Genetic Instability at the *agouti* Locus of the Mouse (*Mus musculus*). I. Increased Reverse Mutation Frequency to the A^w Allele in *A/a* Heterozygotes

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ABSTRACT

We have compiled the reverse mutation rate data to the *white bellied agouti* (A^w) allele in heterozygous *A/a* mice and shown it to be increased by a factor of at least 350 in comparison to the reverse mutation rate in homozygous *a/a* mice. Employing tightly linked flanking restriction fragment length polymorphism DNA markers, we have shown that reversion to A^w is associated with crossing over in the vicinity of the *agouti* locus. The *non-agouti* (*a*) allele has been recently shown to contain an 11-kb insert within the first intron of the *agouti* gene. Together with our present results, these observations suggest possible mechanisms to explain the reversion events.

THE *agouti* locus on chromosome *2* of the mouse is characterized by an extensive series of phenotypically distinct mutant alleles (SILVERS 1979). The *agouti* gene controls the switch from eumelanin to phaeomelanin pigment expression in the developing hair in a tissue and developmental time-specific manner. The locus was identified very early in mouse genetic studies and is an anchor locus for chromosome *2* linkage studies.

Much is known about the mutational events at the *agouti* locus since it has been included in the marker loci assayed in the mouse germ cell specific-locus mutation test (RUSSELL 1951). Such studies were designed to assay forward mutations which occur spontaneously or following irradiation or chemical mutagen treatment. One interesting observation has been the occurrence of reverse mutations from the recessive *non-agouti* (*a*) allele. SCHLAGER and DICKIE (1971) have presented a compilation of extensive breeding results from The Jackson Laboratory production stocks (mostly inbred strains) and have shown the *non-agouti* allele to be relatively unstable and capable of mutating to a number of alternative alleles including wild-type *white bellied agouti* (A^w). We have also shown that irradiation as well as ethylnitrosourea (ENU) treatment increases the reverse mutation rate from *non-agouti* to *white bellied agouti* by a factor of approximately 15 (FAVOR *et al.* 1987a, 1991). Both the spontaneous mutation rates estimated by SCHLAGER and DICKIE (1971) and our results for irradiation and chemical mutagen treatment were for animals homozygous *a/a*. However, in extensive breeding studies of heterozygous *A/a* animals we noted a considerable occurrence of *white bellied agouti* mutations. Here we present an estimation of the spontaneous mutation rate to A^w in breeding experiments of *A/a* mice and show the observed mutation rate to be drastically increased in comparison to the spontaneous mutation rate to A^w in homozygous *a/a* mice. Further, employing flanking DNA markers closely linked to the *agouti* locus we show that the mutations to A^w in heterozygous *A/a* mice are associated with crossing over in the vicinity of the *agouti* locus. These results taken together with the recent molecular characterization of the A^w, *A* and *a* mutant alleles (BULTMAN *et al.* 1992; MILLER *et al.* 1993) suggest possible mechanisms for the observed instability of the *agouti* locus.

MATERIALS AND METHODS

Estimation of mutation rates to A^w: Mice screened for A^w mutations all originated from crosses of presumed recessive specific locus, dominant visible and dominant cataract mutants which were recovered in a series of ENU (FAVOR 1983, 1986; FAVOR *et al.* 1990) or radiation experiments (J. FAVOR, unpublished) as well as the historical control of the laboratory from 1974 to present. For these groups, mutagenically treated or untreated wild-type male (102 × C3H)F_1 hybrid mice were mated to untreated tester stock females. The tester stock females were homozygous recessive *non-agouti* (*a/a*), *brown* (*b/b*), *chinchilla–pink eyed dilution* (c^{ch}–*p*/c^{ch}–*p*), *dilute–short ear* (*d–se/d–se*) and *piebald* (*s/s*). Resultant offspring were expected to be heterozygous at the tester stock marker loci and homozygous wild type at dominant visible and dominant cataract loci. F_1 offspring were examined for phenotypic variants characteristic of mutations occurring at the marker loci as well as dominant visible and dominant cataract loci. When recovered, F_1 mice expressing a phenotypic variant indicative of a recessive specific locus, dominant visible or a dominant cataract mutation were subjected to a genetic confirmation test as well as a characterization for viability by outcrossing to homozygous wild-type (102 × C3H)F_1 hybrid mice. Confining our interests to the *agouti* locus all such crosses are of the type *A/a* × *A/A* and all resultant offspring should be phenotypically agouti.

A series of experiments were carried out in our laboratory in which we screened for specific locus and dominant cataract mutations following irradiation (FAVOR *et al.* 1987a) or ENU treatment (FAVOR *et al.* 1991) but we deviated from the standard experimental design by varying the genotype of the mu-

tagenically treated male. We include here the offspring of presumed specific locus, dominant visible and dominant cataract mutations in the DBA/2 experimental series. For these experiments male strain DBA/2 mice were irradiated or treated with ENU and crossed to tester stock females, and offspring were screened for recessive mutations at tester stock marker loci as well as dominant visible and dominant cataract loci. Since strain DBA/2 is homozygous *non-agouti* (*a/a*), *brown* (*b/b*) and *dilute* (*d/d*) the resultant offspring of the cross DBA/2 × tester stock would also be expected to be homozygous *a/a b/b d/d*. Focusing on the *agouti* locus, outcrosses of presumed specific locus, dominant visible or dominant cataract mutations to (102 × C3H)F_1 hybrid mice is of the sort *a/a* × *A/A*. All resultant offspring are expected to be heterozygous *A/a* and to be phenotypically agouti. Further outcrosses of the resultant F_2 mice are of the sort *A/a* × *A/A* and the offspring should all express the agouti phenotype.

Since A^w is dominant to both the *a* and the *A* alleles, the occurrence of A^w mutations would be evident in all offspring originating from the genetic confirmation crosses as well as the outcrosses of F_2 offspring in the DBA/2 experiments. A^w mutations were genetically confirmed by an allelism test to the *agouti* locus. Briefly, presumed mutants were outcrossed to homozygous *a/a* mice and at least 20 offspring were classified for transmission of the mutant white bellied agouti phenotype. Resultant presumed A^w/a mice from these outcrosses were then backcrossed to homozygous *a/a* mice and at least 20 offspring were classified to determine the segregation of the A^w or *a* alleles. The confirmed A^w mutations were maintained by crossing to either homozygous *non-agouti* or homozygous *agouti* mice. The homozygous *non-agouti* mice used for such crosses were derived from the tester stock. The homozygous *agouti* mice used for such crosses were (102 × C3H)F_1 hybrids. All tester stock, inbred strains and F_1 hybrids of inbred strains were obtained from stocks maintained in Neuherberg.

Mutants: A total of 40 independent A^w mutations occurring in outcrosses of presumed specific locus, dominant visible or dominant cataract mutations from control or mutagenicity experiments conducted at Neuherberg were included for analysis. The crosses in which the 40 A^w mutations occurred were all of the sort *A/a* × *A/A*. Also included for analysis were two independent A^w mutations recovered in the offspring of the DBA/2 mutagenicity experiments (*i.e.*, from the cross DBA/2 × tester stock) (Favor *et al.* 1987a, 1991) and an A^w mutation which occurred in the stock of strain C57BL/6 maintained at Neuherberg. In addition, an A^{vy} and an a^t mutation which also occurred in strain C57BL/6 mice at Neuherberg, as well as two a^{tet}, and one each of a^i, a^e and a^d forward mutations which were induced in mutagenicity experiments conducted at Neuherberg (*i.e.*, from the cross (102 × C3H)F_1 × tester stock) were included for analysis.

Laboratory procedures: High molecular weight genomic DNA was extracted from mouse spleen, liver or kidney, as described elsewhere (Favor *et al.* 1987b), or by employing the AutoGen 540 DNA extractor (AutoGen Instruments, Inc., Beverly, Massachusetts) after proteinase K treatment of homogenized tissue. Genomic DNAs were digested to completion with 5–10 units restriction endonuclease/μg DNA under conditions recommended by the suppliers (Boehringer Mannheim, Appligene). The DNA samples were size fractionated on 0.8% agarose gel in TEA buffer at 300–400 V-hr. The DNA fragments were transferred to a Zeta probe membrane (Bio-Rad) in 10 × SSC and hybridized according to manufacturer's instructions. The DNA probes used for hybridization were ^{32}P-labeled by nick translation (specific activity of *ca.* 10^8 cpm/μg).

TABLE 1

Mutation rate (MR) to A^w observed in the offspring from *A/A*, *a/a* and *A/a* parental mice

Cross[a]	Mutations	Offspring	MR × 10^{-5}
A/A × *A/A*	0	67,395	
a/a × *a/a*	10	8,167,854	0.1
A/a × *A/A*	6	17,556	34.2

[a] The first two entries are data from Schlager and Dickie (1971).

The DNA probes employed included the pKECS/PCS clone, which contains an 0.8-kb *Hin*dIII/*Kpn*I fragment from the 5′ flanking region of the *Psp* gene (Madsen and Hjorth 1985), and the pRI subclone, which contains a 1.1-kb *Eco*RI fragment from the 3′-flanking region of the *Emv-15* insertion site (Lovett *et al.* 1987). The *Psp* locus is located proximal to the *a* locus while the *Emv-15* locus is distal to *a*. The estimated genetic distances between loci are as follows: *Psp–a*, 1.1 cM; *a–Emv-15*, 0.6 cM (Siracusa and Abbot 1992; Siracusa *et al.* *1987, 1989). The estimates of the corresponding physical distances between loci are: Psp–a*, <500 kb; *a–Emv-15*, >600 kb (Barsh and Epstein 1989).

RESULTS

Estimation of the mutation rates to the A^w allele: Mice have been systematically screened for mutations to the A^w allele in control (Schlager and Dickie 1971) as well as radiation or mutagen treated parental generation mice (Favor *et al.* 1987a, 1991). These observations were all based on crosses of homozygous parental genotypes and the mutation rate to the A^w allele was relatively low and observed to occur only in the *a/a* parental mice. In contrast, we observed in offspring from the cross *A/a* × *A/A* a drastically increased mutation rate to the A^w allele (Table 1). These initial results were observed in outcrosses of *A/a* F_1 offspring derived from ENU experiments. In comparison to the spontaneous mutation rates to the A^w allele observed by Schlager and Dickie (1971) for homozygous parental genotypes, the present results for the cross *A/a* × *A/A* indicate a mutation rate at least 350 times higher. If we consider that only the *a* allele is capable of reversion, then on a per allele basis our present results indicate an increased reversion rate in heterozygotes of at least 1400 in comparison to the results for crosses of *a/a* homozygous mice. One explanation of the present results might be that mutagenic treatment in the parental generation of our mutagenicity experiments resulted in an increased mutation rate to A^w first observed in the outcross generation. This phenomenon could be due to delayed mutations which require additional rounds of DNA synthesis before the induced DNA lesions were fixed as mutations. To test this hypothesis, additional offspring for the cross *A/a* × *A/A*, in which the heterozygous animals were descendant from control or mutagen treated parental animals were screened for mutations to the A^w allele. Table 2 summarizes the results. There was a consistent increase in the mutation rate to the A^w allele regardless of

TABLE 2

Mutations to A^w observed in the offspring from the cross $A/a \times A/A$ or $a/a \times A/A$ parental mice

Group[a]	Mutations	Offspring	MR[b] $\times 10^{-5}$
Hybrid F_1 ($A/a \times A/A$)			
Control	9	7483	120.0
ENU	3	6832	43.9
Radiation	2	2708	73.9
DBA/2 F_1 ($a/a \times A/A$)			
ENU	0	3917	
Radiation	0	2247	
DBA/2 F_2 ($A/a \times A/A$)			
ENU	4	2303	173.7
Radiation	2	4627	43.2

[a] Parental genotypes of the cross from which offspring were derived are given in parentheses.
[b] MR = mutation rate.

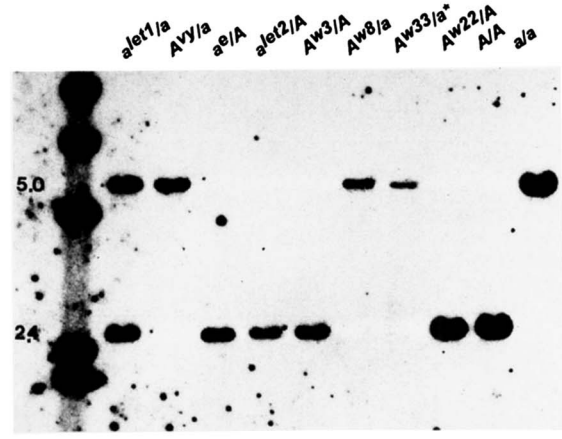

FIGURE 1.—Southern blot analysis of the *Hin*dIII restriction fragments hybridizing to the pRI probe and their association with various mutant alleles at the *agouti* locus. The 2.4-kb fragment (*Emv-15*[a]) is associated with the *A* allele-bearing chromosome as well as mutations derived from the *A* allele (a^{let1}, a^{let2}, a^e). The 5.0-kb fragment (*Emv-15*[b]) is associated with the *a*-bearing chromosome from the tester stock (here designated *a*) as well as from strain C57BL/6 (here designated *a**). Mutations derived from the *a* allele (A^{w8}) or the *a** allele (A^{vy}, A^{w33}) also exhibit the 5.0-kb fragment. The A^{w3} and A^{w22} mutations were recovered from the cross $A/a \times A/A$ and are linked to *Emv-15*[a]

whether the F_1 mice were derived from control, ENU- or radiation-treated parental mice. These observations confirm our initial results and furthermore indicate that there is no association between mutagenic treatment of parental generation mice with an increased mutation rate to the A^w allele in confirmation crosses with F_1 generation offspring. Table 2 also includes observations of confirmation crosses from a series of mutagenicity experiments in which homozygous a/a DBA/2 mice were mutagenically treated and crossed to tester stock mice also homozygous a/a. There was no increased mutation rate to the A^w allele in genetic confirmation crosses of the sort $a/a \times A/A$ in the F_1 generation offspring. This observation does not support the hypothesis that A^w alleles result from delayed mutations following ENU treatment. However, in further outcrosses of the subsequently recovered F_2 heterozygous A/a mice an increased mutation rate to the A^w allele was observed. Together, the results indicate a consistent association of an increased mutation rate to the A^w allele when mice were heterozygous A/a at the *agouti* locus.

Genetic confirmation of the A^w mutations: All A^w reversions occurred as single events for a particular mating, where the minimal number of offspring observed per mating was 20. Each newly occurring presumed A^w reversion expressed the normal white bellied agouti phenotype. There was no indication that the A^w mutations occurred as mosaics based upon the phenotype expressed by the mutants. This was supported by the segregation data observed in the outcrosses of presumed A^w/A heterozygotes to homozygous a/a mice. At least 20 offspring were observed for each presumed A^w mutation and there was no significant deviation from the expected ratio of 50% A^w/a : 50% A/a offspring. Finally, the presumed A^w mutations were confirmed as alleles at the *agouti* locus by backcrossing the presumed A^w/a mice to a/a homozygotes. At least 20 offspring were classified from the backcross of each presumed A^w mutation. For each presumed A^w mutation tested only two phenotypic classes segregated in the progeny, white bellied agouti and non-agouti, and there was no significant deviation from the expected ratio of 50% A^w/a : 50% a/a. It was therefore concluded that all recovered mutants were indeed A^w alleles.

Molecular analyses of the mutational events: Employing the pRI probe linked to the *Emv-15* proviral insertion site on chromosome 2 we distinguished the chromosome carrying the *A* allele derived from strains 102 or C3H and the chromosome bearing the *a* allele from the tester stock according to the methods of LOVETT *et al.* (1987) and SIRACUSA *et al.* (1987). Briefly, restriction fragment length polymorphisms (RFLPs) between the parents of the mutants, (102 × C3H)F_1 or DBA/2 and tester stock, were detected by Southern blot analysis following digestion with *Hin*dIII, *Kpn*I or *Pst*I. Only the *Hin*dIII pattern is shown (Figure 1). Taking advantage of this RFLP, we analyzed a number of mutants at the *agouti* locus, which arose spontaneously in inbred strains or were induced by radiation or chemical treatment. The restriction fragment patterns identified could be correlated with the known strain and allelic origin of each specific mutation at the *agouti* locus (Figure 1; Table 3). The 2.4-kb *Hin*dIII fragment which hybridizes to the pRI probe (which we here designate *Emv-15*[a]) is specific to the chromosome bearing the *agouti* (*A*) allele and to mutations which were derived from the *A* allele (a^{let1}, a^{let2}, a^i, a^e, a^d); the 5.0-kb *Hin*dIII fragment (which we here designate *Emv-15*[b]) is specific to the

TABLE 3

Characterization of mutations at the *agouti* locus of the mouse for RFLPs in the vicinity of the *Psp* locus and the *Emv-15* insertion site

Allele[a,b]	Haplotype[c]		
a (Tester stock)	Psp^b	*a*	$Emv\text{-}15^b$
*a** (C57BL/6)	Psp^a	*a**	$Emv\text{-}15^b$
a (DBA/2)	Psp^b	*a*	$Emv\text{-}15^b$
A (102×C3H) F_1	Psp^a	*A*	$Emv\text{-}15^a$
A^{w8} (DBA/2)	Psp^b	A^w	$Emv\text{-}15^b$
A^{w21} (DBA/2)	Psp^b	A^w	$Emv\text{-}15^b$
A^{w33} (C57BL/6)	Psp^a	A^w	$Emv\text{-}15^b$
A^{vy} (C57BL/6)	Psp^a	A^{vy}	$Emv\text{-}15^b$
a^t (C57BL/6)	Psp^a	a^t	$Emv\text{-}15^b$
a^{let1} (102 × C3H)F_1	Psp^a	a^{let}	$Emv\text{-}15^a$
a^{let2} (102 × C3H)F_1	Psp^a	a^{let}	$Emv\text{-}15^a$
a^i (102 × C3H)F_1	Psp^a	a^i	$Emv\text{-}15^a$
a^e (102 × C3H)F_1	Psp^a	a^e	$Emv\text{-}15^a$
a^d (102 × C3H)F_1	Psp^a	a^d	$Emv\text{-}15^a$
40 independent A^w (A/a × A/A)	Psp^b	A^w	$Emv\text{-}15^a$

[a] Parental genotypes in which the mutations arose are given in parentheses.

[b] The results on line two indicate the haplotype of strain C57BL/6-Neu, which carries the *a* allele, to differ from the haplotypes of tester stock and DBA/2 (also *a*-bearing strains). In order to avoid confusion we designate the *non-agouti* allele in strain C57BL/6-Neu as *a** throughout text, tables and figures, but do not imply an independent origin of the *non-agouti* alleles carried by tester stock, C57BL/6-Neu or DBA/2.

[c] Mice were typed at three loci, *Psp*, *agouti* and *Emv-15*. RFLP alleles at the *Psp* locus were determined by *Rsa*I digestion of genomic DNA and hybridization to the 0.8-kb *Hin*dIII/*Kpn*I fragment of the 5′-flanking region of the *Psp* gene (MADSEN and HJORTH 1985). Psp^a = 1.7-kb restriction fragment normal for the *A*-bearing chromosome of strains 102/E1 and C3H/E1 as well as the *a*-bearing chromosome of strain C57BL/6-Neu. Psp^b = 0.5- and 1.0-kb restriction fragments normal for the *a*-bearing chromosome of tester stock and DBA/2 mice. RFLP alleles at the *Emv-15* locus were determined by *Hin*dIII digestion of genomic DNA and hybridization to the 1.1-kb *Eco*RI fragment from the 3′-flanking region of the *Emv-15* insertion (LOVETT *et al.* 1987). $Emv\text{-}15^a$ = 2.4-kb restriction fragment normal for the *A*-bearing chromosome in strains 102/E1 and C3H/E1. $Emv\text{-}15^b$ = 5-kb restriction fragment normal for the *a*-bearing chromosome of tester stock and strains DBA/2 and C57BL/6-Neu.

chromosome carrying the *non-agouti* (*a*) allele in the tester stock and strains C57BL/6 and DBA/2 as well as for the mutations (A^{w8}, A^{w21}, A^{w33}, A^{vy}, a^t) which arose by mutation of the *non-agouti* allele. It is interesting to note that the a^{let}/a mice carry both the $Emv\text{-}15^a$ and $Emv\text{-}15^b$ alleles, indicating that the a^{let} deletion does not involve the DNA sequences immediately surrounding the proviral integration site.

We analyzed the 40 independent A^w mutants which arose spontaneously in heterozygous A/a animals, taking advantage of the fact that the *A* allele bearing chromosome derived from the (102 × C3H)F_1 parental mice could be distinguished from the *a* allele-bearing chromosome derived from the tester stock or strain DBA/2. For each A^w mutation, heterozygotes over both the *A* and *a* alleles (the *a* allele derived from the tester stock) were bred and analyzed by Southern blot analysis. The A^w mutation in all cases was linked to the $Emv\text{-}15^a$ allele associated with the *A* allele-bearing chromosome (Table 3, Figure 2). This result is surprising, since previous reports indicate A^w mutations occur only from the *a* allele (SCHLAGER and DICKIE 1971; SIRACUSA *et al.* 1987). To explain this contradiction we postulated crossing over as a possible mechanism for mutation to the A^w allele in heterozygous animals. The evidence for this hypothesis was provided by a new set of Southern blot analyses utilizing a genetic marker flanking the *agouti* locus relative to the *Emv-15* insertion site.

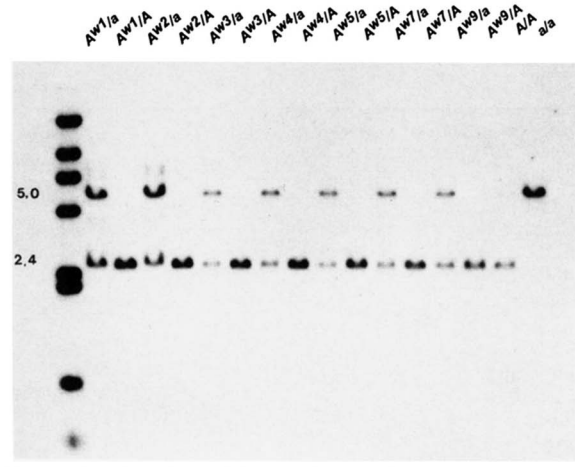

FIGURE 2.—Southern blot analysis of the *Hin*dIII restriction fragments hybridizing to the pRI probe for the series of A^w mutations recovered in the offspring of the cross $A/a \times A/A$. For each A^w mutation A^w/A and A^w/a heterozygotes were bred and analyzed. All A^w mutations derived from the cross $A/a \times A/A$ are associated with the 2.4-kb fragment ($Emv\text{-}15^a$) normally associated with the *A*-bearing chromosome.

An RFLP distinguishing the *A*- and *a*-bearing chromosomes, utilizing as hybridization probe a DNA fragment containing the 5′-flanking region of the *Psp* gene, was identified after genomic DNAs were tested with 18 restriction enzymes: a 1.7-kb *Rsa*I fragment was shown to be associated with the chromosome bearing the *A* allele (which we designate Psp^a) while for the chromosome bearing the *a* allele a restriction pattern of 0.5- and 1.0-kb fragments (which we designate Psp^b) was observed (Table 3; Figure 3). The change in restriction fragment pattern is likely due to the presence of an additional *Rsa*I site specific to the chromosome bearing the *a* allele carried by tester stock mice which is within the 1.7-kb fragment associated with the *A*-bearing chromosome. Interestingly, strain C57BL/6, which is also homozygous *non-agouti*, carries the Psp^a allele. To avoid confusion in the tables and figures, we have designated the C57BL/6 *non-agouti* allele (*a**). It should be emphasized that we do not mean to infer an independent origin of the *non-agouti* allele in the tester stock and in our C57BL/6 strain.

We performed Southern blot analyses on DNAs isolated from mutants at the *agouti* locus which arose either spontaneously or were induced by mutagenic treatment and for which the allele from which the mutation arose was known. Again, in all cases the mutation was

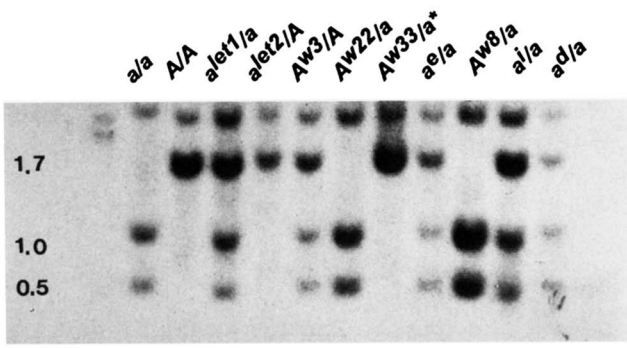

FIGURE 3.—Southern blot analysis of the *Rsa*I restriction fragments which hybridize to the Psp probe. The *A*-bearing chromosome as well as mutations derived from the *A* allele (a^{let1}, a^{let2}, a^e, a^i, a^d) are associated with the 1.7-kb restriction fragment (Psp^a). The *a*-bearing chromosome as well as mutations derived from the *a* allele (A^{w8}) are associated with the 0.5- and 1.0-kb restriction fragments (Psp^b). The *a**-bearing chromosome from C57BL/6 and mutations derived from the *a** allele (A^{w33}) are associated with the Psp^a allele. Mutations (A^{w3}, A^{w22}) recovered in the offspring of the cross $A/a \times A/A$ are associated with the Psp^b allele.

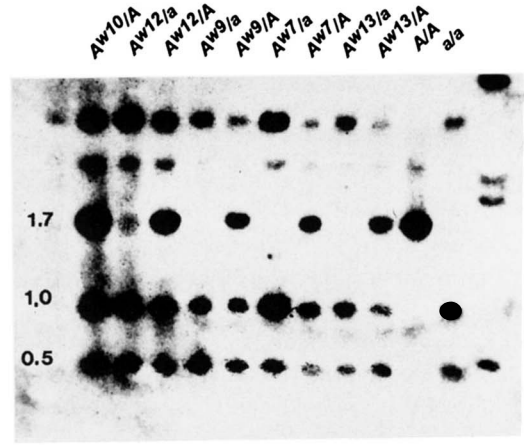

FIGURE 4.—Southern blot analysis of the *Rsa*I restriction fragments hybridizing to the *Psp* probe for the series of A^w mutations recovered in offspring of the cross $A/a \times A/A$. All A^w mutations derived from the cross $A/a \times A/A$ are associated with the 0.5- and 1.0-kb restriction fragments (Psp^b) normally associated with the *a*-bearing chromosome.

shown to be linked to the same *Psp* allele carried by the chromosome from which the mutant *agouti* allele originated (Table 3, Figure 3).

We next subjected the 40 independent A^w mutations which arose in heterozygous A/a mice to Southern blot analysis to characterize the A^w-bearing chromosome in the *Psp* region. The RFLP pattern of each A^w mutation was determined for heterozygotes over either *A* or *a* (the *a* allele derived from tester stock). A clear pattern could be discerned. Of the 40 A^w mutants which arose in A/a heterozygotes all were shown to be linked to the Psp^b allele (Table 3; Figures 3 and 4). Taken together the results indicate that all A^w mutations which arose in the cross $A/a \times A/A$ are linked in the proximal direction to the Psp^b allele associated with the *a*-bearing chromosome; in the distal direction the A^w mutations are linked to the $Emv\text{-}15^a$ allele associated with the *A*-bearing chromosome. Thus, in all cases the A^w mutations which arose in the cross $A/a \times A/A$ are associated with a crossover event between the *Psp* locus and the *Emv-15* integration site in the vicinity of the *agouti* locus.

The overall distance between the *Psp* locus and the *Emv-15* insertion site is estimated to be 1.7 cM (SIRACUSA *et al.* 1989). To test for an unusually high rate of crossing over within the region, normal segregant offspring carrying the *A* or *a* alleles derived from the cross of heterozygous A/a mice to homozygous a/a mice were analyzed for their RFLP patterns at the vicinity of the *Psp* locus and the *Emv-15* insertion site. Two crossovers were identified in 80 offspring analyzed (Table 4), which is not at deviance with the genetic distance expected. The crossover events observed were both the haplotype Psp^a-*a*-$Emv\text{-}15^b$. Based on the *Psp* and *Emv-15* flanking markers, these crossovers are the reciprocal crossover haplotypes to the crossover haplotypes associated with A^w mutations recovered in the crosses of A/a heterozygotes to homozygous A/A mice (Table 3; Figures 2 and 4). It will be interesting to determine in future studies if these are truly reciprocal crossover products, which might indicate a hotspot for crossing over within the *agouti* region of chromosome 2.

TABLE 4

Analysis for crossover events in the vicinity of the *agouti* locus of the mouse in the cross $A/a \times a/a$ employing RFLP markers for the *Psp* locus and the *Emv-15* insertion site

No. of offspring	Haplotype[a]		
Non-crossovers			
40	Psp^a	*A*	$Emv\text{-}15^a$
38	Psp^b	*a*	$Emv\text{-}15^b$
Crossovers			
0	Psp^b	*A*	$Emv\text{-}15^a$
2	Psp^a	*a*	$Emv\text{-}15^b$

[a] Mice were typed at the three loci, *Psp*, *agouti* and *Emv-15*. Southern blot procedures and designations of the *Psp* and *Emv-15* RFLP alleles are as given in Table 3.

DISCUSSION

Stability and structure of mutant alleles at the *agouti* locus: Our results indicate a greatly increased reverse mutation frequency to the A^w allele in the offspring of A/a heterozygous mice and all A^w revertants are associated with a crossover event in the vicinity of the *agouti* locus. An extensive series of phenotypically distinguishable mutant alleles has been isolated at the *agouti* locus (see SILVERS 1979; GREEN 1989). Most mutant alleles were isolated in laboratory breeding colonies. In wild populations of the house mouse the A^w allele predominates, although there are subpopulations carrying the *a* or *A* alleles. We interpret these observations to indicate the A^w allele to be wild type and that both the *a* and *A* alleles

are mutants derived from the A^w allele. The fact that reversion to A^w was observed only in offspring of a/a mice and never in offspring of A/A mice would imply that the original forward mutation A^w to a is associated with a DNA alteration at the *agouti* locus capable of reversion. In contrast, the original forward mutation A^w to A is associated with a DNA alteration which is not capable of reversion to A^w or occurs at such a low frequency that reversion has not yet been observed in laboratory breeding colonies.

The *agouti* gene has been isolated recently (BULTMAN *et al.* 1992; MILLER *et al.*, 1993). The normal gene product is an mRNA of approximately 0.8 kb, and the translation product predicted from the open reading frame is 131 amino acids. A structural domain within the *agouti* gene product suggests that it functions as a signal, which in view of the phenotypes resultant from *agouti* mutant alleles, affects the synthesis of eumelanin and phaeomelanin pigment. The expression pattern of the *agouti* gene product in a/a, A/A and A^w/A^w homozygotes has been characterized. There was no *agouti* gene expression in either dorsal or ventral skin samples of a/a mice. In A/A and A^w/A^w mice there were comparable levels of mRNA expression in the dorsal skin samples, whereas in ventral skin samples there was a higher level of expression of the normal gene product in A^w/A^w mice as compared to the A/A mice. These observations indicate that the level of *agouti* gene expression correlates with the degree of phaeomelanin expression in the hairs. Further, it indicates that the A mutant allele is sufficiently intact to allow normal gene expression, at least in the dorsal skin region. The DNA alteration resulting in the A allele likely resides in the 5′-regulatory region of the *agouti* gene and results in reduced levels of the normal mRNA transcript in the ventral skin. BULTMAN *et al.* (1992) have further characterized the genomic gene structure of several *agouti* mutant alleles including a and A. In comparison to the A allele, the a allele contains an extra 11-kb DNA segment within the first intron of the *agouti* gene. Unfortunately the A^w allele was not characterized. However, Northern blot studies indicate the A and A^w alleles both express a normal sized *agouti* gene product (MILLER *et al.* 1993), which would indicate that the A mutant allele is not due to a large rearrangement within the *agouti* gene. We propose that the extra 11-kb DNA sequence in the first intron of the *agouti* gene to be the DNA alteration resulting in the *non-agouti* mutant allele. This proposal is compatible with the observed instability of the a allele, since the reversion of the a allele could be envisaged to be due to the loss of the extra DNA sequence. In fact, this proposal is partially supported by the molecular characterization of an a^t mutation which arose spontaneously in the cross SEC/E × C57BL/E, both strains being homozygous a/a (BULTMAN *et al.* 1992). Thus, the a^t mutation arose from the a allele and the authors have shown that the extra DNA insert associated with the a allele is altered to a 5.5-kb insert within the first intron of the *agouti* gene in the a^t mutant allele.

Mechanisms of reversion to the A^w allele in crosses of A/a heterozygotes: We premise our considerations for the reversion to A^w in A/a heterozygotes on the assumption that the DNA alteration resulting in the A mutation is in the 5′-regulatory region of the *agouti* locus and that the extra 11-kb DNA sequence in the first intron of the *agouti* gene results in the a mutation. Our present results indicate that all reversions to the A^w allele in crosses of A/a heterozygotes are associated with homologous crossing over. We propose that the site of crossing over is within the *agouti* locus between the site of the DNA alteration resulting in the A mutation and the insertion site of the 11-kb sequence associated with the a allele. The proposed mechanism of reversion to A^w in A/a heterozygotes is schematically diagrammed in Figure 5. With a single crossover event between the mutational sites of the A and a alleles, a crossover product would be recovered with the wild-type 5′-regulatory sequence derived from the a mutant allele as well as a normal first intron derived from the A allele. Thus, a fully functional *agouti* gene would be expected. Further, the proposed crossover event would be expected to carry the Psp^b allele associated with the a-bearing chromosome in the proximal direction to the *agouti* locus as well as the $Emv\text{-}15^a$ allele associated with the A-bearing chromosome in the distal direction to the *agouti* locus. The reciprocal crossover product would result in a chromosome bearing the mutated 5′-regulatory sequence derived from the A mutant allele as well as the 11-kb insert within the first intron of the *agouti* gene. This proposed "double mutant" allele at the *agouti* locus would be expected to be functionally identical to the *non-agouti* allele. In fact we observed two reciprocal crossover products and both were associated with a *non-agouti* allele and had the haplotype Psp^a-"a"-$Emv\text{-}15^b$ (Table 4). Thus, a postulated single crossover event within the *agouti* locus fully explains the results in offspring of A/a mice which we have observed.

The present results also may be used to estimate the genetic distance between the mutational sites of the A and a alleles. The A^w reversions in offspring of A/a mice represent crossovers within this region. The reciprocal crossover product is expected to be associated with a *non-agouti* allele and in the cross $A/a \times A/A$ would not be detected. Therefore, the best estimate of the genetic distance between the A and a mutational sites is twice the frequency of A^w reversions in these crosses. The frequency of A^w offspring observed in the cross $A/a \times A/A$ is 26/41,509 (Tables 1 and 2). Thus, the best estimate of the genetic distance between the A and a mutational sites is $2 \times 0.00063 = 0.0012$, *i.e.*, 0.1 cM.

Mechanisms of reversion to the A^w allele in crosses of a/a homozygotes: We premise our considerations for

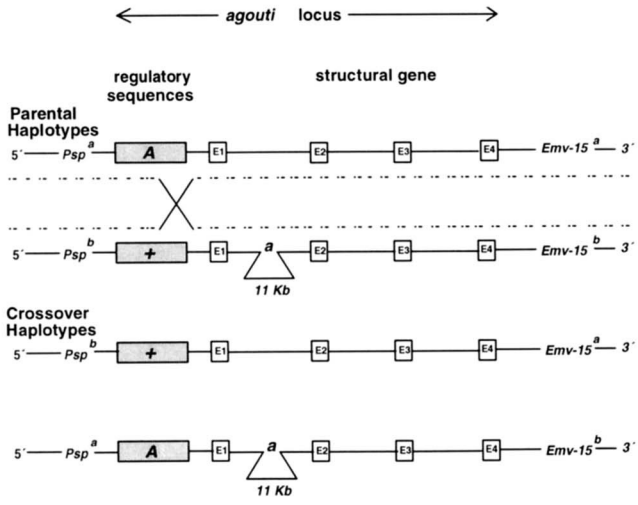

FIGURE 5.—Schematic representation of the structure of the *agouti* gene of the mouse (BULTMAN *et al.* 1992) and the proposed crossover events which we observed to be associated with A^w mutational events in the cross $A/a \times A/A$. The *agouti* locus is comprised of 5′-regulatory sequences (hatched box) and four exons (E1–E4). The parental haplotype Psp^a-*A*-*Emv-15*^{a} is assumed to be associated with a forward mutation to *A* in the 5′-regulatory sequences of the *agouti* locus resulting in the misregulation of expression of the *agouti* gene in ventral skin. The parental haplotype Psp^b-*a*-*Emv-15*^{b} is associated with the forward mutation *a* due to the extra 11-kb sequence in the first intron of the *agouti* structural gene. A single crossover between the mutational sites of the *A* and *a* alleles would result in a fully functional *agouti* gene carrying the 5′-regulatory sequences from the *a*-bearing chromosome and a normal *agouti* structural gene from the *A*-bearing chromosome and should result in reversion to the A^w allele. This crossover should also be associated with crossing over of the flanking markers and result in the haplotype Psp^b-A^w-*Emv-15*^{a}, which we observed in all 40 independent A^w mutations recovered in the crosses $A/a \times A/A$ (Table 3). The reciprocal crossover product is expected to carry the mutated 5′-regulatory sequences from the *A*-bearing chromosome, the 11-kb insertion within the first intron of the *agouti* structural gene from the *a*-bearing chromosome and be functionally identical to the *a* allele. This crossover product would be the haplotype Psp^a-*a*-*Emv-15*^{b}. Two such haplotypes were observed in the analysis of 80 offspring in the cross $A/a \times a/a$ (Table 4).

the mechanisms of instability of the *non-agouti* allele on the assumption that the extra 11-kb DNA sequence contained in the first intron of the *agouti* gene is the mutational event resulting in the *non-agouti* mutation. There are a number of precedents for unstable forward mutations due to the insertion of extra DNA in the vicinity of an affected gene and include tandem duplication, transposon insertion and possibly amplified short nucleotide repeat sequences.

The best characterized example of tandem duplication associated with an unstable forward mutation is *Bar-eyed* in Drosophila (STURTEVANT 1925). The *Bar-eyed* mutation has been shown to be due to an extensive duplication observable at the cytogenetic level (see TSUBOTA *et al.* 1989), is unstable and reverts to wild type via unequal crossing over. The reciprocal crossover product yielding a triplication of the sequence is also recovered as the mutation *Double-Bar*, and this product is also associated with crossover of flanking markers. In the mouse, the most unstable mutant allele recovered to date, *pink-eyed unstable* (p^{un}) has also been shown recently to be due to a tandem duplication of approximately 70 kb (GONDO *et al.* 1993). Reversion of the p^{un} allele is associated with complete loss of the duplicated 70-kb sequence yielding a single, unduplicated 70-kb sequence, which the authors interpret to be due to homologous unequal crossing over. Since the first intron of the *agouti* gene is larger than 11 kb (BULTMAN *et al.* 1992), it is possible that the *a* allele is due to a tandem duplication of an 11-kb sequence within the first intron and that the instability of the *a* allele in *a/a* homozygotes is due to unequal crossing over which results in the loss of one of the copies of the duplicated sequence.

An alternative mechanism to explain the instability of the *a* allele would be the proposal that the *a* allele is due to the insertion of a transposable element within the first intron of the *agouti* gene. The original *dilute* (d^v) mutation carried in a number of inbred strains of the house mouse including strain DBA/2 has been shown to be due to the insertion of an ecotropic specific proviral sequence (JENKINS *et al.* 1981). Reversion of the d^v allele in d^v/d^v homozygotes has been shown to occur at a frequency similar to the reversion rate to A^w in *a/a* homozygotes (SCHLAGER and DICKIE 1971). Reversion of d^v occurs via intrachromosomal homologous crossing over between the proviral long terminal repeat (LTR) sequences (HUTCHISON *et al.* 1984; FAVOR *et al.* 1987b) resulting in the excision of the proviral sequence, although a single LTR sequence remains at the insertion site. Since the insertion resulting in the *a* allele is within the first intron of the *agouti* gene, it would not be precarious to propose that insertion of the entire 11-kb sequence disrupts normal function of the *agouti* gene whereas the proviral excision leaving a single relict LTR within the first intron is associated with reversion to an allele with normal gene function.

Finally, the genome of all eukaryotes so far studied is replete with amplified short nucleotide repeat sequences [see for example JEFFREYS *et al.* (1985), HAMADA *et al.* (1982) and STALLINGS *et al.* (1991)] including the mouse (DIETRICH *et al.* 1992). Such sequences normally reside in non-coding DNA sequences of the genome and exhibit a high frequency of somatic and germinal mutation (JEFFREYS *et al.* 1988). The mutational process is not solely due to unequal homologous crossover although results are conflicting and may be due to specific differences of the particular amplified sequences studied or the particular minisatellite sites considered (CHANDLEY and MITCHELL 1988; WOLFF *et al.* 1989). To date minisatellites in general have not been shown to be associated with mutation of neighboring genes, although if a sequence were to be greatly amplified up to

11 kb, an effect on the function of a neighboring gene could be expected. For the special case of trinucleotide repeats, five examples of an association with mutation have been demonstrated recently, fragile X syndrome (Fu *et al.* 1991), Huntingtons's disease (Duyao *et al.* 1993), myotonic dystrophy (Brook *et al.* 1992), spinobulbar muscular atrophy (La Spada *et al.* 1991), and spinocerebellar ataxia type 1 (Orr *et al.* 1993). The sequences may be relatively short, variable and were shown to be contained within the open reading frame of the affected gene. The wild-type allele contains a small number of trinucleotide repeats and when amplified results in a highly unstable mutation (Imbert *et al.* 1993). Further amplification of the repeated sequence results in more severe forms of the mutant alleles. If the *a* allele were to be due to an amplified short nucleotide sequence, one would expect a high level of instability both within and between strains carrying the *a* allele. This should be easily testable now that appropriate molecular access to the *agouti* locus is possible.

Frequency of reversion to the A^w allele: Our results indicate a reversion frequency to A^w in A/a mice at least 350 times the reversion frequency observed in a/a mice, and all reversions to A^w in A/a heterozygotes are associated with crossover of flanking markers. These observations are compatible with a proposed mechanism of reversion to A^w in A/a heterozygotes following a single crossover within the *agouti* locus between the mutational sites of the *A* and *a* alleles. By contrast, reversion to A^w in a/a homozygotes is proposed to be associated with the loss of the 11-kb insertion within the first intron of the *agouti* gene which is responsible for the *a* allele. As outlined above, a proposed mechanism to explain the loss of the 11-kb sequence associated with the *a* allele in a/a homozygotes depends upon the nature of the 11-kb inserted sequence, *i.e.*, unequal crossing over if the 11-kb sequence is a tandem duplication, excision of a transposable element, or instability of an amplified short nucleotide sequence. In any event, the mechanism involved in reversion to A^w in A/a heterozygotes is likely different than the mechanism of reversion to A^w in a/a homozygotes. Thus, the observed differences in the frequencies of reversion to A^w in A/a heterozygotes as compared to the reversion frequency in a/a homozygotes likely resides in differences in the mechanisms by which the reversions occur.

Conclusions: Although speculative, our proposed mechanisms of instability of the *agouti* locus in A/a heterozygotes and a/a homozygotes are now testable since molecular access has been made possible by the cloning of the *agouti* gene. We have a large collection of A^w revertants as well as other mutant alleles at the *agouti* locus. Future studies will be able to determine an association of DNA structural changes associated with mutant alleles of the *agouti* locus. These analyses should also be useful in associating DNA structural changes with an alteration in gene function. Finally, a characterization of the nature of the 11-kb insertion associated with the *a* allele may be informative to determine why the forward a mutation is unstable in homozygous a/a mice.

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