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Loss of heterozygosity at the *dilute–short ear* (*Myo5a–Bmp5*) region of the mouse: mitotic recombination or double non-disjunction?†

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Summary

The occurrence of homozygous-viable *dilute–short ear* (*Myo5a–Bmp5*) double mutants in mouse specific locus mutation experiments has generally been assumed to be the result of double non-disjunction such that the mutant inherits two copies of chromosome 9 carrying the recessive alleles from the test-stock. A homozygous viable *Myo5a–Bmp5* double mutant was recovered recently in our laboratory. We were able to genetically analyse both the *Myo5a–Bmp5* region and proximal and distal markers in the original mutant as well as in offspring of the original mutant. Our results indicate the mutational event to be due to mitotic recombination and not double non-disjunction.

1. Introduction

The specific locus mutation test of the mouse was developed to screen efficiently for germ line mutations at seven autosomal loci distributed among five chromosomes (Russell, 1951). Virtually all known aspects of genome instability following mutagenic insult resulting in transmitted germ line mutations in mammals are based upon specific locus mutation experiments. Equally important has been the vast array of mutant alleles generated in such mutagenesis experiments, the study of which has provided information about the nature of induced mutations as well as the organization of the mouse genome.

The tightly linked chromosome 9 markers *dilute* (*Myo5a*) and *short ear* (*Bmp5*) have proved to be especially useful since the newly occurring mutant alleles can usually be genetically and/or phenotypically distinguished from the marker alleles and can easily be maintained for analysis. Indeed, less than 10 years after the first publication on the specific locus mutation test results, genetic analyses of mutations in the *Myo5a–Bmp5* region were presented (Russell & Russell, 1960). Detailed genetic (Russell, 1971) and molecular (Rinchik *et al.*, 1985, 1986) analyses of mutations at the *Myo5a–Bmp*5 region followed and provided important information pertaining to the genetic organization of the region and the nature of the alterations of the genome resulting in mutant alleles.

One class of mutations in the region, *Myo5a–Bmp5* double mutants, has been of particular interest. Most have been shown to be due to a deletion involving both loci (Russell, 1971; Rinchik *et al.*, 1985, 1986). However, one group of *Myo5a–Bmp5* presumed double mutations was identified to be homozygous-viable, indistinguishable from the $Myo5a^{d}$–$Bmp5^{se}$ alleles carried by the test-stock, and remained elusive to further genetic or molecular analyses. As an initial hypothesis, the mechanism involved in the occurrence of such mutations was assumed to be double non-disjunction such that the original mutant inherited two copies of chromosome 9 from the test-stock parent and no copies from the homozygous wild-type parent (Russell & Russell, 1960; Russell, 1971). A number of other mechanisms could also account for the homozygous-viable double mutants, including simultaneous mutations at both loci, gene conversion or mitotic recombination, but methodologies were not in place to analyse and differentiate among the mechanisms when the mutants were recovered.

With the recent characterization of the mouse genome for highly polymorphic microsatellite markers

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(Dietrich *et al*., 1994) this situation has changed and an analysis of newly occurring mutants for flanking genetic markers is now possible and practical. Due to eventual crossing over it was imperative that an original homozygous-viable *Myo5a–Bmp5* double mutant as well as the immediate segregants from the original mutant be analysed. We were fortunate to have recovered such a mutation recently (Ehling *et al*., 1997) and we undertook the appropriate analyses. Results presented here indicate the mechanism of occurrence of the double mutant to be mitotic recombination and not double non-disjunction.

2. Materials and methods

(i) *Original mutant*

A female mouse expressing the whole-body recessive phenotype for the closely linked chromosome 9 markers *Myo5a* and *Bmp5* was recovered in the offspring of male, homozygous wild-type (102 × C3H) F1 hybrid mice treated with 10 mg 1-(2-chloroethyl)-3-cycohexyl-1-nitrosourea per kilogram body weight and mated to untreated test-stock females (Ehling *et al*., 1997). The test-stock is homozygous for the recessive visible markers *a*, *non-agouti*; *Tyrp1*^{b}, *brown*; *Tyr*$^{c\text{-}ch}$, *chinchilla*; *Myo5a*^{d}, *dilute*; *p*, *pink-eyed dilution*; *Ednrb*^{s}, *piebald*; *Bmp5*^{se}, *short ear*. The loci *Myo5a* and *Bmp5* are tightly linked (0·12 cM) on chromosome 9 and the loci *p* and *Tyr* are linked (15 cM) on chromosome 7 (Roderick *et al*., 1996). In the absence of mutation, the resultant offspring from the cross are expected to be heterozygous at the seven marker loci and to express the wild-type phenotype.

(ii) *Genetic characterization crosses*

The genetic analysis of the original mutant and the outcross progeny followed the procedures outlined and discussed by Russell & Russell (1960). The original mutant was crossed to a stock *Myo5a*$^{d\text{-}op}$*–+/Myo5a*^{d}*–Bmp5*^{se} and offspring were classified for phenotype at the *Myo5a* and *Bmp5* loci to determine whether the original mutant carrier was due to a deletion of the entire *Myo5a–Bmp5* region. This hypothesis would be supported by the observation that the original mutant was heterozygous for a *Myo5a* mutant allele that does not complement the *Myo5a*$^{d\text{-}op}$ allele and expresses the opisthotonus phenotype. In addition, the original mutant was outcrossed to homozygous wild-type mice, resulting offspring being collected and crossed to the *Myo5a*$^{d\text{-}op}$*–+/Myo5*^{d}*–Bmp5*^{se} stock as outlined above.

All mice utilized in the specific locus mutation experiment, as well as in the genetic and molecular analyses (see below), were obtained from colonies maintained in Neuherberg.

(iii) *Genotyping for microsatellite markers*

Genomic DNA extraction, polymerase chain reaction (PCR) amplifications and agarose gel electrophoresis of PCR products were carried out as previously described (Favor *et al*., 1997). Briefly, DNA was extracted from the liver. All PCR amplifications were carried out according to the manufacturer's specifications (Boehringer Master Mix Taq polymerase, Mannheim, Germany) with the following amplification programme: 4 min 94 °C denaturation followed by 30 cycles of (1 min 94 °C denaturation; 1 min 55 °C annealing; 2 min 72 °C extension) and a final extension at 72 °C for 5 min. Amplification products were electrophoretically separated (100 V, 1·5–2 h) in 3% or 5% agarose gels (BioZym, SeaKem LE) and visualized by ethidium bromide staining. The specific primers to the Mit microsatellite markers (Dietrich *et al*., 1994) were synthesized and provided by Utz Linzner, GSF-Institute of Pathology/BIODV, Neuherberg.

3. Results

(i) *Genetic characterization*

In the cross 'original mutant' × *Myo5a*$^{d\text{-}op}$*–+/Myo5a*^{d}*–Bmp5*^{se}, a total of 40 offspring were produced. Seventeen offspring expressed the dilute, short ear phenotype and 23 offspring were observed with dilute, normal ear phenotype, indicating the original mutant to be homozygous recessive at the *Myo5a* and *Bmp5* loci. Further, none of the 23 dilute, normal ear offspring expressed the opisthotonus phenotype associated with null alleles at the *Myo5a* locus, indicating that the original mutant was not heterozygous for a deletion covering the *Myo5a* and *Bmp5* loci.

A further test of 18 offspring recovered from the outcross of the original mutant to homozygous wild-type mice supported this conclusion. These outcross mice were crossed to the *Myo5a*$^{d\text{-}op}$*–+/Myo5a*^{d}*–Bmp5*^{se} stock and the offspring were phenotypically classified as outlined above. For all 18 matings at least one offspring expressing the dilute, normal ear phenotype was observed before ending the mating. None of the dilute, normal ear offspring expressed the opisthotonus phenotype. Together these results strongly suggest that the original mutation is not due to a multi-locus deletion of the *Myo5a–Bmp5* region.

(ii) *Haplotype analyses by genotyping for chromosome 9 microsatellite markers*

We first tested a number of chromosome 9 Mit microsatellite markers for their appropriateness for haplotype analyses to differentiate between chromosomes derived from the test-stock or (102 × C3H) F1

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Table 1. *Characterization of allele sizes for the chromosome 9 microsatellite markers carried by test-stock and (102 × C3H) F1 mice*

	PCR fragment length (bp)	
Marker	Test-stock	(102 × C3H) F1
D9Mit297	104	112
D9Mit4	124	140
D9Mit12	93	82
D9Mit18	180	210

hybrid mice. For a marker to be informative the allele carried by the test-stock must differ from the allele carried by the strains C3H and 102. For convenience we imposed the further constraint that the alleles carried by strains C3H and 102 should be the same. Four markers were chosen (Table 1) based upon the above criteria as well as chromosomal position (Imai, 1997), amplification efficiency and allele size differences. The markers *D9Mit297* (chromosomal position cM 15) and *D9Mit4* (chromosomal position cM 29) are proximal to the *Myo5a–Bmp5* region (chromosomal position cM 42), while the markers *D9Mit12* (chromosomal position cM 55) and *D9Mit18* (chromosomal position cM 71) are distal to the *Myo5a–Bmp5* region. Genotype analyses indicated the original mutant to be heterozygous for the two proximal markers and homozygous for the test-stock allele for the two distal markers.

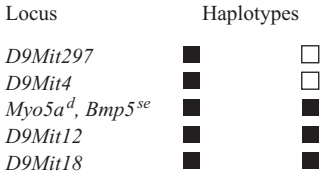

Fig. 1. Putative chromosome 9 haplotypes carried by the original *Myo5a–Bmp5* double mutant. Black squares designate the allele carried by test-stock; white squares designate the allele carried by strains C3H and 102.

As an initial hypothesis, we considered that the two proximal markers for which the original mutant was heterozygous were in coupling (Fig. 1). To test this hypothesis 19 offspring from the outcross of the original mutant with a (102 × C3H) F1 mouse were genotyped for the four informative chromosome 9 Mit markers. Results confirm this hypothesis (Fig. 2). There were 16 haplotypes in which the proximal markers were in coupling and three haplotypes in which the proximal markers were in repulsion recovered in the outcross offspring that segregated from the original *Myo5a–Bmp5* double mutant. Since the haplotypes for the proximal markers in coupling were more abundant we conclude these to be the parental

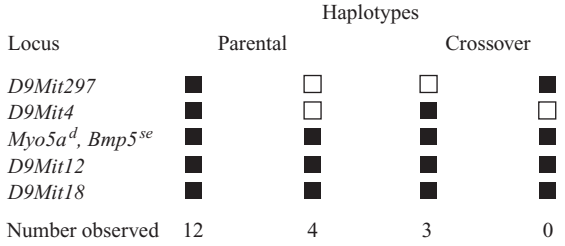

Fig. 2. Segregation analysis of the chromosome 9 haplotypes inherited from the original mutant in 19 offspring derived from the outcross of the original *Myo5a–Bmp5* double mutant to a (102 × C3H) F1 hybrid mouse. Although in this cross the *Myo5a* and *Bmp5* alleles could not be typed they are inferred to be $Myo5a^{d}$ and $Bmp5^{se}$ from the initial genetic analyses, indicating the original mutant to be homozygous $Myo5a^{d}$–$Bmp5^{se}$/$Myo5a^{d}$–$Bmp5^{se}$. Allele designations as in Fig. 1.

haplotypes. It follows that the less abundant haplotypes in which the proximal markers were in repulsion represent crossover haplotypes. The crossover frequency between *D9Mit297* and *D9Mit4* was 16%, which is in agreement with the expected genetic distance (Dietrich *et al.*, 1994). It should be noted, however, that there is a distortion in the segregation frequency of the two parental haplotypes ($\chi^2 = 4{\cdot}0$).

4. Discussion

A number of mechanisms have been proposed to explain the occurrence of *Myo5a–Bmp5* double mutants in specific locus mutation experiments (Russell & Russell, 1960). They include: (1) two simultaneous mutational events at the *Myo5a* and *Bmp5* loci, (2) a single deletion event of the *Myo5a–Bmp5* region, (3) a single mutational event in the vicinity of the *Myo5a–Bmp5* region that affects the expression of both the *Myo5a* and *Bmp5* loci, (4) gene conversion, (5) mitotic recombination, or (6) double non-disjunction such that the original mutant carries two copies of chromosome 9 from the test-stock parent and no copy from the (102 × C3H) F1 hybrid parent.

Previous genetic analyses of *Myo5a–Bmp5* double mutations have identified two classes (Russell & Russell, 1960). Those *Myo5a–Bmp5* double mutants in which the mutational event was shown to be homozygous-lethal or did not complement the $Myo5a^{d\text{-}op}$ allele were concluded to be the result of a deletion of the entire *Myo5a–Bmp5* region. Detailed genetic (Russell, 1971) and molecular (Rinchik *et al.*, 1985, 1986) analyses have confirmed this conclusion. The second class of *Myo5a–Bmp5* double mutants consisted of mutational events in which the 'mutant allele' was not homozygous-lethal, could complement the $Myo5a^{d\text{-}op}$ allele and was indistinguishable from the $Myo5a^{d}$–$Bmp5^{se}$ alleles carried by the test-stock.

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This genetic information is consistent with the hypothesis that the homozygous-viable mutational event associated with the second class of *Myo5a–Bmp5* double mutations is not a deletion of the entire region, nothing more.

The haplotype analysis of the original *Myo5a–Bmp5* double mutant and a segregation analysis of the haplotypes in the outcross progeny allow further differentiation among the six alternative mechanisms proposed for the mutational event resulting in a *Myo5a–Bmp5* double mutation. The first four proposed mechanisms involve a single mutation, two independent mutations or a gene conversion event in the *Myo5a–Bmp5* region resulting in a chromosome derived from the (102 × C3H) F1 hybrid which does not complement the $Myo5a^{d}$–$Bmp5^{se}$ mutant alleles carried by the test-stock chromosome. In these cases the original mutant should be heterozygous for all flanking genetic markers where the allele carried by the test-stock differs from the allele carried by (102 × C3H) F1 hybrids, both proximal and distal to the *Myo5a–Bmp5* region, and the haplotypes carried by the original mutant should be in coupling. This was clearly excluded by our observations. For double non-disjunction of chromosome 9, the original *Myo5a–Bmp5* mutant would be homozygous for the test-stock alleles at all informative flanking markers. This mechanism was also excluded by our observations. Mitotic recombination may result in loss of heterozygosity for all markers distal to the recombination event. If the *Myo5a–Bmp5* double mutation were the result of a mitotic recombination event, the site of crossover must have been proximal to the *Myo5a–Bmp5* region. We would expect the original mutant to be heterozygous for the informative flanking markers proximal to the site of crossover and homozygous for the test-stock alleles $Myo5a^{d}$, $Bmp5^{se}$ and all informative distal flanking markers. Further, the haplotypes of the original mutant would be in coupling. Our results are consistent with this prediction and we conclude that the mitotic recombination event occurred distal to *D9Mit4* and proximal to the *Myo5a–Bmp5* region in a heterozygous zygote cell from the cross test-stock × (102 × C3H) F1 hybrid.

If loss of heterozygosity due to mitotic recombination were to occur, two descendant populations of genetically distinct cells will result. One population of cells will be fixed for the recessive alleles and the second population of cells will be fixed for the wild-type alleles. Should the mitotic recombination event occur at the 1-cell stage zygote, embryonic development will originate from two genetically distinct cell populations that are either homozygous wild-type or homozygous recessive. If, however, the mitotic recombination event were to occur at a later cleavage stage, embryonic development will proceed from three genetically distinct cell populations: the homozygous wild-type and homozygous recessive cells resulting from the crossover event as well as heterozygous cells that were descended from zygotic cells in which recombination did not occur.

Since the development of the mouse embryo proceeds from a small subpopulation of early embryonic cells, two scenarios are to be expected based on sampling error in which homozygous recessive cells are included in the embryo. First, the resulting embryo could be derived from a single cell type fixed for the recessive alleles. This apparently occurred in the mutant analysed here since it was a whole-body dilute, short ear phenotype and it was genetically confirmed to be homozygous $Myo5a^{d}$–Bmp^{se}/$Myo5a^{d}$–$Bmp5^{se}$. Alternatively, a resulting embryo could be derived from a mixture of cell types derived from the three genetically distinct cell populations with the genotypes $Myo5a^{d}$–$Bmp5^{se}$/$Myo5a^{d}$–$Bmp5^{se}$, +−+/+−+ or $Myo5a^{d}$–$Bmp5^{se}$/+−+. Although we have hypothesized that the original mutant that we report here was homozygous recessive, we cannot exclude the possibility that it was in fact mosaic with a very small contribution of homozygous wild-type and/or heterozygous cells. We have previously reported two *Myo5a–Bmp5* mosaic mutants (Favor & Neuhäuser-Klaus, 1994). Both mutants were shown to be gonosomic mosaics and the *Myo5a–Bmp5* mutational event could not be distinguished from the $Myo5a^{d}$–$Bmp5^{se}$ marker alleles carried by the test-stock. Unfortunately, the original mutants could not be genetically analysed for flanking markers.

We believe the present study to be the first to provide evidence for the occurrence of mitotic recombination in the early zygotic cell stages of the mouse such that the germ line of the affected embryo was involved and allowed a genetic analysis of the event. This is not surprising for a very rare event, considering the time, labour and cost requirements to conduct mutation experiments in the mouse compared with other laboratory genetic organisms. Indeed, in a detailed analysis of the *Myo5a–Bmp5* region, Russell (1971) listed a total of 235 mutational events of which 144 were single mutations at the *Myo5a* locus, 47 were single mutations at the *Bmp5* locus, 38 were deficiencies of the *Myo5a–Bmp5* region and only six were homozygous viable *Myo5a–Bmp5* double mutations and possibly due to mitotic recombination. The experience in Neuherberg is similar, where a total of six original mutants of this class have been reported (Ehling *et al*., 1982, 1997; Graw *et al*., 1986; Ehling & Neuhäuser-Klaus, 1989, 1994, 1995). The second limitation to the demonstration of mitotic recombination in the mouse has been the availability of the means to analyse flanking genetic markers in the original mutants. This has only recently become practical with the characterization of the mouse genome for microsatellite loci (Dietrich *et al*., 1994).

Finally, although genetic segregation analyses of the putative crossover products were not possible mitotic recombination has been previously implicated in the mouse (Carter, 1952; Wallace, 1964; Grüneberg, 1966; Geissler *et al*., 1981; West, 1992). Stronger evidence for mitotic recombination was presented by an analysis of flanking genetic markers in the affected cells (Bremner & Balmain, 1990; Panthier *et al*., 1990; Henson *et al*., 1991; De Sepulveda *et al*., 1995). The occurrence of twin spots in mice heterozygous for coat colour loci in which the descendants of the two daughter cells following mitotic recombination are phenotypically distinct from each other as well as from the heterozygous non-recombinant cell type also represents strong evidence for mitotic recombination (Russell, 1964; Bateman, 1967; Fahrig & Neuhäuser-Klaus, 1985; Fisher *et al*., 1986). All four cases involved the mouse chromosome 7 coat colour loci *p* and/or *Tyr*. These loci are also included in the specific locus mutation test but, to our knowledge, no *p–Tyr* double mutant or mosaic has ever been reported. The discrepancy between the occurrence of homozygous-viable double mutants for the *Myo5a–Bmp5* region of chromosome 9 but not for the *p–Tyr* region of chromosome 7 is especially interesting since mitotic recombination of chromosome 7 apparently can occur in somatic cells in later stages of embryonic development. This discrepancy is probably due to the distribution of imprinting domains among the mouse chromosomes.

A detailed summary for the mouse is given in the MRC-Mammalian Genetics Unit Imprinting Database (http://www.mgu.har.mrc.ac.uk/imprinting/implink.html). Essentially, a number of regions of mouse chromosome 7 are imprinted and only embryos carrying both a maternal and a paternal copy will survive (Searle & Beechey, 1990; Ferguson-Smith *et al*., 1991; Guillemot *et al*., 1995; Beechey *et al*., 1997). Non-disjunction or mitotic recombination at an early zygotic stage will result in uniparental disomy for a chromosome or chromosome region that is not compatible with survival. This appears not to be the case for mouse chromosome 9, although a single gene has recently been identified which undergoes imprinting (Plass *et al*., 1996). However, embryos which are uniparental disomics for the entire or partial chromosome 9 are fully viable.

Evidence for mitotic recombination requires a haplotype analysis via genotyping of flanking genetic markers. For multicellular diploid organisms, this consists of an analysis of the original mutant as well as a haplotype segregation analysis of its offspring. Although these requirements have in the past rarely been fulfilled, molecular biological techniques have provided adequate evidence for the occurrence of mitotic recombination and indicated this mechanism to be of relevance in human heritable disease and carcinogenesis (Gallie & Worton, 1986; Müller & Scott, 1992; Sengstag, 1994; Ramel *et al*., 1996; Gupta *et al*., 1997). The increased powers of analysis provided by molecular biological techniques, the ever-increasing progress in the molecular and genetic characterization of the mouse genome, and the possibilities of segregation analysis in the mouse should open up the possibility of differentiating among the various mechanisms of mutation in future studies.

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