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Molecular and genetic characterization of a radiation-induced structural rearrangement in mouse chromosome 2 causing mutations at the limb deformity and agouti loci

(chromosomal translocation/chromosomal inversion/radiation-induced mutations)

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ABSTRACT **Molecular characterization of mutations in the mouse, particularly those involving agent-induced major structural alterations, is proving to be useful for correlating the structure and expression of individual genes with their function in the whole organism. Here we present the characterization of a radiation-induced mutation that simultaneously generated distinct alleles of both the limb deformity (*ld*) and agouti (*a*) loci, two developmentally important regions of chromosome 2 normally separated by 20 centimorgans. Cytogenetic analysis revealed that an interstitial segment of chromosome 17 (17B-17C; or, possibly, 17A2-17B) had been translocated into the distal end of chromosome 2, resulting in a smaller-than-normal chromosome 17 (designated 17^{del}) and a larger form of chromosome 2 (designated 2^{17}). Additionally, a large interstitial segment of the 2^{17} chromosome, immediately adjacent and proximal to the insertion site, did not match bands 2E4-2H1 at corresponding positions on a normal chromosome 2. Molecular analysis detected a DNA rearrangement in which a portion of the *ld* locus was joined to sequences normally tightly linked to the *a* locus. This result, along with the genetic and cytogenetic data, suggests that the alleles of *ld* and *a* in this radiation-induced mutation, designated ld^{In2} and a^{In2}, were associated with DNA breaks caused by an inversion of an interstitial segment in the 2^{17} chromosome.**

Molecular characterization of mutations is a powerful way of correlating the structure and expression of genes with their function in the organism. In mice, mutations produced in chemical- and radiation-mutagenesis programs are important tools for the characterization of the mammalian genome. Many of these mutations are major structural chromosomal alterations, namely, deletions, inversions, or translocations, and are particularly useful for studying gene function in the mouse. For example, deletion mutations arising mainly from exposure to radiation are being used as tools in complementation (1) and saturation-mutagenesis (2) experiments to define and study the function of individual genes located in several different chromosomal segments. DNA probes mapping to the deleted regions are being used to derive physical maps of deletion sections of DNA and to clone and characterize the genes associated with these regions (3).

Here we describe the genetic, cytogenetic, and molecular characterization of a complex radiation-induced chromosomal rearrangement in the mouse associated with two phenotypes controlled by chromosome 2 loci. These loci, *ld* (limb deformity) and *a* (agouti), normally map 20 centimorgans (cM) apart. The *ld* locus was originally defined more than 30 years ago by two independently arising spontaneous mutations, one at the Oak Ridge National Laboratory and the other at The Jackson Laboratory (4–6). This locus, which became accessible at the molecular level with probes derived from an insertional mutation in a transgenic mouse (7), is associated most notably both with a skeletal abnormality affecting the fore- and hindlimbs of the animal and with kidney defects (4–9). The *a* locus regulates the distribution of the eumelanin and phaeomelanin pigments in individual hairs as well as over the entire coat (10, 11). Additionally, analysis of radiation-induced mutations has revealed the presence of genes within or near the *a* locus that regulate embryonic development (1, 10–12). Despite a considerable effort, using genetic mapping and molecular "walking" techniques over the past several years, the *a* locus has not yet been characterized at the molecular level, nor have any of the other developmentally regulated genes near the *a* locus been cloned (13–16). The radiation-induced mutation described here involves an inversion of chromosome 2 that has allowed us to use probes from the *ld* locus to "jump" to a region tightly linked to the *a* locus. Consequently, we have been able to derive DNA probes within or very close to the genes associated with this developmentally important region of the genome.

METHODS AND MATERIALS

Mutagenesis, Genetic, and Cytogenetic Analysis. A ^{137}Cs source was used to generate γ-radiation, delivered at a dose rate of 0.7 R/min (1 R = 0.258 mC/kg). (C3H/Rl × 101/Rl)F_1 males of *a* locus genotype A/A^w were irradiated with 300 R and subsequently mated to (SEC/Rl × C57BL/E)F_1 females of genotype a/a; $+/b$; $+/c^{ch}$; $+ \; +/d \; se$ (*a*, nonagouti; *b*, brown; c^{ch} chinchilla; *d*, dilute; *se*, short ear). The mutant described in this report was conceived 41 weeks after irradiation and was, therefore, derived from an irradiated spermatogonial stem cell. All stocks of mice employed in the radiation experiment, as well as those used in subsequent crosses of the mutant and his descendents, were bred at the Oak Ridge National Laboratory. All methods for cytogenetic analysis were as described (17). The *Mus spretus/Mus musculus* backcross experiments were conducted as described (18).

Northern and Southern Blot Analysis. For Southern blots, 10 μg of genomic liver DNA was digested with restriction enzymes, electrophoresed through agarose gels, and blotted and UV cross-linked to GeneScreen by using standard procedures (19). For Northern blots, poly(A)$^+$ RNA, prepared from 500 μg of total RNA, was electrophoresed in formal-

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dehyde gels and blotted and UV cross-linked to GeneScreen by using standard procedures (19, 20). All blots were probed with random-hexamer-labeled double-stranded restriction fragments (21).

Cloning a DNA Breakpoint Within the *ld* Locus. A total of 250 μg of genomic liver DNA from a male heterozygous for the radiation-induced rearrangement was digested to completion with *Eco*RI and size-fractionated on a 10–40% sucrose gradient (19, 20). An EMBL4 phage library, prepared with fractions containing 6.5- to 7.5-kilobase (kb) fragments, was screened by standard procedures using a region of the *ld* locus as a probe (19, 20). The fragments of interest were subcloned into the pGEM4 vector (Promega), restriction mapped, and partially sequenced as described (19, 20).

RESULTS

Mutant Recovery and Genetic Analysis. Among 2870 male offspring of irradiated (C3H/Rl × 101/Rl)F_1 males (A/A^w) mated to (SEC/Rl × C57BL/E)F_1 females (a/a), there was one nonagouti male, presumed to be a/a^*, where a^* represents a mutation involving the a locus on either the A or A^w chromosome. In crosses to a/a mice, this mutant male produced 52 nonagouti offspring and no other types. In crosses to A/A stocks, 17 offspring were obtained, and all were agouti. Crosses to A/a mice yielded 6 agouti and 10 nonagouti offspring. These results indicate that a^* is recessive to A and is either equivalent or recessive to a.

The mutant was revealed to be "partially sterile" due to embryonic death of some of his progeny. In matings with 12 normal females, 29% of the conceptuses were found to die at around the time of implantation. Among surviving sons of the mutant, 19 were also "partially sterile" (with, again, 29% of their conceptuses dying at around implantation), 2 were completely sterile, and 22 had normal fertility.

Homozygotes were obtained from matings of partially sterile males to daughters of partially sterile males. Such homozygotes of either sex were found to be fully fertile, allowing establishment of a homozygous stock. The phenotype of the homozygous animals resembles a^j/a^j ("jet," probably equivalent to a^e/a^e, extreme nonagouti); a^j is recessive to a. Crosses of homozygotes to a^j/a^j produced only jet offspring.

Unexpectedly, these homozygous a^*/a^* jet-colored animals also had severely deformed fore- and hindlimbs. Close examination of alizarin red S-stained skeletons from 19 homozygous, young adult mice revealed an abnormality where, in all cases, there was an absence of at least two digits on each extremity, radioulnar synostosis, complete fibular aplasia, and aplasia or hypoplasia of some carpals and many tarsals. Some variable features were also observed, which included boney syndactyly and brachydactyly, fusions of various carpals and tarsals, malpositioning of the patella, and hindfeet that did not point in the correct direction. In extreme cases, the hindfeet contained only a single digit or the foot articulated with a projection from the side of the tibia instead of from the distal end of the tibia. The defects were bilateral but generally asymmetric. The radioulnar synostosis could often be interpreted as also involving radial hypoplasia except near the elbow, and, in some mice, there appeared to be no radius.

In addition to the skeletal malformations, kidney defects were also observed in homozygous progeny from crosses involving heterozygotes and homozygotes in various combinations. The kidneys of 11 post-weaning mice, 9 of which expressed the limb defect, were analyzed. Six of the 9 animals with the limb defect had renal abnormalities consisting of various degrees of hydronephrosis and hydroureter, and 1 animal had unilateral aplasia and hypoplasia. None of the animals with normal limbs had kidney defects. Additionally, kidneys were examined in 133 fetuses at day 17, of which 80 clearly expressed the limb defect. In this case, 17 of the 80 fetuses with the limb defect had urinary-tract defects, including dilation of one or both ureters (10 fetuses), renal hypoplasia (5 fetuses), and renal agenesis (2 fetuses). None of the fetuses with normal limbs showed any kidney defects.

Since the limb and kidney defects resembled the phenotype of the *ld* mutation, various crosses were made between mice carrying the radiation-induced mutation described above and mice of a stock carrying the allele ld^{OR} (4). The results of these crosses clearly reveal that the radiation-induced mutation is allelic to *ld* (Table 1). Therefore, this mutation involves alterations at two different loci, *a* and *ld*, which are normally separated by 20 cM on the standard genetic map (22).

Cytogenetic Analysis Reveals a Chromosomal Rearrangement. The partial sterility of the original mutant and of some of his descendents suggested that the radiation-induced mutation might have resulted from a chromosomal rearrangement. Therefore, karyotypes were prepared of Giemsa-banded metaphase chromosomes from four phenotypically normal heterozygous adults (two males and two females) and from two adult homozygous males expressing both the extreme-nonagouti and limb-deformity conditions. Although high-resolution Giemsa-banding analysis has failed to provide a definitive cytogenetic interpretation of this mutation, it is clear that all karyotypes show a longer-than-normal chromosome 2 (designated 2^{17}) and a shortened chromosome 17 (designated 17^{del}) (Fig. 1*A*). The banding pattern suggests that an interstitial segment of chromosome 17, involving bands 17B-17C, integrated into the subdistal region of chromosome 2 proximal to band 2H2 (Fig. 1*B*). (An alternative interpretation is that bands 17A2-17B are inserted in the same location but in an inverted fashion.) In addition, a segment of the 2^{17} chromosome immediately proximal to the insertion site does not match the 2E4-2H1 segment of the normal chromosome 2 and is presumably rearranged (Fig. 1).

Altered Gene Expression at the *ld* Locus. We were interested in analyzing how this mutation affects the expression of a gene at the *ld* locus whose structure and patterns of expression will be described in detail elsewhere (R.P.W., R. Maas, R. Zeller, T. Vogt, and P. Leder, unpublished results). In brief, this gene is structurally very complex and is capable of producing a family of mRNAs by alternate tissue-specific splicing and by the utilization of multiple polyadenylylation sites. Expression of this gene is normally detectable in numerous sections of the developing fetus as well as in severaltissues of the adult (ref. 9; R.P.W. *et al.*, unpublished results). The composite structure of some RNA transcripts

Table 1. Manifestation of allelism between R^* and ld^{OR} as expressed by limb morphology of progeny

Cross	Mice, no. Abnormal	Mice, no. Normal
$\frac{R}{R} \times \frac{ld^{OR}}{ld^{OR}}$	48	0
$\frac{R}{ld^{OR}} \times \frac{R}{ld^{OR}}$	73	0
$\frac{ld^{OR}}{ld^{OR}} \times \frac{R}{ld^{OR}}$	78	0
$\frac{ld^{OR}}{+} \times \frac{R}{R}$	58	49

The R* designation refers to the radiation-induced mutation Is*l*Gso.

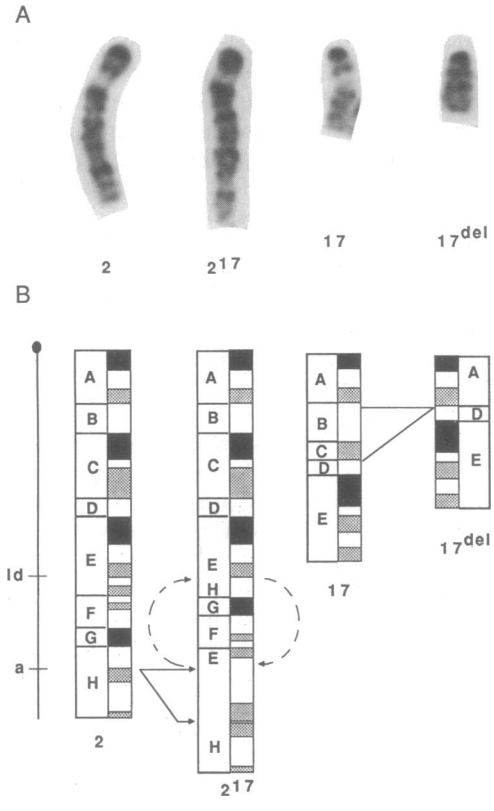

FIG. 1. Structure of the rearranged chromosomes 2 and 17 in the radiation-induced mutation. (*A*) Chromosomes from Giemsa-banded mitotic metaphase karyotypes. From left to right, normal chromosome 2, rearranged chromosome 2 (designated 2^{17}), normal chromosome 17, and deleted chromosome 17 (designated 17^{del}). (*B*) G-banding diagrams illustrating the proposed structure for the radiation-induced chromosomal rearrangement involving chromosomes 2 and 17 (see text). Broken curved arrows show the position of the inverted 2E4-2H1 segment of chromosome 2. Solid linear arrows between chromosomes 2 and 2^{17} show the position of the interstitial segment of chromosome 17 that has integrated into the distal portion of chromosome 2, immediately adjacent and distal to the 2E4-2H1 inversion. The probable positions of the *a* and *ld* loci, relative to the banded map, are shown to the left of the diagram for the normal chromosome 2.

produced from this gene was derived from overlapping cDNA clones and is shown in Fig. 2*A*.

By using a small 0.7-kb section of *ld* locus cDNA as a probe (Fig. 2*A*), the analysis of RNA from the adult brain, kidney, testes, salivary gland, and small intestine revealed that the expression of this gene is markedly altered in animals homozygous for the radiation-induced mutation (Fig. 2*B*). With the exception of a very small transcript in the testis, none of the normal transcripts, covering a range of sizes up to approximately 13 kb, were detectable in any of the mutant samples (Fig. 2*B*). However, aberrant-sized transcripts were detectable in the brain and testes of the mutant animals.

DNA Breakpoint in the *ld* Locus. To test the hypothesis that the altered expression of the gene at the *ld* locus was caused by a radiation-induced DNA structural rearrangement, we searched for an altered-size DNA fragment unique to the mutant allele and not detectable in the DNA from either the C3H or the 101 inbred parents. When the same region of cDNA that had been used for the Northern blot in Fig. 2*B* was employed as a probe, an altered-size DNA fragment was observed with several restriction enzymes, including *Eco*RI, *Xba* I, and *Hin*dIII (Fig. 3*A*). The observation that multiple enzymes generated a DNA fragment unique to the mutant allele suggested that this region of the *ld* locus had undergone a structural rearrangement.

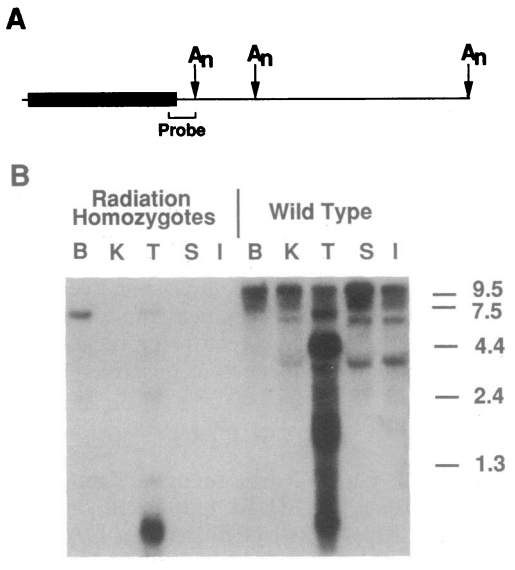

FIG. 2. RNA expression from a complex gene located at the *ld* locus. (*A*) Structure of mRNAs expressed from this gene as derived from overlapping cDNA clones (R.P.W. *et al.*, unpublished results). Solid box represents a putative protein-coding region; narrow lines represent noncoding regions. The designation A_n refers to the sites of polyadenylylation. (*B*) Northern blot of poly(A)$^+$ RNA from the adult brain (lanes B), kidney (lanes K), testes (lanes T), salivary gland (lanes S), and small intestine (lanes I) of the radiation-induced homozygous mutant (on the left) and wild-type (on the right) animals. A total of 500 μg of total RNA was oligo(dT)-selected and loaded into each lane of the gel. The probe was a 0.7-kb fragment of cDNA corresponding to the sequence immediately 5′ to the first polyadenylylation site (in *A*). Numerals refer to RNA size markers in kb.

To characterize the structural rearrangement, the normal 6.5-kb fragment and the corresponding mutant 7.5-kb *Eco*RI fragment (Fig. 3*A*) were cloned and analyzed (Fig. 3*B*). Hybridization and nucleotide-sequencing experiments revealed that the two clones diverged in sequence at a position approximately 0.9 kb from the 3′ end of the normal 6.5-kb fragment (Fig. 3*B*; S.J.B., C. Tancongco, and R.P.W., unpublished results). Therefore, the 7.5-kb fragment arose from a structural alteration of the *ld* locus.

Mouse–hamster somatic cell hybrids were used to determine whether this structural alteration represented a DNA breakpoint region in the 2^{17} chromosome associated with the inserted sequences from chromosome 17. A DNA probe, corresponding to a 0.2-kb restriction fragment unique to the region 3′ to the breakpoint on the mutant 7.5-kb fragment (Fig. 3*B*), was hybridized to DNA from somatic cell hybrids containing, along with other mouse chromosomes, either both chromosomes 2 and 17 (hybrid line BEM 1-4) or just mouse chromosome 17 (hybrid line R44-1) (23). Results showed that the probes hybridized to chromosome 2 but not to chromosome 17 (data not shown). Therefore, the alteration in the *ld* locus represented by the cloned 7.5-kb fragment did not involve the DNA breakpoint associated with the insertion of the chromosome 17 sequences.

In additional experiments, this same probe, derived from the region 3′ to the breakpoint on the 7.5-kb fragment, was hybridized to DNA from 93 *M. spretus/M. musculus* backcross animals (ref. 18; R. van der Meer-de Jong, M.E.D., R.P.W., L. Stubbs, C. Hetherington, and B.L.M.H., unpublished results). *Msp* I digestion of the DNA allowed us to differentiate the *M. spretus* from the *M. musculus* hybridizing alleles and to determine the genetic distance of the sequence 3′ to the breakpoint on the 7.5-kb fragment relative to the normal *ld* and *a* loci, which had been mapped previously in this manner (R. van der Meer-de Jong *et al.*, unpublished results). As shown in Fig. 4, 13 backcross animals (14%, with

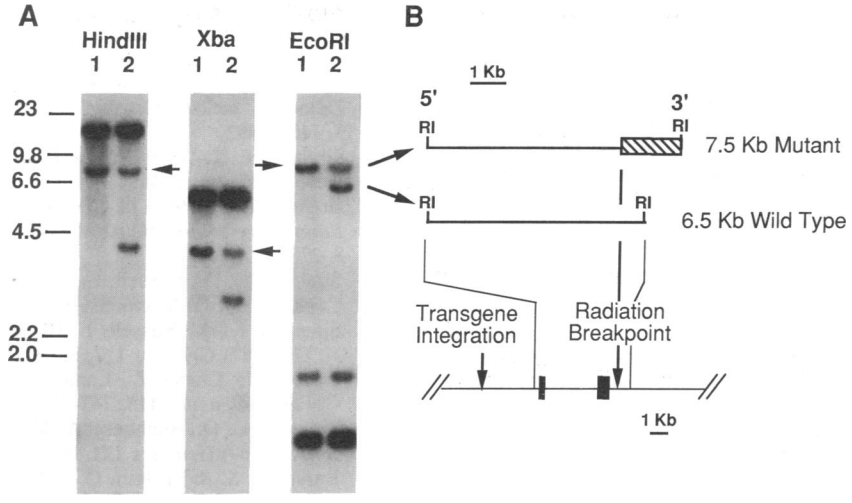

FIG. 3. Identification of rearranged DNA fragments at the *ld* locus of the radiation-induced mutation. (*A*) Genomic DNA, prepared from the liver of mice homozygous (lanes 1) or heterozygous (lanes 2) for the radiation-induced mutation, was digested with *Xba* I, *Hin*dIII, or *Eco*RI, transferred to a nylon membrane, and probed with the 0.7-kb fragment shown in Fig. 2*A*. Several genomic fragments were detected with this cDNA probe; arrows identify DNA fragments unique to the mutant locus. Numerals refer to DNA size markers in kb. (*B*) Structure of the cloned 7.5-kb mutant and corresponding normal 6.5-kb *Eco*RI fragments. The boxed region at the 3′ end of the 7.5-kb clone represents a segment of the rearranged portion of the *ld* locus in the mutant allele. Below this, the position of the 6.5-kb normal fragment on a larger region of genomic DNA is also shown, along with the position of the radiation-induced breakpoint in the mutant allele and the transgene integration site in the insertional mutant, ld^{Hd} (7). Small solid boxes represent two exons of the gene on the genomic DNA (R.P.W. *et al.*, unpublished results).

95% confidence limits 7.7 and 22.7) were recombinants between the breakpoint sequence on the 7.5-kb fragment and the *ld* locus. This is comparable to the established 20 cM distance between *ld* and *a* loci (22). However, there were no recombinants between this sequence and the *a* locus (upper 95% confidence limit of zero recombination frequency, 4%) indicating that the sequence 3′ to the breakpoint on the mutant 7.5-kb fragment is normally tightly linked to the *a* locus. Therefore, the *ld* locus was joined to sequences at or very close to the *a* locus to form the structurally altered 7.5-kb *Eco*RI fragment on the mutant allele.

DISCUSSION

A mouse mutation that is allelic to both *ld* and *a* arose in a radiation-mutagenesis experiment. Cytogenetic analysis revealed that a short interstitial segment of Chromosome 17 (17B-17C; or, possibly, inverted 17A2-17B) was inserted into the distal region of chromosome 2, proximal to band 2H2 and that a large segment in the 2^{17} chromosome, immediately proximal to the insertion site, did not match the 2E4-2H1 region of the normal chromosome 2 (Fig. 1). Molecular analysis revealed that a region at or near the *a* locus had been joined to the *ld* locus in the mutant allele. Based on the genetic, cytogenetic, and molecular findings presented here, we are proposing that the nonmatching interstitial segment, probably containing bands 2E4-2H1, was inverted on the 2^{17} chromosome, with DNA breaks and sequence disruptions in the *ld* and *a* loci. Also, based on the cytogenetic analysis, we are speculating that the 17B-17C (or possibly, 17A2-17B) interstitial segment of chromosome 17 had integrated at a point immediately adjacent and distal to the 2E4-2H1 inverted region of the 2^{17} chromosome. On the basis of the available evidence concerning this structural rearrangement, we have designated the mutation Is(17;In2)*ld*,a^{j}1Gso. For convenience, the name of the rearrangement will be abbreviated to Is*l*Gso, and the genotype at the mutant loci will be designated as $Id^{In2}a^{jIn2}$.

	No. of Backcross Animals					
	48	32	8	5	0	0
limb deformity	S	B	B	S	S	B
agouti	S	B	S	B	S	B
breakpoint sequence	S	B	S	B	B	S

FIG. 4. Analysis of 93 N2 progeny derived from a (C57BL/10-Nimr+/Sey^{MH} × *Mus spretus*)F_1 × C57BL/10Nimr backcross (ref. 18; R. van der Meer-de Jong *et al.*, unpublished results). Genotypes at the *a* locus were scored by examination of coat color [agouti versus black (nonagouti)]. For the genotype analysis of the *ld* locus, genomic DNA samples were digested with *Hin*dIII and probed with a 1.5-kb *Sau*3A–*Pvu* II fragment located approximately 7 kb on the 3′ side of the transgene integration site in the ld^{Hd} allele (7). For genotyping of the breakpoint sequence, samples were digested with *Msp* I and probed with a 0.2-kb *Pvu* II fragment ("breakpoint sequence") derived from the region on the 3′ side of the breakpoint on the cloned 7.5-kb mutant *Eco*RI shown in Fig. 3*B*. B, C57BL/10 allele; S, *M. spretus* allele. Each column represents the genetic composition of the chromosome inherited from the F_1 parent for the three loci. The numeral above each column represents the number of progeny that inherited a given chromosome.

The DNA breakpoint within the ld^{In2} allele corresponds to a position that is approximately 8 kb on the 3′ side of the site where the transgene integrated in the insertional allele, ld^{Hd} (7) (summarized in Fig. 3*B*). The close proximity of the structural alteration in each of these alleles not unexpectedly leads to a similar disruption in the expression of a complex gene at the *ld* locus. In experiments to be described elsewhere (D. Maas *et al.*, unpublished results), the analysis of RNA from several adult tissues indicated that the alteration in the expression of this gene in the ld^{In2} allele is similar to that observed with the ld^{Hd} allele. The fact that the expression of this gene is disrupted by both of these independent mutations of *ld* provides compelling evidence that this particular transcription unit represents the gene that is directly related to the mutant phenotype.

The inversion in the structurally altered chromosome 2 in the Is*l*Gso rearrangement has allowed us to jump directly from the cloned region of the *ld* locus to a region closely linked to, or within, the *a* locus. The position of this cloned region relative to the *a* locus, in addition to its association with the *a* locus phenotype of the mutation described here,

leads us to hypothesize that the breakpoint region on the 7.5-kb mutant fragment (Fig. 3) is very close to, or possibly within, the gene directly associated with the extreme-nonagouti ("jet") phenotype. If correct, this is of considerable significance in that it will now provide us with molecular probes to initiate a study of the molecular biology of the genes situated at the *a* locus. However, before any detailed analysis can be performed, it will be necessary to map this putative agouti-region probe relative to the mutations at this locus. A number of agent-induced mutations that have been generated over the past several decades should be particularly useful for this purpose.

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