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Dominant hemimelia and *En-1* on mouse chromosome 1 are not allelic

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Summary

Previous studies have shown that *En-1*, a homeobox-containing gene, maps close to or at the *Dh* locus in the mouse. Since homeobox-containing genes are key genes in the control of development the close proximity of *En-1* to the developmentally significant gene *Dh* raised the possibility that the *Dh* mutation represented a mutant allele of *En-1*. A genetic analysis involving *En-1*, *Dh*, and other chromosome 1 markers (*Emv-17*, *ln* and *Pep-3*) shows that although *Dh* and *En-1* are closely linked they are separable by recombination (4/563). The likely gene order and recombination frequencies of these loci are: *ln* ($5{\cdot}2 \pm 0{\cdot}9$) *Emv-17* ($1{\cdot}1 \pm 0{\cdot}4$) *Dh* ($0{\cdot}7 \pm 0{\cdot}4$) *En-1* ($3{\cdot}0 \pm 0{\cdot}7$) *Pep-3*. This shows that *Dh* is not a mutant allele of *En-1*.

1. Introduction

In the mouse there are two homeobox-containing *Engrailed* genes. These genes, *En-1* and *En-2*, share extensive sequence similarity to the *Drosophila engrailed* gene (Joyner *et al.* 1985; Joyner & Martin, 1987), which is known to be important in establishing and maintaining the segmented body plan during embryogenesis (Kornberg, 1981*a, b*; Lawrence & Struhl, 1982). *En-1* has previously been cloned and mapped, via recombinant inbred strain analysis, to the central portion of chromosome 1 (Hill *et al.* 1987; Joyner & Martin, 1987). This finding localised the *En-1* gene close to, or at the locus of the developmentally significant gene, Dominant hemimelia (*Dh*).

The *Dh* mutation is a member of the luxoid group of mouse mutants which are characterized by a twisting of the fore- or hind-limbs and the reduction or loss of certain long bones (Gruneberg, 1963). Members of this group, which includes luxate (Carter, 1951; 1953; 1954) and luxoid (Forsthoefel, 1958, 1959), also exhibit a wide variety of visceral abnormalities. In heterozygous form, *Dh* causes preaxial abnormalities of the hind limb, asplenia, a reduced number of pre-sacral vertebrae and ribs, a shortened coelom, a small and defective digestive tract and abnormalities of the urogenital system (Searle, 1964; Green, 1967). In homozygous form the abnormalities are more severe and are usually fatal. In 1967 Green demonstrated that, in both *Dh*/+ and *Dh*/*Dh* animals, the earliest morphological defect was in the splanchnic mesoderm at 9·5 days and she postulated that the pleiotropic effects of the *Dh* mutation were all traceable to this tissue. This hypothesis assumes that the *Dh* gene interferes with the normal structural arrangement of the cells of the splanchnic mesoderm. One of the major problems with the functional analysis of mammalian homeobox-containing genes has been the lack of associated developmental mutations, which were so vital in establishing the importance and function of the *Drosophila* genes (Nusslein-Volhard & Wieschaus, 1980). The observation that a homeobox gene (*En-1*) mapped in the vicinity of a developmental mutation (*Dh*) was therefore very provocative. It prompted the question of whether *Dh* represented a mutant allele of *En-1* and was the impetus for the work described below. The overall aim of this study was to determine whether *Dh* and *En-1* were separable by meiotic recombination and thus determine whether these genes were in fact allelic.

2. Materials and methods

(i) *Generation and maintenance of mice stocks*

FZT strain mice were obtained from MRC Radiobiology Unit, Harwell where they had kindly been

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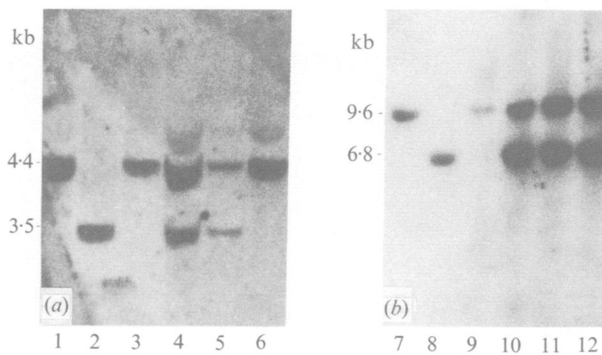

Fig. 1. Southern blot showing restriction fragment length polymorphism for *En-1* and *Emv-17*. (*a*) Genomic DNA digested with *Taq* I and hybridized to pEN1471. *En-1* polymorphisms produced 3·5 kb (*En-1^b*) and 4·4 kb (*En-1^d*) bands. (*b*) Genomic DNA digested with *Eco*R I and hybridized to pPS1·25. *Emv-17* polymorphisms produced 6·8 kb (*Emv-17^a*) and 9·6 kb (*Emv-17^b*) bands. DNA in lanes 1, 2, 7 and 8 were from 'type-strains' DBA/2 (lanes 1 and 7) and C57BL/6 (lanes 2 and 8); DNA in other lanes was from mice produced in the DH and DHF crosses used for the genetic linkage analysis. The high molecular weight bands in lanes 4 and 6 were the result of incomplete enzymatic digestion.

recovered from frozen stocks by Mr Peter Glenister. FZT mice are segregating for both *Dh* and *ln* on chromosome 1 and were used to produce two stocks of mice. Heterozygous (*ln Dh*/+ +) FZT individuals were crossed to inbred C57BL/OlaWs mice and used after 2–4 backcross generations (DH stock; also *ln Dh*/+ +). A homozygous *ln*/*ln* subline of FZT was later derived (*ln Dh*/*ln*+). This was used in place of the DH stock in the later crosses because it obviated the need for testcrosses to ensure that the *ln* allele was retained. In each stock the *ln-Dh* chromosome also carried *En-1^d*, *Pep-3^b* and *Emv-17^b*. For the linkage analysis heterozygous mice were produced by crossing DH or *ln*/*ln* FZT animals to C57BL/OlaWs. These two groups of heterozygotes are designated 'DH' and 'DHF' respectively. Each has the same genotype for the five chromosome 1 alleles used in the analysis: *ln Emv-17^b Dh En-1^d Pep-3^b*/+ *Emv-17^a* + *En-1^b Pep-3^a*.

The LIII strain of mice, which is an outbred linkage testing stock for chromosome 1, was obtained from MRC Radiobiology Unit, Harwell. LIII mice are homozygous for *ln* and wild type for *Dh*. The mice were tested for their *Pep-3*, *Emv-17* and *En-1* genotypes. Those mice which were homozygous at all three loci were used to maintain stocks. The genotype of the LIII mice used as the homozygous parents in the linkage study was: *ln Emv-17^b* + *En-1^d Pep-3^b*/*ln Emv-17^b* + *En-1^d Pep-3^b*.

(ii) *Assay of peptidase-3 in blood*

Peptidase-3 is a red blood cell (rbc) enzyme, the different allozymes of which can be distinguished electrophoretically (Lewis & Truslove, 1969; Chapman, Ruddle & Roderick, 1971). Haemolysates were prepared from washed, packed rbc. Blood from the tail was spun in a microfuge (13500 rpm, 2 min) and the plasma removed. The rbc were then washed twice in 0·9% saline before adding $\frac{1}{2}$ volume of distilled water. The lysate was then frozen and thawed twice to ensure complete lysis and adequate liberation of the enzyme. Electrophoresis was performed on 60 × 76 mm, Titan III Cellulose acetate plates (Helena Laboratories, Gateshead, UK). These plates were pre-soaked in Tris-Borate-EDTA buffer (0·09 M Tris, 0·05 M boric acid, 0·002 M-EDTA) for 20 min. The plates were removed from the TBE buffer immediately before use and gently blotted to remove any excess buffer. 10 μl of haemolysate was loaded per well of the multiwell loading plate (Helena Labs) and then applied to the cathodal end of the cellulose plate using the Super Z applicator (1 application = 0·25 μl). The plate was then placed sample side down across the zip-zone electrophoresis chamber (Helena Labs) and 200 V were applied for 30 min after which they were stained to assay for peptidase activity by the method of Dr Jo Peters (MRC Radiobiology Unit, personal communication). The stain comprised 2 ml 0·1 M phosphate buffer pH 7·0 (2·28 g $K_2HPO_4.3H_2O$, pH to 7·0), 20 μl $MnCl$ 3·15 g/100 ml, 40 μl *Crotalus adamanteus* snake venom 10 mg/ml (Sigma), 40 μl peroxidase 10 mg/ml (Sigma), 80 μl L-leucine-L-tyrosine 10 mg/ml (Sigma), 80 μl 4-amino-9-ethylcarbazole (1% in dimethyl formamide made up fresh just before use, Sigma). Components were added in the above order and then mixed gently with 2 ml of 2% agar and the entire mixture poured evenly over the electrophoresed cellulose plate and allowed to set. The plate was then wrapped in cling-film and incubated at 37 °C for 1–2 h. Peptidases stain up reddish brown on a yellow background.

(iii) *Preparation of tail tip DNA*

Mice were anaesthetised and the terminal 1 cm of tail removed into ice cold 0·9% saline using a pre-heated scalpel blade. The tail tip was chopped up and placed in an Eppendorf tube containing 700 μl of homogenization buffer (50 mM Tris, pH 8·0, 100 mM-EDTA, 100 mM-NaCl, 1% sodium dodecyl sulphate). This was incubated overnight at 55 °C in the presence of 35 μl proteinase K (10 mg/ml in distilled H_2O) following which 20 μl of RNase was added and this was then incubated at 37 °C for a further 1–2 h. The tube was then filled with phenol, shaken vigorously and then centrifuged for 15 min in a microfuge. The aqueous phase along with the interface was removed to a fresh tube and filled up with phenol:chloroform (1:1) shaken and centrifuged for 15 min. Again the aqueous phase along with the interphase was removed to a fresh tube and filled up with chloroform, shaken

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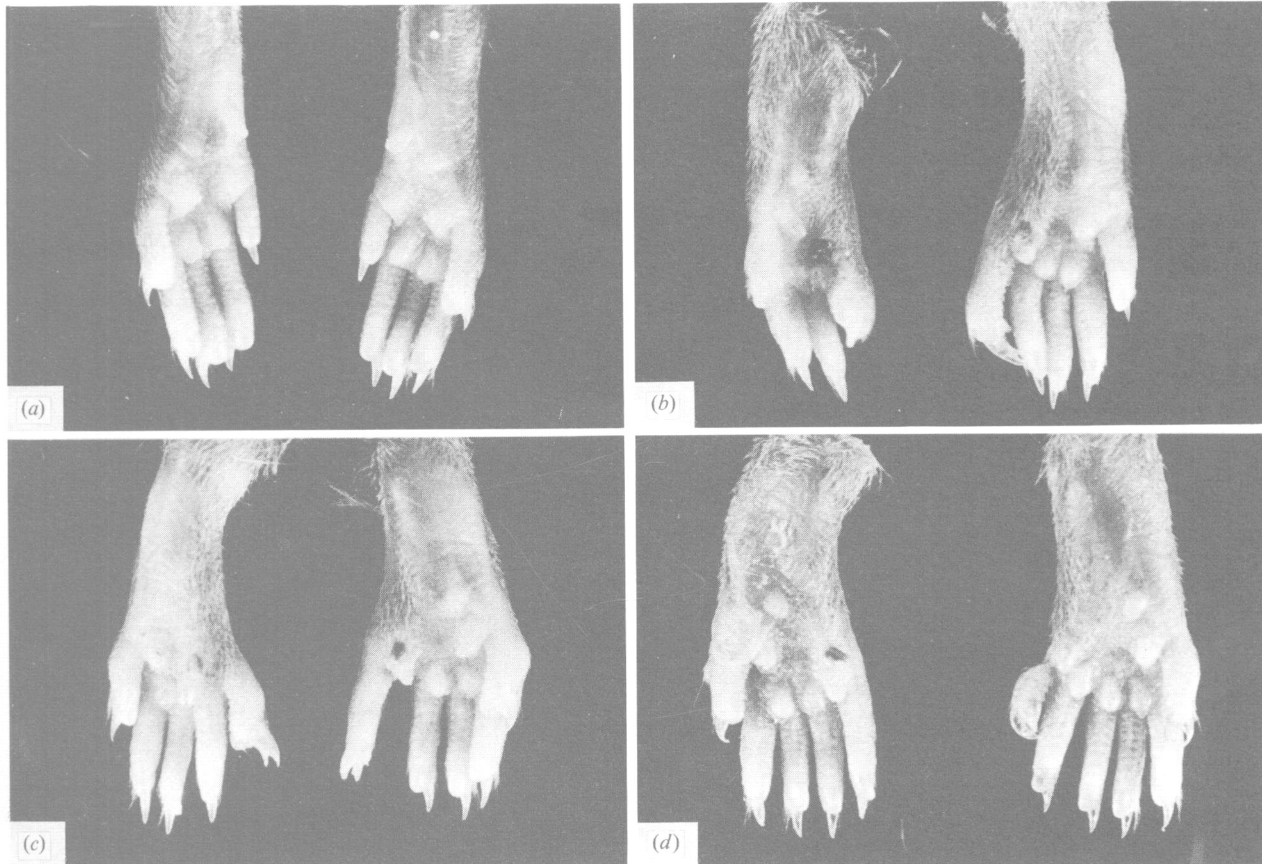

Fig. 2. Underside view of hind feet (left and right refer to left and right of mouse). (*a*) Normal (+/+) hind feet, (*b–d*) from *Dh*/+ mice. (*b*) Oligodactyly of left foot, lengthening of right hallux. (*c*) Thickening/lengthening of left hallux, lengthening of right hallux, note the abnormal nail on both affected digits. (*d*) Lengthening of left hallux, polydactyly preaxial to the right hallux.

and centrifuged for 15 min. The aqueous phase was removed to a fresh Eppendorf, this time leaving any interphase behind, filled up with isopropanol, and the tube was inverted several times until the DNA formed a stringy precipitate whereupon it was spooled out from the tube using a glass Pasteur pipette (the pipette had been flamed to seal the end). Spooled DNA was then dipped in 70% ethanol, air dried and finally resuspended in 100–200 μl of TE buffer (10 mM Tris, 1 mM-EDTA). As the yield between samples varied the DNA concentration was checked by reading the optical density at 260 nm.

(iv) *Southern blot hybridization*

Southern blot hybridization of genomic DNA was performed as previously described (see Hill *et al.* 1987). The probes employed in this study were pEN1471 which is a 2·6 kb *Bam*H I–*Eco*R I genomic fragment which hybridises to *En-1* and pPS1·25 which is a 1·25 kb *Pst* I–*Sst* II fragment of genomic sequences flanking the 5′ region of the *Emv-17* provirus of RF/J mice (Buchberg *et al.* 1986). Examples are shown in Fig. 1.

(v) *Statistics*

χ^2 tests and other calculations were performed on an Apple Macintosh Computer.

3. Results

(i) *Phenotype of the* Dh *mutation*

Classification of all offspring generated in this study, with regard to whether they carried the *Dh* gene, was achieved initially by the examination of the hind limbs. Searle (1964) had previously reported that the expressivity of the gene with regard to the hind-limb abnormalities was variable and included: (1) slight thickening/lengthening of the hallux (big toe), (2) polydactyly pre-axial to the hallux (i.e. extra toe anterior to the limb axis), (3) oligodactyly (loss of digits) and (4) luxation (dislocation) and reduction in the length of one or both hind limbs.

The limb abnormalities exhibited by the mice generated in this study certainly confirm the variable expression and included all of the above (see Fig. 2), although the most common abnormality was a lengthening of the hallux. Although the long bones of

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the hind limbs were not directly examined, Fig. 3 shows mice which had long bone abnormalities; this again showed a variable expression. The abnormalities of either the digits or the long bones were not necessarily the same on both limbs.

Asplenia is a constant feature of the *Dh* mutation in both the hetero- and homozygous animals. On the basis of spleen classification Searle found the *Dh* mutation to be 100% penetrant but only 96% penetrant on the basis of hind limb abnormalities. As the initial classification of *Dh*/+ mice in this study was based on hind limb abnormality the above observations would suggest that some *Dh*/+ animals would go undetected. However all offspring were checked for the presence or absence of spleen (approximately 550 animals). There was complete concordance with regard to hind-limb abnormalities and asplenia.

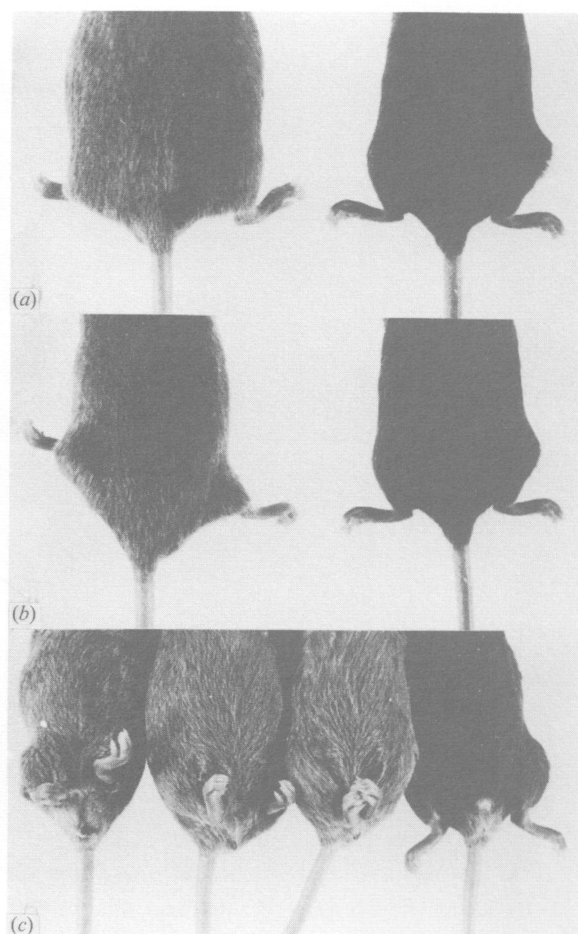

Fig. 3. (*a*) and (*b*) show dorsal view of *Dh*/+ (left) and +/+ (right) mice. (*c*) A ventral view of three *Dh*/+ (left) and one +/+ (far right) mouse. Note the variable expression of the abnormality with regard to the long bones and that the abnormalities are not necessarily the same on both limbs.

(ii) *Recovery of the* Dh *mutation*

Heterozygous *Dh*/+ mice were outcrossed to wild-type (+/+) mice, 50% of the offspring were expected to be heterozygous for *Dh*. The data are summarized in Table 1. *Dh*/+ mice were classified by limb morphology and asplenia at autopsy. From the data presented in Table 1 it can be seen that without exception the number of *Dh*/+ animals recovered from either cross is significantly lower than the numbers expected assuming all *Dh*/+ animals are viable. In contrast, Searle (1964) found that although fewer *Dh*/+ mice were recovered than expected in a standard outcross analysis the difference was not significantly different from the expected (44·7 *vs*. 50% expected). The animals in this study were scored at weaning whereas Searle scored for *Dh* at birth. Searle also reported that the survival rate to weaning for

Table 1. *Inheritance of the* Dh *mutation and expectations for autosomal inheritance assuming all heterozygotes are viable*

Parent	Progeny			Observed	Expected	Significance†
	Dh/+	+/+	Total	% *Dh*/+	% *Dh*/+	χ^2
DH cross						
Dh/+ Female	13	39	52	25·0	50	13·00*
Dh/+ Male	65	165	230	28·3	50	43·48*
Total	78	204	282	27·7	50	56·30*
DHF cross						
Dh/+ Female	69	113	182	37·9	50	10·64*
Dh/+ Male	33	66	99	33·3	50	11·00*
Total	102	179	281	36·3	50	21·10*
Combined data						
Dh/+ Female	82	152	234	35·0	50	20·94*
Dh/+ Male	98	231	329	29·8	50	53·77*
Total	180	383	563	32·0	50	73·20*

* $P < 0{\cdot}001$.
† χ^2 tests the significance of departure from the Mendelian expectation if all heterozygotes are viable.

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Table 2. *Total recombination events observed between the* In–Dh–Pep-3 *loci in both the DH and DHF crosses*

Cross: $\frac{\textit{ln Dh Pep-3}^b}{+\ +\ \textit{Pep-3}^a} \times \frac{\textit{ln}\ +\ \textit{Pep-3}^b}{\textit{ln}\ +\ \textit{Pep-3}^b}$

Gene combinations	*ln*	*Dh*	*Pep-3*	Heterozygous parent: Female	Male	Total
Parental type	*ln*	*Dh*	*b*	73	92	165
	+	+	*a*	124	218	342
Recombinant (*ln–Dh*)	*ln*	+	*a*	18	7	25
	+	*Dh*	*b*	4	6	10
Recombinant (*Dh–Pep-3*)	*ln*	*Dh*	*a*	5	0	5
	+	+	*b*	10	6	16
Double recombinants	*ln*	+	*b*	0	0	0
	+	*Dh*	*a*	0	0	0

Recombinant fractions:	Heterozygous female	Heterozygous male	Total
ln–Dh	22/234 = 9·4 ± 1·9	13/329 = 4·0 ± 1·1	35/563 = 6·2 ± 1·0
Dh–Pep-3	15/234 = 6·4 ± 1·6	6/329 = 1·8 ± 0·7	21/563 = 3·7 ± 0·8
ln–Pep-3	37/234 = 15·8 ± 2·4	19/329 = 5·8 ± 1·3	56/563 = 9·9 ± 1·3

Dh/+ animals was 73%. Assuming 44·7% recovery at birth followed by 73% survival to weaning then approximately 32·6% of weanlings would be expected to be *Dh*/+ heterozygotes. Similarly if the frequency of *Dh* was 50% at birth the expected weaning frequency would be 36·5%. In this study the observed frequency at weaning was 180/563 (32·0%) overall which is not significantly different from 32·6% ($\chi^2 = 0{\cdot}10$, $P > 0{\cdot}05$) but is significantly different from 36·5% ($\chi^2 = 4{\cdot}98$, $P < 0{\cdot}05$). The recovery rates for the *Dh*/+ animals in the DH and DHF cross were 27 and 36% respectively neither of which are significantly different from 32·6%. Thus it would seem that although the recovery of *Dh*/+ animals at weaning is slightly lower than expected this difference may be accounted for by a small deficiency at birth plus a reduced viability to weaning.

(iii) *Close linkage of Engrailed-1 to the dominant hemimelia locus on chromosome 1*

A standard outcross analysis was used to determine the recombination frequency between *En-1* and *Dh* as well as three closely linked markers, the endogenous murine leukaemia virus locus *Emv-17*, the coat colour gene leaden (*ln*) and the red blood cell enzyme, Peptidase-3 (*Pep-3*). The specific aims of this analysis were (1) to test whether *Dh* and *En-1* were separable by recombination and, if so, (2) to determine the genetic distance and orientation of *En-1* relative to *Dh*. Heterozygous parental animals were derived from *Dh*/+ mice as described in Materials and Methods. The genotypes of the mice used are shown in Tables 2 and 3.

Tail tissue was collected from each recombinant animal for DNA preparation. Southern blots of tail tip DNA were hybridised with the *En-1* and *Emv-17* probes respectively. The *Emv* and *En* genotype was determined using restriction fragment length polymorphism (RFLP) analysis; *En-1* exhibits a *Taq* I RFLP between C57BL/6 (3·5 kb) and DBA (4·4 kb) mice using the pEN1471 probe whereas *Emv-17* exhibits an *Eco*R I RFLP using the pPS1·25 probe. This probe hybridizes to a 9·6 kb band in DBA strain DNA and a 6·8 kb band in C57BL/6 strain DNA. The *Pep-3* genotype was determined electrophoretically whereas *ln* and *Dh* were determined morphologically.

The linkage study was carried out in two stages. Male and female mice from either the DH or DHF stocks were mated to LIII mice and the offspring were initially scored for *ln*, *Dh* and *Pep-3*. Since there were no statistically significant differences between the results obtained with either the DH or DHF stocks (Higgins, 1991) the data from both crosses were pooled. The results of this recombination analysis are shown in Table 2. From this it can be seen that a total of 563 animals were scored of which 56 showed recombination within the *ln–Dh–Pep-3* region. These 56 recombinant animals were then further analysed at the molecular level. This two step experimental design avoided the necessity for the molecular analysis of all offspring by focusing on those animals known to be recombinant in the *ln–Dh–Pep-3* region. The results of this further analysis are shown in Table 3. From this data it can be seen that although *Dh* and *En-1* are closely linked they are separable by recombination (4/563). The likely gene order and recombination frequencies of these loci are: *ln* (5·2 ± 0·9) *Emv-17* (1·1 ± 0·4) *Dh* (0·7 ± 0·4) *En-1* (3·0 ± 0·7) *Pep-3*. These

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Table 3. *Molecular analysis of recombinant animals observed in the DH and DHF crosses*

Cross: $\dfrac{In\ Emv\text{-}17^b\ Dh\ En\text{-}1^d\ Pep\text{-}3^b}{+\ Emv\text{-}17^d +\ En\text{-}1^b\ Pep\text{-}3^a} \times \dfrac{In\ Emv\text{-}17^b +\ En\text{-}1^d\ Pep\text{-}3^b}{In\ Emv\text{-}17^b +\ En\text{-}1^d\ Pep\text{-}3^b}$

Gene combinations					Heterozygous parent		
In	*Emv-17*	*Dh*	*En-1*	*Pep-3*	Female	Male	Total
In–Dh recombinants							
In	*d*	+	*b*	*a*	15	6	21
+	*b*	*Dh*	*d*	*b*	3	5	8
In	*b*	+	*b*	*a*	3	1	4
+	*d*	*Dh*	*d*	*b*	1	1	2
Dh–Pep-3 recombinants							
In	*b*	*Dh*	*b*	*a*	1	0	1
+	*d*	+	*d*	*b*	1	2	3
In	*b*	*Dh*	*d*	*a*	4	0	4
+	*d*	+	*b*	*b*	9	4	13

No other gene combinations were found.

Recombinant fractions:	Heterozygous female	Heterozygous male	Total
In–Emv-17	18/234 = 7·7 ± 1·7	11/329 = 3·3 ± 1·0	29/563 = 5·2 ± 0·9
Emv-17–Dh	4/234 = 1·7 ± 0·8	2/329 = 0·6 ± 0·4	6/563 = 1·1 ± 0·4
Dh–En-1	2/234 = 0·9 ± 0·6	2/329 = 0·6 ± 0·4	4/563 = 0·7 ± 0·4
En-1 Pep-3	13/234 = 5·6 ± 1·5	4/329 = 1·2 ± 0·6	17/563 = 3·0 ± 0·7
In–En-1	24/234 = 10·3 ± 2·0	15/329 = 4·6 ± 1·2	39/563 = 6·9 ± 1·1
Emv-17–En-1	6/234 = 2·6 ± 1·0	4/329 = 1·2 ± 0·6	10/563 = 1·8 ± 0·6
Emv-17 Pep-3	19/234 = 8·1 ± 1·8	8/329 = 2·4 ± 0·8	27/563 = 4·8 ± 0·9

data supersede the preliminary results published in abstract form (Higgins *et al.* 1990).

4. Discussion

A major goal in mammalian genetics is the construction of a high resolution linkage map which can provide the basis for the construction of molecular maps. The results presented here show that although *Dh* and *En-1* are closely linked together on the same region of chromosome 1, they are separable by recombination and *Dh* maps 0·7 ± 0·4 cM proximal to *En-1* and 1·1 ± 0·4 cM distal to *Emv-17*. These data provide genetic evidence that *Dh* is not simply a mutant allele of *En-1*. This result is substantiated by Martin *et al.* (1990) who were also able to demonstrate recombination between *Dh* and *En-1*. However one major discrepancy between the two studies is the reported genetic map distances. Martin *et al.* mapped *Dh* to a position 0·28 ± 0·28 cM proximal to *En-1* and 0·28 ± 0·28 cM distal to *Emv-17*, thus rendering a genetic distance of 0·55 cM over the entire *Emv-17–En-1* region compared to 1·8 cM estimated in this study. Combining the data from the two studies with regard to recombination between *Dh* and *En-1* (5/925) gives a recombination frequency of 0·54 ± 0·2.

The genetic map of the mouse is approximately 1600 cM and the haploid genome content is approximately 3×10^9 base pairs (bp). If the relationship between physical and genetic distance is constant one would expect that 1 cM would equal approximately 2 Mb. The genetic distance of 0·54 cM between *Dh* and *En-1* would therefore be equivalent to a physical distance of approximately 1·1 Mb. However the mouse genome does not adhere to this average (1 cM = 2 Mb) in a uniform way. A comparison of the physical and genetic maps around the agouti locus suggests that the ratio of physical to genetic distance in different regions exhibits a wide variation. In two adjacent segments of the agouti locus Barsh & Epstein (1990) reported values of < 150 kb/cM and 4 Mb/cM. In accordance with this data *Dh* could be as much as 2 Mb or as little as 80 kb away from *En-1* in molecular terms.

As *Dh* and *En-1* are separable by recombination, *Dh* is not likely to be a mutant allele of *En-1*. This conclusion is supported by the observation that *En-1* mRNA transcripts are of normal size and abundance in +/+, *Dh*/+ and *Dh*/*Dh* embryos (Martin *et al.*, 1990). In +/+ embryos, *En-1* is expressed in several tissues affected by *Dh* (vertebrae, ribs and limb buds) as well as tissues apparently unaffected by *Dh* (CNS) (Davidson *et al.* 1988; Davis & Joyner, 1988; Davis *et al.* 1991). *En-1* is not expressed in the splanchnic mesoderm, which Green (1967) proposed was the primary site of action of the *Dh* gene. Although *En-1*

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is not likely to be directly responsible for the *Dh* mutation, *Dh* and *En-1* may have some developmental relationship. For example, the *Dh* gene could cause ectopic expression of *En-1* in the splanchnic mesoderm of *Dh* animals.

In the mouse there are two *En* genes, *En-1* and *En-2*, which share extensive homology both with each other and with their *Drosophila* counterpart (Joyner *et al.* 1985; Joyner & Martin, 1987). We have shown that *En-1* maps 0·7 cM distal to *Dh* on chromosome 1 and Martin *et al.* (1990) reported that *En-2* maps 1·1 cM proximal to hemimelic extra toes (*Hx*) on chromosomes 5. *Dh* and *Hx* both cause similar phenotypic abnormalities. *Dh* causes pre-axial abnormalities of the hind limb and a reduction or absence of the tibia; *Hx* is a dominant mutation which causes preaxial polydactyly and hemimelia (Dickie, 1968; Knudsen & Kochhar, 1981). That each of the *En* genes show genetic linkage with essentially similar developmental mutations is certainly very provocative. Although the significance of these linkages has not been ascertained it is inviting to suggest that it is more than coincidence. The vertebrate genome is believed to have undergone several duplications during evolution (Lundin, 1979; Nadeau, 1989) and these linkages, on chromosomes 1 and 5 respectively, may represent a duplicated conserved linkage group. Both the *En-1–Dh* and *En-2–Hx* linkage groups are associated with integrated ecotropic provirus loci (*Emv-17* and *Emv-1* respectively), thus possibly extending the putative homologies between these regions (Martin *et al.* 1990). It has been suggested that a common ecotropic proviral integration site existed in the ancestral genome segment, but that the actual proviral sequences would not be part of this group as their insertion is a relatively recent event (Jenkins *et al.* 1982). Although ecotropic proviruses are found all over the genome it appears that integration occurs near developmentally active genes (Shih *et al.* 1980). If this region of the genome does represent a conserved linkage group it would suggest that *Dh* and *Hx*, *Hm* are paralogous genes. If so, the molecular cloning of either *Dh* or *Hx*, *Hm* may facilitate the cloning of the other.

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