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Molecular characterization of a radiation-induced reverse mutation at the dilute locus in the mouse

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Summary

Irradiation has been employed successfully to increase the reverse mutation rate at the agouti and dilute loci in the mouse. The dilute allele has previously been shown to be due to the insertion of an ecotropic-specific murine leukaemia virus in the vicinity of the dilute locus, and its instability to be due to the excision of the proviral sequence (Jenkins *et al.* 1981). Molecular analysis of the recovered radiation-induced revertant at the dilute locus indicated excision of all but approximately 500 bp of the proviral sequence. The proviral sequence remaining in the mouse genome hybridizes to a probe specific for the proviral long terminal repeat (LTR) sequence. Previous characterization of two spontaneous reverse dilute mutations indicated precise proviral excision of all but a single LTR, and suggests homologous recombination between the proviral LTR sequences as the mechanism of proviral excision (Hutchison, Copeland & Jenkins 1984). The present results indicate that radiation and increases the reverse mutation rate at the dilute locus acts by a similar mechanism, and suggest that mutagenic treatment may be employed to produce genetic variants of interest.

1. Introduction

The unstable dilute mutation carried by DBA/2 and related strains of the mouse has been shown to be due to the insertion of an ecotropic-specific MuLV DNA sequence (Jenkins *et al.* 1981). Analysis of two independent revertants has shown a precise excision of the proviral sequence leaving one exact copy of the LTR (Hutchison, Copeland & Jenkins, 1984), and strongly supports the suggestion of homologous recombination between the LTR sequences as the mechanism of proviral excision (Copeland, Hutchison & Jenkins, 1983). In a recent germ-cell mutation experiment in mice (Favor, Neuhäuser-Klaus & Ehling, 1987) one reverse mutation was recovered at each of the agouti and dilute loci. Since the dilute allele has been shown to be associated with an ecotropic-specific murine retroviral sequence, molecular probes were available to characterize the reversion event.

2. Materials and Methods

High-molecular-weight (HMW) DNA was isolated from spleens of individual mice according to standard procedures. Briefly, the tissues were homogenized in the presence of proteinase K and extracted with phenol:chloroform:isoamyl-alcohol. The HMW nucleic acids were ethanol-precipitated, re-dissolved in 10 mM Tris-HCl (1 mM EDTA, pH 7·5) and treated with pancreatic RNase, proteinase K, and extracted with phenol:chloroform:isoamyl-alcohol. After ethanol precipitation DNA was dissolved in 10 mM Tris-HCl and dialysed against 10 mM Tris-HCl. Some 10 μg of the DNA was digested to completion with an excess of Eco R1 restriction endonuclease. The DNA fragments were separated on 0·7% agarose gels, transferred on to a nitrocellulose filter and hybridized to a ^{32}P-labelled DNA probe (Amersham nick translation kit) as described by Southern (1975). The hybridizations were carried out in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 20 mM-Na_2HPO_4/NaH_2PO_4, 2·5% dextran sulphate and 0·1 μg/ml salmon sperm DNA. Filters were washed under stringent conditions (0·1 × SSC and 0·1% SDS solution at 65 °C) and autoradiographed at −70 °C with Kodak XOMAT AR film. DNA probes employed included a retroviral *env* region, specific for ecotropic murine retroviral sequences (Chattopadhyay *et al.* 1980), a *v-mos* oncogene probe (Van Beveren *et al.* 1981), a 3 kb genomic sequence which was subcloned as a Pst 1 endonuclease fragment from the 18 kb Eco

R1 sequence containing the *Emv-3* insert (Copeland, Hutchison & Jenkins, 1983) and a probe specific for the U3 region between the Pst 1 and the Kpn 1 restriction sites of the AKR MuLV LTR (Etzerodt *et al.* 1984).

3. Results

(i) *Origin of mutations*

The original mutants were recovered among 15931 offspring of the cross T-stock × DBA/2, in which the DBA/2 male parents were irradiated with 3 + 3 Gy. The T-stock (Russell, 1951), is genetically *a/a*; *b/b*; c^{ch}/c^{ch}; *d,se/d,se*; *p/p*; *s/s* (non-agouti; brown; chinchilla; dilute and short ear; pink-eyed dilution; piebald), while DBA/2 has the genotype *a/a*; *b/b*; *d/d* (non-agouti; brown; dilute). Resultant offspring from the cross T-stock × DBA/2 are expected, in the absence of a newly occurring mutation, to be genetically *a/a*; *b/b*; $c^{ch}/+$; *d,se/d*, +; *p/+*; *s/+*, and to express the non-agouti, brown, dilute phenotype. The reverse mutation at the agouti locus was recovered as a mouse expressing the white-bellied agouti, brown, dilute phenotype. The reverse mutation at the dilute locus resulted in a mouse with the phenotype non-agouti, brown, non-dilute. The independent parental crosses from which the reverse mutants arose each produced 40 or more offspring. This information is consistent with the hypothesis that the mutations were not pre-existing spontaneous mutations segregating in the stocks. Schlager & Dickie (1971) have studied the spontaneous forward and reverse mutation frequencies in the mouse at a set of loci including agouti and dilute. Their observed spontaneous reverse mutation frequency from *a* to a^+ was 34/8, 167, 854 and from *d* to d^+ was 9/2, 286, 472. The presently observed reverse mutation frequencies at the agouti and dilute loci following parental male irradiation were, respectively, 15 and 16 times higher than the spontaneous rate, suggesting that radiation treatment increased the reverse mutation rate ($P = 0{\cdot}066$, agouti locus, $P = 0{\cdot}067$, dilute locus; Fisher's exact test). Combining probabilities (Fisher, 1954) yields a *P*-value between 0·05 and 0·025 ($\chi^2 = 10{\cdot}81$, 4 D.F.) and leads to the conclusion that the reversion frequency is increased due to paternal irradiation.

In the T-stock the dilute allele is closely linked (0·16 cM) to the short ear (*se*) allele, while in strain DBA/2 the dilute allele is linked to the wild-type allele at the *se* locus. Therefore, an outcross to a stock of mice with the genotype *d,se/d,se* was used to confirm genetically the suspected reverse mutation at the dilute locus. Results of the cross to the *d,se/d,se* stock indicate that, of 78 offspring examined, 43 were non-dilute with normal external ears and 35 were dilute, short-eared. These results are consistent with the Mendelian expectation for a backcross of a heterozygote to a homozygous recessive. More importantly, it indicates that the reverse mutation occurred at the dilute locus linked to the wild-type short-ear allele. Thus the reverse mutation arose in the genome derived from the radiation-treated DBA/2 parent, further supporting the conclusion that radiation treatment increased the frequency of reverse mutation at the dilute locus. The mutation has been maintained by backcrossing to strain DBA/2, outcrossing to the *d,se/d,se* stock, or after backcrossing to DBA/2 by crossing *inter se* to recover homozygotes.

(ii) *Molecular characterization of the reverse mutation at the dilute locus*

Characteristic for DBA/2 and related strains carrying the dilute allele is an insertion into the dilute region of the mouse genome of an ecotropic murine leukaemia retrovirus, designated *Emv-3* (Jenkins *et al.* 1982), Restriction endonuclease analysis of DNA from offspring resulting from the cross of the original mutant to *d,se/d,se* confirms the presence of the *Emv-3* insert in an 18 kb Eco R1 DNA fragment which hybridizes to a probe specific for the ecotropic murine leukaemia retroviral genome (indicated by arrow, Fig. 1). Mice which are homozygous for the dilute allele (Fig. 1, lanes 3, 4 and 5) have similar levels of DNA, which hybridize to the ecotropic-specific probe relative to the amount of a 15 kb Eco R1 DNA fragment, which hybridizes to the probe for the *c-mos* oncogene sequence, employed as an internal control of the amount of DNA applied per lane. In contrast, it was shown by densitometer analysis that the hybridization signal of the ecotropic-specific probe in a heterozygote for the reverse mutation (Fig. 1, lane 2) is one-half the hybridization signal for the *c-mos* sequence and similar to the hybridization signal for the ecotropic-specific probe of a dilute homozygote in which one-half the amount of genomic DNA was applied (Fig. 1, lane 4).

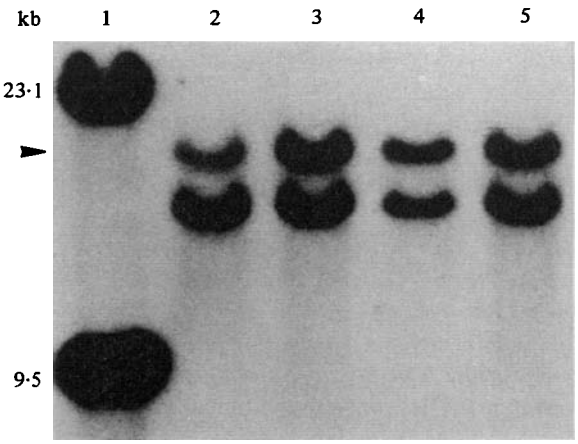

Fig. 1. Characterization of the endogenous ecotropic murine leukaemia proviral insert *Emv-3* in DNA of homozygous dilute (*d/d* or *d,se/d,se*) and a reverse dilute mutation (d^+/d): lane 1, Hind III digested λ DNA standard marker fragments; lane 2, d^+/d; lane 3, *d/d* litter-mate; lane 4, identical to lane 3 but one-half the amount of DNA; lane 5, *d,se/d,se*.

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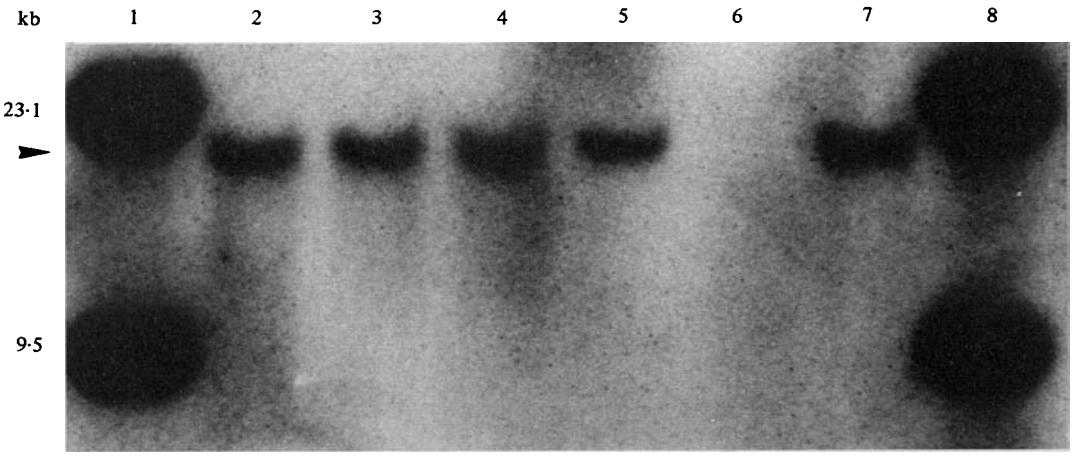

Fig. 2. Characterization of the endogenous ecotropic murine leukaemia proviral insert *Emv-3* in homozygous dilute (DBA/2, T-stock, *d,se/d,se*), heterozygous and homozygous reverse mutants: lanes 1 and 8, standard marker DNA fragments; lanes 2 and 7, DBA/2; lane 3, T-stock; lanes 4 and 5, d^+/d; lane 6, d^+/d^+.

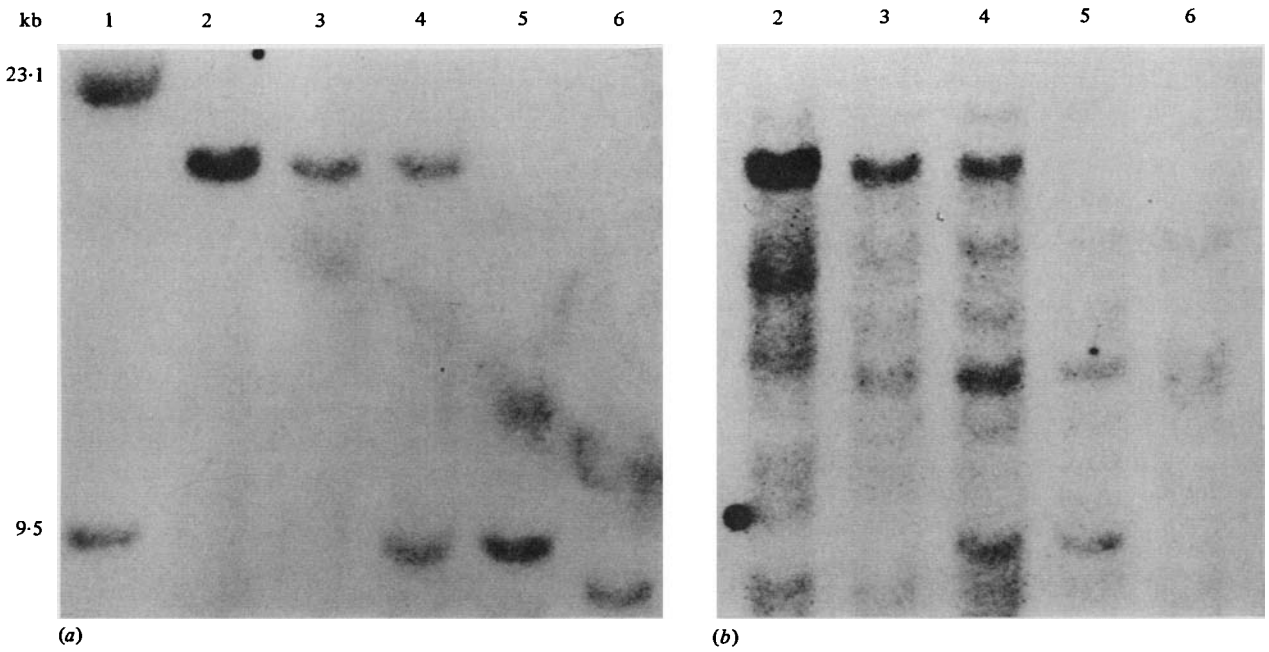

Fig. 3. Characterization of the flanking DNA sequence in the dilute region of the mouse for homozygous dilute (DBA/2, T-stock), heterozygous or homozygous for a reverse mutation at the dilute locus, and homozygous wild-type (NMRI) mice: lane 1, standard marker DNA fragments; lane 2, T-stock d/d; lane 3, DBA/2 d/d; lane 4, d^+/d; lane 5, d^+/d^+; lane 6, NMRI $+/+$. (*a*) The filter was hydridized to a probe specific for a 3-kb dilute region genomic sequence flanking the *Emv-3* insert. (*b*) Association of the dilute region in mutant dilute, revertant and wild-type alleles with a retroviral LTR sequence. The DNA blot is identical to that used in Fig. 3*a*. The nitrocellulose filter was dehybridized and subsequently hybridized to a probe specific for the U3 region between the Pst 1 and the Kpn 1 restriction sites of the AKR MuLV LTR (Etzerodt *et al.* 1984).

These results are consistent with the interpretation that a loss of the *Emv-3* insertion of the ecotropic murine leukaemia retroviral sequence resulted in the reverse mutation at the dilute locus, as was demonstrated for spontaneous reverse mutations at this locus (Copeland, Hutchison & Jenkins, 1983; Jenkins *et al.* 1981).

To test this hypothesis, homozygous d^+/d^+ individuals were recovered by intercrossing heterozygotes and were analysed for the presence of the *Emv-3* insert of the ecotropic murine leukaemia retroviral genome. Fig. 2 shows that homozygotes for the reverse mutation (lane 6) contain no DNA sequence which hybridizes with the ecotropic-specific probe, although the parental genotypes DBA/2 and T-stock from which the reverse mutation arose as well as the *d,se/d,se* stock with which the mutation is maintained all contain the *Emv-3* insert. This supports the hypothesis that the radiation-induced reverse mutation at the dilute locus is associated with the loss of the *Emv-3* insertion of the murine ecotropic retrovirus, known to be inserted close to or within the dilute locus and to

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result in the dilute mutation carried by strain DBA/2 and other related strains of mice (Copeland, Hutchison & Jenkins, 1983; Jenkins *et al.* 1981).

The remaining flanking DNA sequence in the d^+ reverse mutation was molecularly characterized by hybridization to a 3 kb genomic DNA probe specific for the dilute region. DNA from mice homozygous for the dilute allele (Fig. 3*a*, lanes 2 and 3) or heterozygous (Fig. 3*a*, lane 4) contain an 18 kb DNA Eco R1 restriction fragment which hybridizes to the genomic dilute region-specific probe. Mice heterozygous (Fig. 3*a*, lane 4) or homozygous (Fig. 3*a*, lane 5) for the reverse mutation contain a 9·5 kb Eco R1 fragment which hybridizes to the genomic dilute region-specific probe. By comparison, mice homozygous for the wild-type allele at the dilute locus (Fig. 3*a*, lane 6), contain a 9·0 kb DNA fragment which hybridizes to the genomic dilute region-specific probe. These results indicate that approximately 0·5 kb from the proviral sequence remained in the flanking DNA region upon excision of the *Emv-3* provirus. This was supported by results from a hybridization employing a murine retroviral long terminal repeat-(LTR) specific probe. Both an 18 kb Eco R1 restriction DNA fragment of the dilute mutant allele (Fig. 3*b*, lanes 2, 3 and 4) and a 9·5 kb fragment of the revertant allele (Fig. 3*b*, lanes 4 and 5) hybridize to the LTR sequence, whereas the 9·0 kb fragment of the wild-type allele (Fig. 3*b*, lane 6) does not hybridize to the LTR-specific probe. Results are thus consistent with the hypothesis that the reverse dilute mutation recovered after parental radiation involves excision of all but one LTR copy of the *Emv-3* retroviral insert. Hutchison, Copeland & Jenkins (1984) have shown that exactly one copy of the 523 bp LTR from the *Emv-3* insert remained in two spontaneous dilute revertants. The authors suggest homologous recombination as a mechanism which would explain the precise nature of the excision event. That the DNA sequence of the radiation-induced revertant allele is approximately 500 bp longer than the wild-type allele and also hybridizes to an LTR-specific probe is consistent with this hypothesis. However, confirmation must await DNA sequence analysis of the revertant.

4. Discussion

At least two mechanisms of crossing over between homologous LTR sequences resulting in a precise excision of all but one LTR sequence are possible. An unequal crossover between homologous chromosomes or between sister chromatids would lead to one crossover product containing one copy of the LTR sequence and the other crossover product to contain the sequence LTR–internal sequence–LTR–internal sequence–LTR. Alternatively, an intrachromosomal crossover event between the LTR sequences, as demonstrated for delta–delta recombination in yeast (Roeder & Fink, 1980), would result in a chromosome with one LTR sequence and a circularized proviral sequence with one LTR. In Drosophila it has been shown that X-irradiation increases the reversion frequency of unstable alleles at the yellow, scute, singed and white loci (Eeken & Sobels, 1986; Green, 1961). It could be demonstrated that these unstable alleles are due to the insertion of transposable elements (Eeken, 1982; Modolell, Bender & Meselson, 1983; Rasmuson *et al.* 1981). By employing a chromosome inversion or a ring chromosome in Drosophila, it was shown that the inhibition of crossing over between homologous chromosomes or sister chromatids did not influence the rate of reversion of unstable alleles (Green, 1961, 1967). These results would argue for an intrachromosomal crossover event as the mechanism of precise proviral excision.

The demonstration of unstable mutations is a useful criterion with which to identify alleles possibly associated with the insertion of transposable elements. For example, the non-agouti allele has also been shown to revert spontaneously (Schlager & Dickie, 1971) and the reversion frequency was increased following parental irradiation (Favor, Neuhäuser-Klaus & Ehling, 1987). However, if the mechanism of excision of the transposable elements resulting in the loss of all but one terminal repeat should prove to be the rule, then it should be emphasized that only those alleles will be observed to be unstable in which a proviral insertion occurs at a site such that gene expression is disrupted when the entire proviral sequence is present but not when a single terminal repeat sequence, as large as *c.* 0·5 kb, is present. Certainly other mutations also exist associated with the insertion of a transposable element such that upon excision the remaining terminal repeat sequence continues to disrupt gene expression. Thus, unstable alleles represent only one class of mutation associated with the insertion of transposable elements.

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References

Chattopadhyay, S. K., Lander, M. R., Rands, E. & Lowy, D. R. (1980). Structure of endogenous murine leukemia virus DNA in mouse genomes. *Proceedings of the National Academy Science (USA)* **77**, 5774–5778.

Copeland, N. G., Hutchison, K. W. & Jenkins, N. A. (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.

Eeken, J. C. J. (1982). The stability of mutator (MR)-induced X-chromosomal recessive visible mutations in Drosophila melanogaster, *Mutation Research* **96**, 213–224.

Eeken, J. C. J. & Sobels, F. H. (1986). The effect of X-irradiation and formaldehyde treatment of spermatogonia

on the reversion of an unstable, P-element insertion mutation in Drosophila melanogaster. *Mutation Research* **175**, 61–65.

Etzerodt, M., Mikkelsen, T., Pedersen, F. S., Kjeldgaard, N. O. & Jorgensen, P. (1984). The nucleotide sequence of the Akv murine leukemia virus genome. *Virology* **134**, 196–207.

Favor, J., Neuhäuser-Klaus, A. & Ehling, U. H. (1987). Radiation-induced forward and reverse specific locus mutations and dominant cataract mutations in treated strain BALB/c and DBA/2 male mice. *Mutation Research* **177**, 161–169.

Fisher, R. A. (1954). *Statistical Methods for Research Workers*. Edinburgh: Oliver & Boyd.

Green, M. M. (1961). Back mutation in Drosophila melanogaster. I. X-ray-induced back mutations at the yellow, scute and white loci. *Genetics* **46**, 671–682.

Green, M. M. (1967). The genetics of a mutable gene at the white locus of Drosophila melanogaster. *Genetics* **56**, 467–482.

Hutchison, K. W., Copeland, N. G. & Jenkins, N. A. (1984). Dilute-coat-color locus of mice: nucleotide sequence analysis of the d^{+2J} and d^{+Ha} revertant alleles. *Molecular and Cellular Biology* **4**, 2899–2904.

Jenkins, N. A., Copeland, N. G., Taylor, B. A. & Lee, B. K. (1981). Dilute (d) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. *Nature* **293**, 370–374.

Jenkins, N. A., Copeland, N. G., Taylor, B. A. & Lee, B. K. (1982). Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. *Journal of Virology* **43**, 26–36.

Modollel, J., Bender, W. & Meselson, M. (1983). Drosophila melanogaster mutations suppressible by the suppressor of Hairy-wing are insertions of a 7·3-kilobase mobile element, *Proceedings of the National Academy of Science (USA)* **80**, 1678–1682.

Rasmuson, B., Westerberg, B. M., Rasmuson, A., Gvozdev, V. A., Belyaeva, E. S. & Ilyin, Y. V. (1981). Transpositions, mutable genes, and the dispersed gene family Dm225 in Drosophila melanogaster. *Cold Spring Harbor Symposia on Quantitative Biology* **45**, 545–551.

Roeder, G. S. & Fink, G. R. (1980). DNA rearrangements associated with a transposable element in yeast. *Cell* **21**, 239–249.

Russell, W. M. (1951). X-ray-induced mutations in mice. *Cold Spring Harbor Symposia on Quantitative Biology* **16**, 327–336.

Schlager, G. & Dickie, M. M. (1971). Natural mutation rates in the house mouse. Estimates for five specific loci and dominant mutations. *Mutation Research* **11**, 89–96.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

Van Beveren, C., van Straaten, F., Galleshaw, J. A. & Verma, I. M. (1981). Nucleotide sequence of the genome of a murine sarcoma virus. *Cell* **27**, 97–108.