments. However, using probes distributed along 95 kb of cloned DNA surrounding the *Psp* gene, we have not found cleavage sites in genomic DNA for any of the restriction enzymes shown in Figure 3.

**Complementation relationships of the $a^x$ allele:** Among the four well-characterized homozygous lethal agouti alleles—$A^y$,$a^x$,$a^{16H}$, and $a^l$, the combinations $A^y/a^x$,$A^y/a^{16H}$, and $a^{16H}/a^l$ are viable, and $A^y/a^l$ is lethal (PAPAIOANNOU and MARDON 1983; LYON, FISHER and GLENISTER 1985). To determine the status of the combinations $a^x/a^{16H}$ and $a^x/a^l$, we crossed $a^x/a$ with $a^{16H}/a^l$ animals and distinguished the $a^x/-$ from the $a/-$ progeny by following the segregation of *Msp*I and *Hin*dIII RFLPs at the *Emv-15* locus (LOVETT *et al.* 1987). The $a^x$, $a^{16H}$, and $a^l$ alleles are associated with a 1.7-kb *Msp*I fragment detected with a probe from the 5′ region of the *Emv-15* insertion site and with a 2.6-kb *Hin*dIII fragment detected with a probe from the 3′ region of the *Emv-15* insertion site. The *a* allele, however, is associated with 1.2-kB *Msp*I and 5.4-kB *Hin*dIII fragments in the same regions. A diagram of the breeding experiment and a representative autoradiograph from seven progeny are shown in Figure 4. Of 41 progeny from this first cross shown in Figure 4a, eight were homozygous for the 1.7-kb *Msp*I and 2.6-kb *Hin*dIII fragments and had, therefore, inherited the $a^x$ allele from their parent (Table 1). In general, agouti alleles that lead to phaeomelanin production tend to be dominant over those that lead to eumelanin production. In accord with this observation, 33 $a/-$ mice had yellow pinna hairs characteristic of non-agouti allele, and the eight $a^x/-$ mice had black pinna hairs. Dorsal and ventral coat color patterns of $a/a^{16H}$ and $a/a^l$ are more difficult to distinguish, and genotypic identification of the $a/-$ mice therefore could not be made unambiguously on the basis of phenotype. Nonetheless, the deviation of the observed segregation ratio from 0.5 ($P < 0.01$), expected if all potential combinations from the cross were viable, suggested that both $a/a^{16H}$ and $a/a^l$ animals were represented among the 33 progeny with yellow pinna hairs and that either the $a^x/a^{16H}$ or the $a^x/a^l$ combination was lethal and therefore would be not present among the eight progeny with black pinna hairs.

To investigate the identity of the allele or alleles opposite $a^x$ in the eight $a^x/-$ animals, we determined whether the *Psp* probe would detect the $a^l$-specific 775-kb *Not*I and 600-kb *Bss*HII restriction fragments. All of the $a^x/-$ progeny were homozygous for the 850-kb *Not*I fragment (Figure 4c) and 675-kb *Bss*HII fragment (Figure 4d), identifying their genotype as $a^x/a^{16H}$. These identities have been confirmed genetically in the three of the eight animals that were test bred to $a^e/a^e$ homozygotes (Table 1). Viability of $a^{16H}/a^x$ mice thus defines a third recessive lethal complementation group represented by $a^{16H}$, and our failure to recover any $a^x/a^l$ mice ($P < 0.01$) suggests that the $a^l$-deletion includes sequences of both the $A^y$ and $a^x$ complementation groups (Figure 5). Of four $a/-$ animals tested in a similar fashion, one carried the $a^l$-specific high molecular weight restriction fragments and three did not (Table 1). When test bred to an $a^e/a^e$ mouse, the presumptive $a/a^l$ mouse produced four of 13 progeny with an extreme non-agouti phenotype.

## DISCUSSION

The agouti locus is one of the oldest known and most well studied mouse coat color genes (CUENOT 1905; SILVERS 1979). Some alleles exhibit intricate tissue-specific patterns of expression; others are susceptible to position effects (PHILLIPS 1966) and heritable variability of expression (WOLFF and PITOT 1973). These and other observations have prompted many investigators to speculate about the "complex" structure of the locus (SILVERS 1979; SIRACUSA *et al.* 1987a). In this regard, most intriguing are the existence of recessive alleles that arrest embryonic development long prior to the time when agouti gene action is thought to effect a switch in melanosome synthesis. Although extensive breeding experiments have suggested occasional instances of intragenic complementation (WALLACE 1954) and pseudoallelism (WALLACE 1965), viability has not been genetically separated from an effect on coat color in any of the recessive lethal agouti alleles.

We have found a DNA alteration that is likely to represent a 75-kb deletion in the $a^l$ (non-agouti lethal) allele. In the *Psp* hybridization pattern of $a^l$ DNA, high molecular weight restriction fragments generated by the "rare-cutting" enzymes *Bss*HII, *Not*I, *Nru*II and *Sac*II are each shorter than their normal counterpart by 75 kb. Although formal proof that these changes are due to a deletion will require isolation of deleted sequences, it is difficult to otherwise explain the altered hybridization pattern. Because there are no sites for "rare-cutting" enzymes within 25 kb on either side of the *Psp* gene, and because the smallest $a^l$-specific fragment is 675 kb (for *Bss*HII and *Sac*II), the deletion endpoints must lie between 25 kb and 575 kb away from *Psp*. The absence of rare-cutting restriction sites either close to *Psp* or within the deleted sequences suggests that these regions may

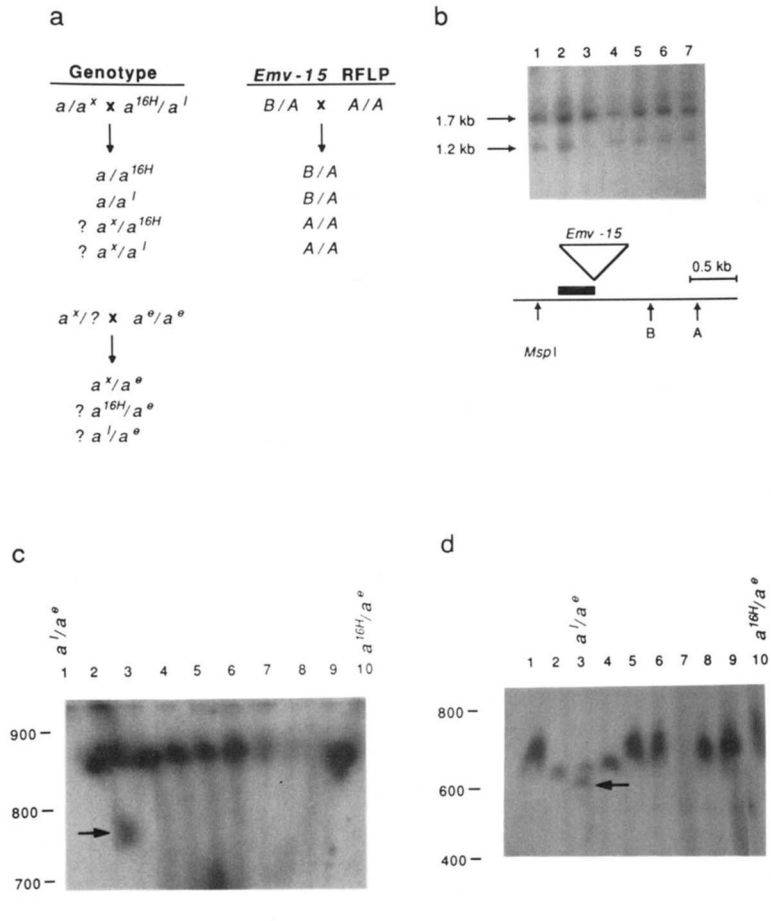


FIGURE 4.—Complementation analysis of homozygous lethal agouti mutations using molecular markers at the *Emv-15* and *Psp* loci. (a) Breeding diagram as described in the text. The "A" RFLP corresponds to a 1.7-kb *Msp*I and 2.6 kb *Hin*dIII fragment from the 5′ and 3′ regions of the *Emv-15* insertion site, respectively (LOVETT *et al.*, 1987). The "B" RFLP corresponds to a 1.2-kb *Msp*I and 5.4 kb *Hin*dIII fragment in the analogous regions. (b) Lanes 1–7 represent the first seven progeny recovered in the experiment. Tail DNA was digested with *Msp*I, blotted, and hybridized to a radiolabeled 0.5-kb *Pst*I fragment from the 5′ flanking region of the *Emv-15* insertion site. An *Msp*I partial cleavage product specific for the "B" RFLP comigrates with the 1.7-kb band specific for the "A" RFLP, which accounts for the reduced intensity of the 1.2-kb band relative to the 1.7-kb band in the "*A/B*" heterozygotes. Putative "*A/A*" animals that lacked the 1.2-kb *Msp*I band were tested for the absence of a 5.4-kb *Hin*dIII fragment during a second hybridization with a 1.1-kb *Eco*RI fragment from the 3′ flanking region of the *Emv-15* insertion site. (c) Lane 1 represents a known $a^l/a^e$ animal and lane 10 represents a known $a^{16H}/a^e$ animal. Lanes 1–9 represent $a^x/-$ animals from the initial cross. Spleen cell DNA from each animal was digested with *Not*I and fractionated by orthogonal pulsed field electrophoresis as described in MATERIALS AND METHODS. The *Psp* probe detects a characteristic 775-kb *Not*I fragment in DNA from $a^l/a^e$ aniaml (arrow). (d) Lane 3 represents a known $a^l/a^e$ animal and lane 10 represents a known $a^{16H}/a^e$ animal. Lanes 1, 2 and 4–9 represent $a^x/-$ animals from the initial cross. Spleen cell DNA from each animal was digested with *Bss*HII and fractionated by orthogonal pulsed field electrophoresis as described in MATERIALS AND METHODS. The *Psp* probe detects a characteristic 600-kb *Bss*HII fragment in DNA from the $a^l/a^e$ animal (arrow).

**TABLE 1**

**$F_1$ progeny of $a/a^x \times a^{16H}/a^l$**

| Coat color phenotype[a] | No. of $F_1$ mice | *Emv-15* RFLP[b] | | Total tested for $a^l$ genotype[c] | No. of tested $F_1$ mice by genotype | | | |
|---|---|---|---|---|---|---|---|---|
| | | *B/A* ($a/a^{16H}$ or $a/a^l$) | *A/A* ($a^x/a^{16H}$ or $a^x/a^l$) | | $a/a^{16H}$ | $a/a^l$ | $a^x/a^{16H}$ | $a^x/a^l$ |
| Yellow pinna hairs, black dorsum, non-agouti ventrum | 33 | 33 | | 4 | 3 | 1 (1)[d] | 0 | 0 |
| Black pinna hairs, black dorsum, non-agouti ventrum | 8 | | 8 | 8 | 0 | 0 | 8 (3) | 0 |

[a] Phenotypes as determined at the time of weaning. The non-agouti ventrum appeared similar to that of C57BL/6J-$a/a$ mice, and was slightly paler than the extreme non-agouti ventrum of AEJ/Gn-$a^e/a^e$ mice.

[b] The "A" RFLP represents a 1.7-kb *Msp*I fragment and a 2.6-kb *Hin*dIII fragment; the "B" RFLP represents a 1.2-kb *Msp*I fragment and a 5.4-kb *Hin*dIII fragment (LOVETT *et al.* 1987). See the legend to Figure 4 for details.

[c] Mice were tested for the presence of the $a^l$ allele by pulsed field gel electrophoresis of spleen cell DNA after digestion by *Bss*HII or *Not*I and hybridization to the *Psp* probe. See Figure 4 for examples.

[d] The number in parentheses refers to those mice tested for the $a^l$ allele by test breeding to $a^e/a^e$ animals. Absence of the $a^l$-allele was inferred if more than seven offspring were produced and none exhibited an extreme non-agouti phenotype.

be relatively devoid of CpG islands, frequently found at the 5′ ends of many mammalian housekeeping genes (BIRD 1987; GARDINER-GARDEN and FROMMER 1987).

The $a^l$ allele was found among the offspring of an X-irradiated C3H/HeH × 101/H $F_1$ female (of agouti genotype $A/A^w$) mated to a male of agouti genotype $a/a$, and the "null" coat color phenotype was recognized concordantly with homozygous lethality (PHILLIPS 1961; LYON, FISHER and GLENISTER 1985). It is possible that coincidental molecular lesions other than the 75-kb deletion could be responsible for one or

a

| | $A^y$ | $a^x$ | $a^{16H}$ | $a^l$ |
|---|---|---|---|---|
| $A^y$ | − | + | + | − |
| $a^x$ | | − | + | − |
| $a^{16H}$ | | | − | + |
| $a^l$ | | | | − |

b

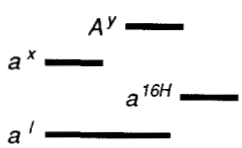


FIGURE 5.—Complementation relationships among the recessive lethal agouti alleles. (a) Viability is indicated with a + sign, and embryonic lethality with a − sign. Previous classical genetic analysis has suggested that $a^l$ only partially complements $a^{16H}$ (LYON, FISHER and GLENISTER 1985); the limited number of animals generated in our experiment did not allow us to address the issue of partial complementation. (b) Complementation map based on the data in panel a; see text for discussion.

more aspects of the $a^l$ phenotype. However, the deletion, embryonic lethality, and the "null" coat color have remained associated through many generations of breeding, and it therefore seems likely that a deletion of 75 kb is sufficient to 1) inactivate agouti coat color gene action completely; and 2) block embryonic development when in combination with either of the two complementary recessive lethal alleles, $A^y$ and $a^x$.

Much effort has been devoted to analyzing the relationship between the *Emv-15* provirus and the $A^y$ allele (COPELAND, JENKINS and LEE 1983). Despite their close genetic linkage (SIRACUSA *et al.* 1987b), altered expression of a transcriptional unit at the proviral insertion site (LOVETT and EPSTEIN 1987), and the precedent of a proviral insertion inducing a coat color mutation at the dilute locus (COPELAND, HUTCHISON and JENKINS 1983), it is now clear that the *Emv-15* insertion is not causally related to yellow coat color (LOVETT *et al.* 1987). In a conventional backcross (SIRACUSA *et al.* 1987a), $A^y$ did not recombine with *Emv-15* in 1222 progeny, yielding a genetic distance of less than 0.30 cM (upper 95% confidence limit between the two loci). But, analysis of recombinant inbred strain distributions and intercrosses between mice heterozygous for *Psp* and agouti alleles has resulted in an estimated genetic distance of 3 cM (upper 95% confidence limit of 6 cM between these markers) (OWERBACH and HJORTH 1980). Given that agouti is more closely linked to *Emv-15* than to *Psp*, it is interesting that it is the *Psp* and not the *Emv-15* probe that detects the $a^l$ deletion. Although the ratio of genetic to physical distance is sometimes assumed to be only a function of chromosome length or centromeric distance, recombinational "hotspots" are well-established in a variety of organisms. Our results underscore the difficulty of relating genetic to physical distance over small areas, and may indicate a preferred site of recombination between *A* and *Psp*, or a region of recombination suppression between *Emv-15* and $A^y$.

The failure of $a^l$ to complement $A^y$ is particularly intriguing when the coat color phenotypes of these alleles are considered. The $a^l$ allele is at the bottom of the dominant-recessive hierarchy and in combination with a homozygous viable "bottom" recessive allele, such as $a^e$ or $a^{4H}$, produces animals that are completely black (LYON, FISHER and GLENISTER 1985). This null phenotype and the molecular evidence of a deletion suggest that zygotes homozygous for $a^l$ are non-viable because they fail to express a gene required for embryonic survival. In contrast, $A^y$ is dominant over all other agouti alleles, and on most backgrounds an $A^y/-$ mouse is completely yellow (SILVERS 1979). Thus, $A^y$-mediated coat color appears to be a neomorphic or hypermorphic effect, but $A^y$-mediated recessive lethality appears to be an amorphic effect (MULLER 1932). Given these considerations, it seems unlikely that a single transcriptional unit is responsible for the effects of the $A^y$ mutation on both coat color and embryonic survival. Indeed, analysis of a large number of breeding experiments has suggested that the $A^y$ mutation is genetically separable from other agouti coat color genes (SIRACUSA *et al.* 1987a). Resolution of these questions and an understanding of agouti gene action should be possible once the genes that control coat color and embryonic viability are isolated. Identification of the $a^l$-deletion represents a first step in this process.

We thank TEODOSIA ZAMORA for excellent technical assistance. This work was supported by research grant HD-03132 from the National Institute of Child Health and Human Development. G.S.B. was supported by a grant from the Weingart Foundation and the National Institute of General Medical Sciences (GM-07085).

## LITERATURE CITED

BIRD, A., 1987 CpG islands as gene markers in the vertebrate nucleus. Trends Genet. **3:** 342–347.

BLATT, C., M. E. HARPER, G. FRANCHINI, M. N. NESBITT and M. I. SIMON, 1984 Chromosomal mapping of murine c-*fes* and c-*src* genes. Mol. Cell. Biol. **4:** 978–981.

BURKE, D., G. F. CARLE and M. V. OLSON, 1987 Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science **236:** 807–812.

CALARCO, P. G., and R. A. PEDERSEN, 1976 Ultrastructural observations of lethal yellow ($A^y/A^y$) mouse embryos. J. Embryol. Exp. Morphol. **35:** 73–80.

CARLE, G. F., M. FRANK and M. V. OLSON, 1986 Electrophoretic separation of large DNA molecules by periodic inversion of the electric field. Science **232:** 65–68.

COPELAND, N. G., K. W. HUTCHISON and N. A. JENKINS, 1983 Excision of the DBA ecotropic provirus in dilute coat-color revertants of mice occurs by homologous recombination involving the viral LTRs. Cell **33:** 379–387.

COPELAND, N. G., N. A. JENKINS and B. K. LEE, 1983 Association of the lethal yellow ($A^y$) coat color mutation with a ecotropic murine leukemia virus genome. Proc. Natl. Acad. Sci. USA **80:** 247–249

CUENOT, L. 1905 Les races pures et leurs combinaisons chez les souris. Arch. Zool. Exp. Gen. **3:** 123–132.

DISTECHE, C. M., and D. ADLER, 1984 Localization of cloned mouse chromosome 7-specific DNA to lethal albino deletions. Somatic Cell Mol. Genet. **10:** 211–215.

EATON, G. J., and M. M. GREEN, 1963 Giant cell differentiation

and lethality of homozygous yellow mouse embryos. Genetica **34:** 155–161.

GARDINER-GARDEN, M., and M. FROMMER, 1987 CpG islands in vertebrate genomes. J. Mol. Biol. **196:** 261–282.

HESTON, W. E., and G. VLAHAKIS, 1961 Influence of the $A^y$ gene on pulmonary tumors in mice by alteration of its effect on normal growth. JNCI **27:** 1189–1196.

LOVETT, M., and C. J. EPSTEIN, 1987 The lethal yellow allele-associated provirus results in the production of chimeric viral-host RNAs. Proc. Natl. Acad. Sci. USA **84:** 2853–2857.

LOVETT, M., Z. Y. CHENG, E. M. LAMELA, T. YOKOI and C. J. EPSTEIN, 1987 Molecular markers for the agouti coat color locus of the mouse. Genetics **115:** 747–754.

LYON, M. F., G. FISHER and P. H. GLENISTER, 1985 A recessive allele of the mouse agouti locus showing lethality with yellow, $A^y$. Genet. Res. **46:** 95–99.

MULLER, H. J., 1932 Further studies on the nature and causes of gene mutations. Proceedings of the Sixth International Congress Genetics, Ithaca, N.Y.

OWERBACH, D., and J. P. HJORTH, 1980 Inheritance of a parotid secretory protein in mice and its use in determining salivary amylase quantitative variants. Genetics **95:** 129–141.

PAPAIOANNOU, V. E., and H. MARDON, 1983 Lethal nonagouti ($a^x$): description of a second embryonic lethal at the agouti locus. Dev. Genet. **4:** 21–29.

PHILLIPS, R. J. S., 1961 A comparison of mutation induced by acute *X* and chronic gamma irradiation in mice. Br. J. Radiol. **34:** 261–264.

PHILLIPS, R. J. S., 1966 A *cis-trans* position effect at the *A* locus of the house mouse. Genetics **54:** 485–495.

RINCHIK, E. M., L. B. RUSSELL, N. G. COPELAND and N. A. JENKINS, 1986 Molecular genetic analysis of the dilute-short ear (*d-se*) region of the mouse. Genetics **112:** 321–342.

ROBERTSON, G. G., 1942 An analysis of the development of homozygous yellow mouse embryos. J. Exp. Zool. **89:** 197–231.

RUSSELL, L. B., M. N. C. MCDANIEL and F. N. WOODIEL, 1963 Crossing over within a "locus" of the mouse (abstr.). Genetics **48:** 907.

SEARLE, A. G., 1968 *Comparative Genetics of Coat Color in Mammals.* Academic Press, New York.

SHAW, P., and U. SCHIBLER, 1986 Structure and expression of the parotid secretory protein gene of mouse. J. Mol. Biol. **192:** 567–576.

SILVERS, W. K., 1979 The agouti and extension series of alleles, umbrous and sable, pp. 6–28 in *The Coat Colors of Mice*, edited by W. K. SILVERS. Springer-Verlag, New York.

SIRACUSA, L. D., L. B. RUSSELL, E. M. EICHER, D. J. CORROW, N. G. COPELAND and N. A. JENKINS, 1987a Genetic organization of the agouti region of the mouse. Genetics **117:** 93–100.

SIRACUSA, L. D., L. B. RUSSELL, N. A. JENKINS and N. G. COPELAND, 1987b Allelic variation within the *Emv-15* locus defines genomic sequences closely linked to the agouti locus on mouse chromosome 2. Genetics **117:** 85–92.

VAN OMMEN, G. J. B., and J. M. H. VERKERK, 1986 Restriction analysis of chromosomal DNA in a size range up to two million base pairs by pulsed field gradient electrophoresis, pp. 113–133 in *Human Genetic Diseases. A Practical Approach*, edited by K. DAVIES. IRL Press, Oxford.

VOLLRATH, D., and R. W. DAVIS, 1987 Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. Nucleic Acids Res. **15:** 7865–7875.

WALLACE, M. E., 1954 A mutation or crossover in the house mouse? Heredity **8:** 89–105.

WALLACE, M. E., 1965 Pseudoallelism at the agouti locus in the mouse. J. Hered. **56:** 267–271.

WOLFF, G. L., and H. C. PITOT, 1973 Influence of background genome on enzymatic characteristics of yellow ($A^y/-$, $A^{vy}/-$) mice. Genetics **73:** 109–123.

WOLFF, G. L., D. W. ROBERTS and D. B. GALBRAITH, 1986 Prenatal determination of obesity, tumor susceptibility, and coat color pattern in viable yellow ($A^{vy}/a$) mice. The yellow mouse syndrome. J. Hered. **77:** 151–158.

Communicating editor: D. BENNETT