Genet. Res., Camb. (1996), **68**, *pp.* 221–231 *With 5 text-figures*

Doublefoot: a new mouse mutant affecting development of limbs and head

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(*Recieved 8 August 1996 and in revised form 10 September 1996*)

Summary

The mutant doublefoot, *Dbf*, of the mouse arose spontaneously, and was shown to be inherited as an autosomal dominant, mapping 9–13 cM proximal to leaden, *ln*, on chromosome 1 and showing no recombination with the microsatellite markers *D1Mit24* and *D1Mit77*. In heterozygotes the phenotype includes many extra toes on all four feet, and the tibia and fibula may be reduced and bowed. The head is shortened and broad and the eyes are held half-closed, and some animals develop hydrocephalus. The tail is kinked and abnormally thick, and the soles of the feet are swollen. Growth is retarded, viability is reduced, and reproduction is impaired in both sexes. Only about 30% of males are normally fertile, and testis weights and sperm counts may be reduced, although this appears not to be the main cause of poor fertility. In females vaginal opening is delayed and oestrous cycles are irregular, although the animals appear to respond to gonadotrophic hormones. Crosses of *Dbf*/+ × *Dbf*/+ are very poorly fertile. Prenatally, *Dbf*/+ heterozygotes can first be recognized at $11\frac{1}{2}$ days gestation by abnormally broad fore limb buds. Putative *Dbf*/*Dbf* homozygotes at $12\frac{1}{2}$ days have similar limbs defects and also split face, due to failure of the maxillae to fuse in the midline. Some homozygotes and a few putative heterozygotes have cranioschisis. At $13\frac{1}{2}$ days, the heads of homozygotes tend to bulge in the frontal region and a bleb of clear fluid is visible medially. At $14\frac{1}{2}$ days *Dbf*/*Dbf* fetuses may have oedema and some are dead. From $15\frac{1}{2}$ days onwards no live *Dbf*/*Dbf* fetuses have been found. The gene maps close to the locus of *Pax3*, but crossovers between *Dbf* and *Pax3* have been found, ruling out the possibility that a gain-of-function mutation in *Pax3* might be involved.

1. Introduction

Naturally occurring mouse mutants provide a valuable resource for obtaining insight into developmental mechanisms. Much remains to be learned concerning the genetic control of limb and head development. Important information will undoubtedly be gained from knockouts of genes homologous with those known to affect developmental patterns in other organisms, such as the homeobox genes or paired-box genes. However, analysis of mouse mutants will still be needed, since these involve a range of partial loss- or gain-of-function mutations, and thus allow the dissection of gene regulation. Moreover, such mutants may provide better models for human genetic disorders. Many mouse mutants affecting limb development are already known. Several result in polydactyly, especially of the hind limbs. These include luxate, *lx*, luxoid, *lu*, Stong's luxoid, *lst*, hemimelic extra toes, *Hx*, dominant hemimelia, *Dh*, and extra-toes, *Xt*. Of these, the *Xt* phenotype has been shown to result from a mutation in the *Gli3* gene (Vortkamp *et al.*, 1992), and to be a homologue of Greig's cephalopolysyndactyly in man. The underlying molecular bases of most of the other forms of polydactyly and other limb defects are not known. We report here a spontaneous mutation, briefly described earlier (Lyon *et al.*, 1989), which from its map position is at a new genetic locus, and which produces striking abnormalities in limb and head development of heterozygotes and homozygotes, including polydactyly of all four feet.

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2. Materials and methods

The animal studies described in this paper were carried out under the guidance issued by the Medical

Research Council in 'Responsibility in the Use of Animals for Medical Research' (July 1993) and Home Office Project Licence no. 30/00875.

All animals were maintained under conventional conditions in the animal house of the MRC Mammalian Genetics Unit, Harwell.

For sperm counts, epididymal counts were obtained using a haemocytometer according to the method of Searle & Beechey (1974). Each caput epididymis was macerated in 0·2 ml of a 1% solution of trisodium citrate. The solution was made up to 2 ml, mixed well and allowed to settle for about 1 min. A drop of suspension was run into each chamber of a Neubauer haemocytometer, after the coverslip had been pressed down to show Newton's rings. Numbers of sperm heads were counted in the four large squares and the large central square, and the numbers shown are the counts obtained.

Preliminary mapping was performed by crossing to a set of linkage testing stocks carrying visible marker genes.

For molecular mapping *Dbf*/+ females were crossed to *M. spretus* males, and the F_1 female offspring backcrossed to C3H/HeH males. DNA was extracted from tails of backcross offspring using standard protocols (Hogan *et al.*, 1986). Primers were obtained from Research Genetics (Huntsville, AL) and polymerase chain reactions (PCR) were carried out in a buffer containing 1·5 mM $MgCl_2$, using an annealing temperature of 55 °C.

3. Results

(i) *Origin*

The original animal was an affected male which resulted from a spontaneous mutation in a cross between a C3H/HeH female and a 101/H male. It is not known whether the mutation occurred on the C3H/H or the 101/H chromosome. The animal had several extra toes on both fore and hind feet. The feet were abnormally broad, and the soles appeared swollen. The long bones of the limbs were shortened, the hind limbs were twisted, and the animal was smaller than its normal litter-mates. In addition, its snout was shortened and its head rather broad. The tail appeared abnormally thick and was softly kinked (Fig. 1).

When this original male was crossed with two unrelated (C3H/HeH × 101/H)F_1 (hereafter 3H1) normal females, he sired 28 similarly affected animals of both sexes and 19 normal animals. This was regarded as preliminary evidence that the phenotype was due to an autosomal dominant mutation, and the name and symbol doublefoot, *Dbf*, were given (Lyon *et al.*, 1989).

(ii) *Genetics*

The inheritance of doublefoot was studied by crosses to unrelated stocks and to 3H1 hybrids, and by linkage tests. In the initial crosses to normal animals (Table 1) an approximate 1:1 ratio of *Dbf*/+ to +/+ offspring was obtained. However, in later crosses with substantial data (Table 1, rows 3a and b) there was a deficiency of *Dbf*/+ offspring, which was statistically significant when the data from reciprocal crosses were pooled. Since the phenotype was striking and could be scored at birth, this deficiency is unlikely to be due to incomplete penetrance or postnatal death. It may be due to intrauterine or neonatal death.

Attempts were made to obtain *Dbf/Dbf* homozygotes by intercrossing *Dbf*/+ heterozygotes. However, no live offspring were obtained from such crosses and instead pregnant females in *Dbf*/+ × *Dbf*/+ crosses were dissected (see below).

Linkage tests were performed by crossing *Dbf*/+ animals with mice from stocks carrying visible marker genes. The results (Table 2) indicated the *Dbf* locus to be on chromosome 1, showing linkage with the markers fuzzy, *fz*, and leaden, *ln*. The order of markers *fz–Dbf–ln* requires the minimum number of double crossovers (five) and is taken to be the correct one. The recombination values obtained from male heterozygotes were markedly lower than those from females (Table 2), as is commonly found in linkage studies with mice. As in the outcrosses presented in Table 1, there was a severe shortage of *Dbf*/+ young, particularly among the offspring of *Dbf*/+ males (female heterozygotes $\chi^2 = 3{\cdot}83$, $P = 0{\cdot}05$; male heterozygotes, $\chi^2 = 30{\cdot}77$, $P < 0{\cdot}00001$). If, as is thought, this shortage is due to reduced viability of *Dbf*/+, rather than incomplete penetrance, then the shortage of *Dbf*/+ should not bias the recombination values.

These linkage data thus indicate that the *Dbf* locus lies 9–13 cM proximal to *ln*. According to the latest mouse genetic map (Lyon *et al.*, 1996) no other loci resulting in similar mutant phenotypes are known in this region of chromosome 1. The locus of dominant hemimelia, *Dh*, which gives a somewhat similar phenotype, resulting in defects of the hind limbs, is also on chromosome 1, but is located at a different point a few centimorgans distal to *ln* (Searle, 1964; Higgins *et al.*, 1992). Thus, *Dbf* can be regarded as a new locus affecting mouse limb development.

To obtain a more precise map position, linkage tests with microsatellite markers were carried out on offspring from an interspecific backcross with *Mus spretus*. The results (Fig. 2) indicated that the *Dbf* locus lies between the markers *D1Mit22* and *D1Mit8*, and is closely linked to *D1Mit24* and *D1Mit77*. The recombination fractions are *D1Mit22* – 13/193 – (*D1Mit24*, *D1Mit77*, *Dbf*) – 9/193 – *D1Mit8*, giving distances of *D1Mit22* – 6·7 ± 1·8 (*D1Mit24*, *D1Mit77*, *Dbf*) – 4·7 ± 1·5 – *D1Mit8*. The order of markers agrees with that given by Miller *et al.* (1996) but the distances differ in that these authors found 4·6 cM between *D1Mit24* and *D1Mit77* in contrast to the absence of recombination found here. As in the

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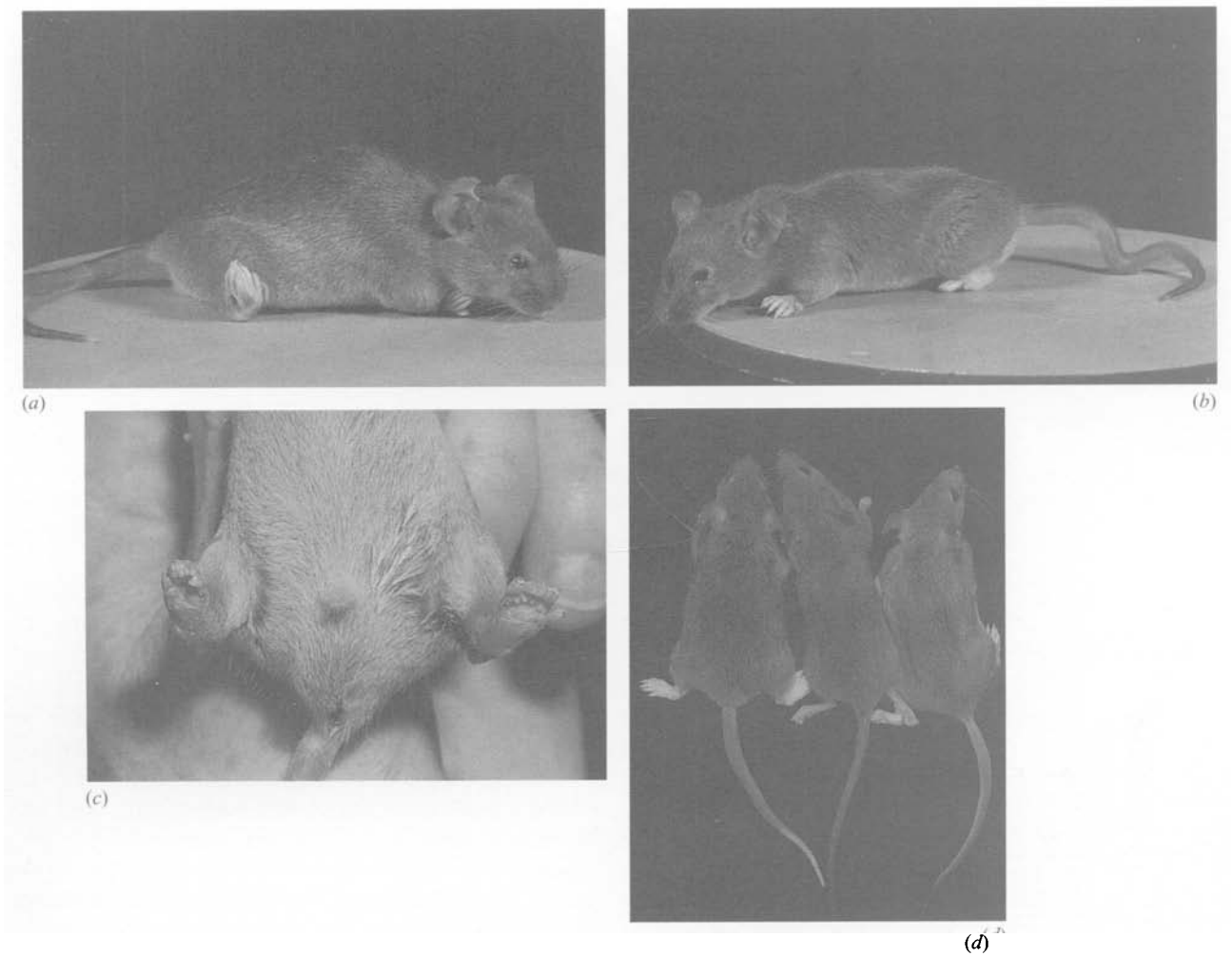

Fig. 1. Typical *Dbf*/+ heterozygotes. (*a*) Twisted fore and hind limbs with ankles and wrists resting on ground rather than feet. (*b*) More normal position of feet but kinked tail and half-closed eyes. (*c*) Abnormally smooth and swollen soles of feet. (*d*) From left to right: typical *Dbf*/+, normal, and *Dbf*/+ × *M. spretus* F_1, showing broad head and tail in typical *Dbf*/+ but normal head and tail in *M. spretus* F_1.

Table 1. *Inheritance of doublefoot in crosses to normal*

	Parents		Offspring *Dbf*	Offspring +	χ^2	*P*
1.	Original male	3H1♀ × *Dbf*/+♂	28	19	1·72	0·19
2.	Outcrosses to other stocks	+/+♀ × *Dbf*/+♂	22	25	0·19	0·66
3.	Outcrosses to 3H1 hybrids					
	(a)	*Dbf*/+♀ × +/+♂	89	108	1·83	0·18
	(b)	+/+♀ × *Dbf*/+♂	83	117	5·78	0·016
	(a)+(b)		172	225	7·08	0·008

χ^2 tests deviation from the expected 1:1 ratio of *Dbf* and + offspring

previous studies, there was a shortage of *Dbf*/+ offspring (*Dbf*, 69; + 124; $\chi^2 = 15{\cdot}7$, $P = 0{\cdot}00007$).

There was a possibility (see Discussion) that *Dbf* might involve a mutation at the *Pax3* locus, which lies just proximal to *D1Mit8* (Fleming *et al.*, 1996). Therefore, animals which were crossovers between *Dbf* and *D1Mit8* were typed for *Pax3*, using a restriction fragment length polymorphism (RFLP) revealed by Southern blotting. Of the 9 animals, 4 were successfully typed, and of these 3 involved a crossover between *Dbf* and *Pax3* and 1 a crossover between *Pax3* and *D1Mit8*. Thus, the *Dbf* mutation is not at the *Pax3* locus.

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Table 2. *Linkage tests of doublefoot with markers on chromosome 1*

	Offspring										
	Dbf				+						
Parents	+ +	*fz* +	+ *ln*	*fz ln*	+ +	*fz* +	+ *ln*	*fz ln*	Total	*Dbf*	+
+ *Dbf* + /*fz* + *ln*♀ × *fz* + *ln*/*fz* + *ln*♂	26	13	6	2	1	6	24	37	115	47	68
fz + *ln*/*fz* + *ln*♀ × + *Dbf* + /*fz* + *ln*♂	27	4	1	1	1	9	20	66	129	33	96

Recombination values:

	Female heterozygotes	%	Male heterozygotes	%
fz–Dbf	40/115	34·8 ± 4·4	26/129	20·2 ± 3·5
Dbf–ln	15/115	13·0 ± 3·1	12/129	9·3 ± 2·6
fz–ln	49/115	42·6 ± 4·6	34/129	26·4 ± 3·9

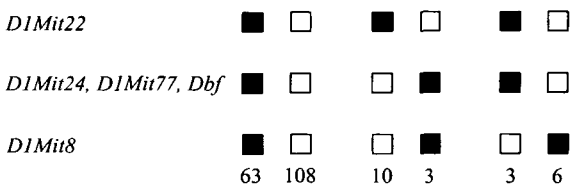

Fig. 2. Haplotypes of offspring obtained in an interspecific backcross of (*Dbf*/+ × *M. spretus*) F_1♀ × C3H/HeH♂. Filled boxes, laboratory mouse allele; open boxes, *M. spretus* allele.

Table 3. *Numbers of digits on fore and hind feet of Dbf*/+ *animals*

No. of digits	Fore feet	Hind feet
5	0	11
6	30	89
7	95	45
8	21	3
9	2	0
Total	148	148

(iii) *Phenotype of Heterozygote*

(*a*) *Morphological Abnormalities*

Abnormalities in the skeleton were studied by inspection and by alizarin preparations.

All affected animals had extra toes but the number of digits on each foot varied (Table 3). On the fore feet the numbers of toes ranged from 6 to 9, with a modal number of 7, whereas on the hind feet numbers ranged from 5 to 8, with a mode of 6. There was no difference between right and left feet.

Typically the toes were arranged in a single group, with the most anterior and most posterior toes shorter than the others, as in a normal foot. However, other patterns were found. In some cases two or more toes were webbed with soft tissue. In other cases the toes were arranged in two groups, with a medial digit shortened, and with only two phalanges, as in a normal digit I. Such patterns were uncommon, however. The question arises whether the broadening of the feet was due to a mirror image duplication of the anterior–posterior axis of the limbs, or to a broadening of a single axis. It was not possible to resolve this question from the size and position of individual toes.

There were some other limb defects. In the hind limbs, the tibia and fibula typically failed to fuse distally and articulated separately with the tarsal bones. In some cases the tibia was considerably reduced and bent, and the fibula was markedly bowed (Fig. 3). This resulted in luxation of the ankle joint with the foot rotated so that the ankle rather than the toes rested on the ground (Figs. 1, 3). In the fore limbs, the radius and ulna typically were of normal shape (Fig. 3), but occasionally were somewhat bent. In some cases the feet were rotated, with the wrists resting on the ground. Occasionally a foramen was present in the scapula. In addition to the skeletal abnormalities the ventral surface of both fore and hind feet of *Dbf*/+ mice appeared swollen and abnormally smooth (Fig. 1).

The skulls of affected mice were abnormally broad in the frontal region. Frequently there was a small gap between the frontal bones anteriorly, filled with an interfrontal bone. Some animals developed hydrocephalus. The onset of this could occur at any age from 2–3 weeks to a few months. Once it occurred it was rapidly fatal.

A further feature of the head was that the eyes were typically kept in a half-closed position, in contrast to the open eyes of normal animals (Fig. 1). The remainder of the axial skeleton was essentially normal, but occasionally there were seven lumbar vertebrae, in place of the normal six. The tails of affected mice were typically kinky, and were sometimes held up over the back. However, in most animals there was no bony abnormality underlying the kinks. Rather the kinks seemed to result from some abnormality of soft tissue.

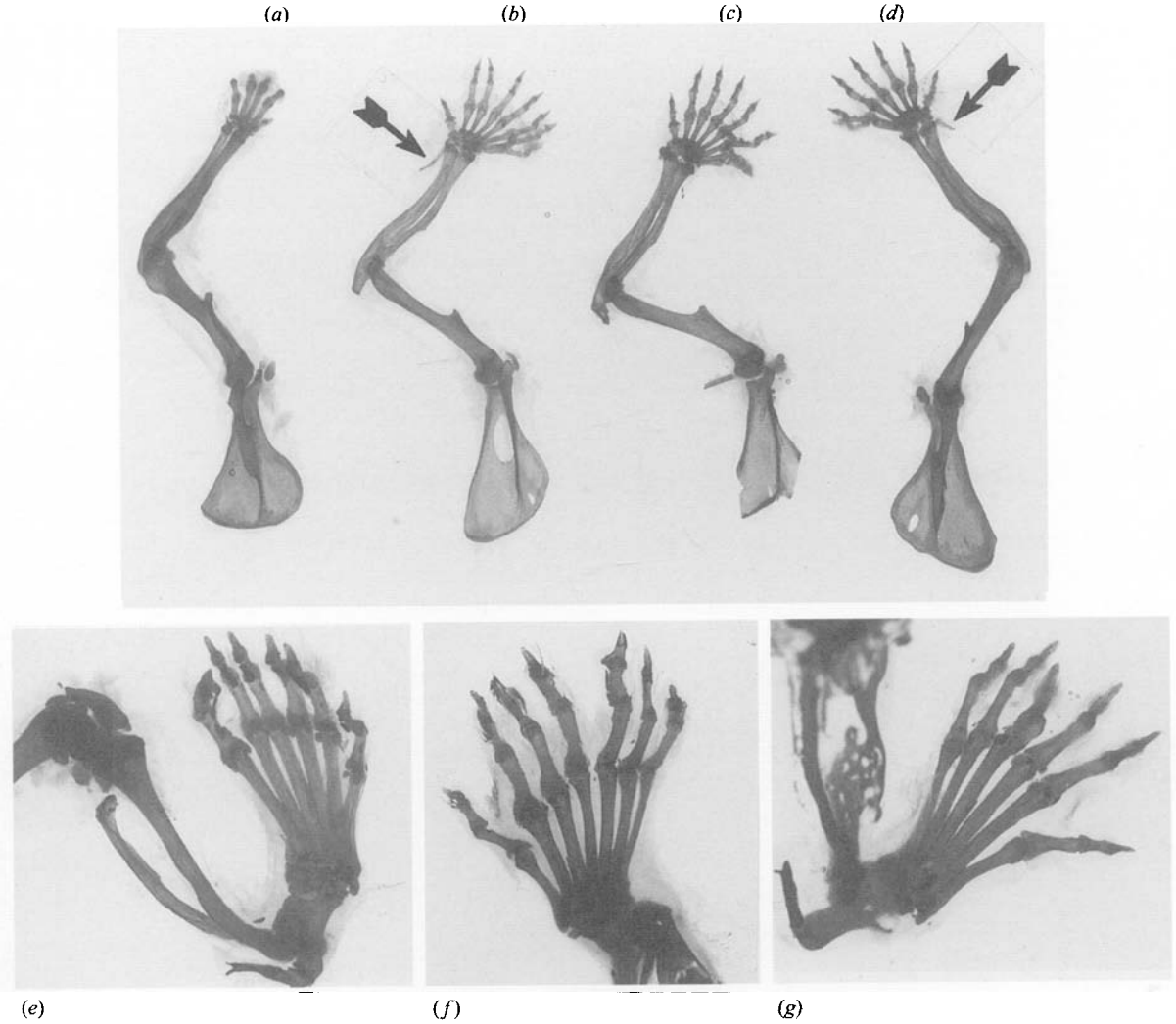

Fig. 3. Alizarin preparations of limbs of *Dbf*/+. (*a*)–(*d*) Fore limbs. (*a*) Normal; (*b*) typical *Dbf*/+ showing eight digits, foramen in scapula and calcified tendon (arrow); (*c*) less typical *Dbf*/+ showing fusion of digits 1 and 2; (*d*) less typical *Dbf*/+ showing webbing but not fusion of digits 1 and 2. (*e*) Hind limb of *Dbf*/+ showing tibia and fibula shortened and bent with gross twisting of ankle. (*f*) Hind foot of *Dbf*/+ with more normal position of ankle. (*g*) Hind foot of *Dbf*/+ showing central digit shorter than outside digits.

In some animals, however, bony kinks due to fusion of vertebrae were also present. In addition, the tails were abnormally thick, and this again appeared to be an abnormality of soft tissue, with no underlying bony basis (Fig. 1). Although the animals sometimes adopted a hunched posture, no bony abnormalities were seen in the vertebral column or ribs.

In the course of genetic studies, *Dbf*/+ females were crossed to *Mus spretus* males. Unexpectedly, the *Dbf*/+ offspring showed a somewhat different phenotype. The abnormalities of the limbs were similar to those seen on the usual genetic background. However, the heads were of normal shape with the eyes held open, and the tails were of normal thickness and not kinked (Fig. 1).

(*b*) *Growth and Reproduction*

To obtain some quantitative idea of the effects of the *Dbf* mutation on growth, affected animals and normal sibs were weighed daily from birth to 18 days, then at 3 weeks and once weekly to 8 weeks. From 12 to 21 pairs of *Dbf*/+ and +/+ controls from 9 litters were weighed at each time. The results showed no significant difference between *Dbf*/+ and +/+ at birth. However, from birth onwards the weight gain of *Dbf*/+ mice was less than that of controls, so that by 18 days affected mice weighed about 25% less than controls. Comparable weight differences persisted up to 8 weeks of age. As in the controls, *Dbf*/+ females weighed less than *Dbf*/+ males (Fig. 4).

Reproductive function was impaired in both sexes. In males, this was manifested by total sterility in some animals, and by only transitory fertility in others. A total of 152 males were crossed with 3H1 hybrid females (one or two females each) in the course of maintaining the stock. Of these only 74 (49%) were fertile, and in 29 of these 74 males (39%) the fertility was only transitory Thus, only about 30% of males showed long-lasting fertility.

In an attempt to find the cause of impaired fertility of males, testis weights and sperm counts were

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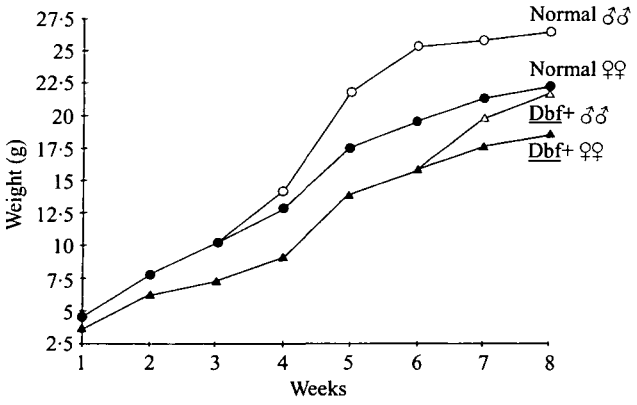

Fig. 4. Mean body weights of *Dbf*/+ and normal sibs aged 1–8 weeks.

measured on a sample of 10 *Dbf*/+ males and 10 normal sibs, aged 8 weeks, which had not been mated. The results (Table 4) showed a statistically significant reduction of both testis weights and sperm counts in *Dbf*/+ males, the mean sperm counts being about half normal. Because the body weight of *Dbf*/+ males is below normal a somewhat lower testis weight would be expected, and the reduction in testis weight could be entirely due to the reduced body weight. Moreover, the reduction in mean sperm counts appears not sufficiently great to cause sterility. However, there was very marked variation in sperm count among individual males. In 3 animals the counts were only about one-fourth that of their normal sibs, whereas in 2 others the counts were near normal. The fertility of these animals was not known and it is possible that those with low sperm counts were sterile and the others partly or wholly fertile. Therefore, testis weights and sperm counts were measured on a further group of *Dbf*/+ males: 3 proven fertile and 3 sterile and of similar age. Only one of the 6 animals had low testis weights and sperm counts (Table 5), and this animal had been proved fertile and had sired several litters.

Table 4. *Comparison of testis weights and sperm counts from Dbf*/+ *and normal males*

	Doublefoot (*Dbf*+)					Normal (+ +)			
	Testis weight (mg)		Sperm count (heads)			Testis weight (mg)		Sperm count (heads)	
Mouse	Left	Right	A	B		Left	Right	A	B
1	66·2	67·9	147	143	11	93·7	108·5	250	231
2	85·4	93·5	93	96	12	117·6	113·7	224	263
3	108·8	97·4	226	214	13	137·0	148·6	236	264
4	96·1	107·2	97	95	14	111·4	122·8	196	222
5	65·3	59·8	63	56	15	153·4	137·3	211	213
6	132·1	129·6	186	176	16	145·2	139·5	201	195
7	107·8	111·0	62	70	17	112·4	132·1	223	225
8	88·7	88·3	38	40	18	132·6	136·8	249	223
9	97·3	89·7	87	103	19	149·3	145·6	198	216
10	101·1	98·7	136	130	20	96·9	84·6	185	196
Mean	94·6 ± 4·4*		112·9 ± 12·4†		Mean	126·0 ± 4·5*		221. ± 5·1†	

A and B denote counts from left and right epididymes.
Student's/Welch's t-test
* Difference in mean testis weights highly significant: $t_{38} = 5{\cdot}0$; $P < 0{\cdot}001$.
† Difference in mean sperm count highly significant: $t_{25{\cdot}9} = 8{\cdot}1$; $P < 0{\cdot}001$.

Table 5. *Testis weights and sperm counts of Dbf*/+ *males of proven fertility and sterility*

Fertile				Sterile			
Testis weights (mg)		Sperm count (heads)		Testis weight (mg)		Sperm count (heads)	
Left	Right	A	B	Left	Right	A	B
55·0	57·8	90	105	107·6	116·6	329	382
110·0	116·0	232	264	112·0	112·0	290	279
114·0	114·0	309	314	106·0	110·0	208	291

A and B denote counts from left and right epididymes.

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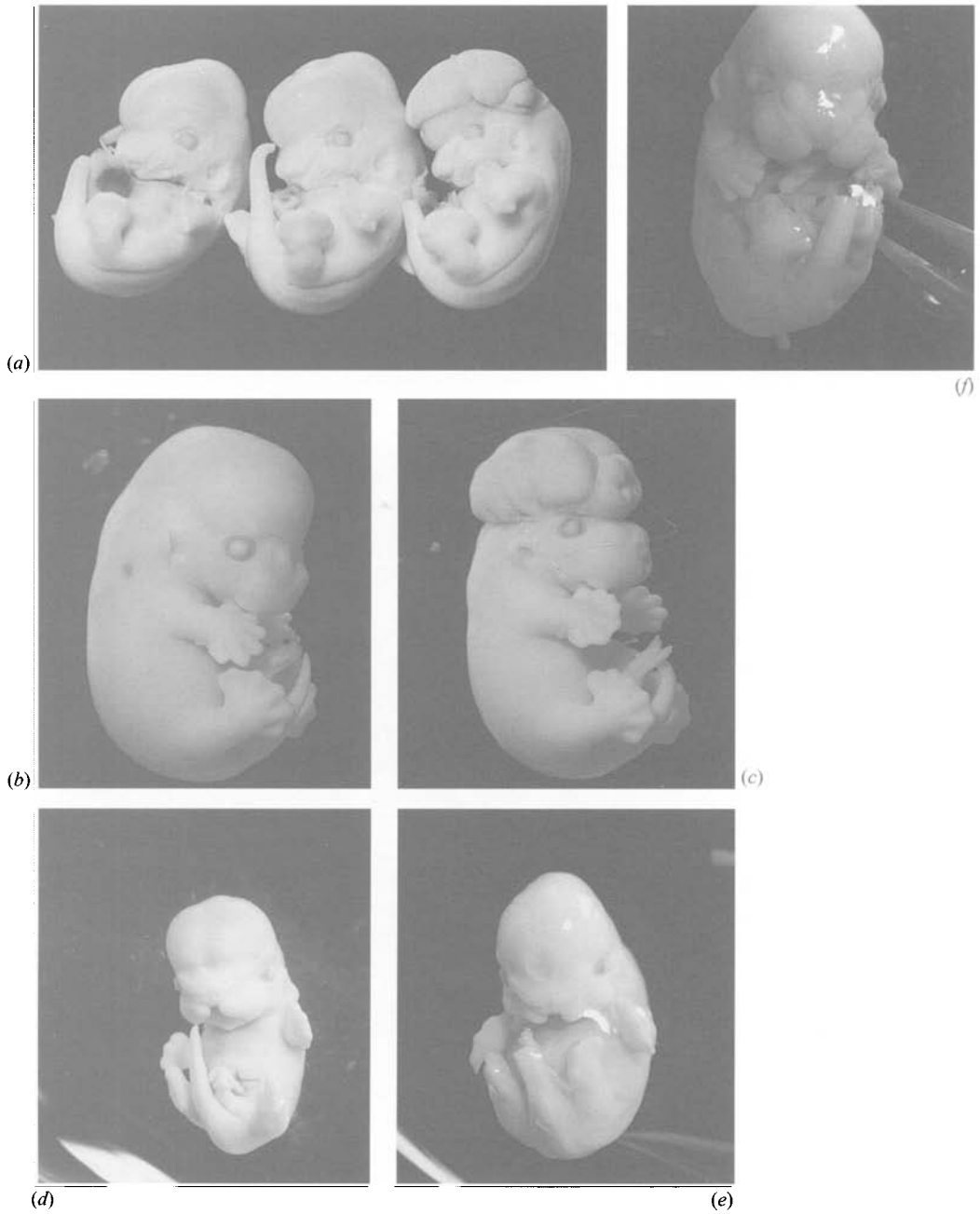

Fig. 5. *Dbf* fetuses. (*a*) Normal (left) and *Dbf*/+ fetuses at $12\frac{1}{2}$ days gestation. Note the abnormally broad footplates in *Dbf*/+ and cranioschisis in one. (*b*), (*c*) *Dbf*/+ fetuses at $13\frac{1}{2}$ days showing extra digits forming and cranioschisis in (*c*). (*d*), (*e*) Putative *Dbf*/*Dbf* fetuses at $13\frac{1}{2}$ days. Note split face and, in (*d*), bulging of head in frontal region and, in (*e*), bleb of clear fluid in frontal region. (*f*) Putative *Dbf*/*Dbf* fetus found dead at about 14-day stage in litter of $16\frac{1}{2}$ days gestation. In (*e*) and (*f*) tails have been removed to show split face.

Thus, it is unlikely that impairment of spermatogenesis is the sole or main cause of the poor fertility of *Dbf*/+ males. Among a group of 27 males placed with two or more 3H1 females and examined daily for 1 month for copulation plugs, 10 failed to make plugs. One other made plugs which did not result in pregnancy, and the remaining 16 made plugs leading to pregnancies. In types of male sterility resulting from impaired spermatogenesis, the males typically mate and make copulation plugs normally. The failure of some *Dbf*/+ males to make plugs indicates some other defect in reproduction, in addition to low sperm count. The limb defects of males which did not make plugs appeared no worse than those of some fertile males and thus it is unlikely that they were physically unable to mate.

In contrast with males, *Dbf*/+ females were usually fertile when crossed with normal 3H1 animals. In crosses made to maintain the stock, 125 of 131 *Dbf*/+ females mated to 3H1 males were fertile, and this must be regarded as in the normal range. However, the litter size was below normal. The mean litter size of 68 litters from *Dbf*/+ females crossed with 3H1 males was $4{\cdot}97 \pm 0{\cdot}24$, whereas in 59 litters from reciprocal crosses the litter size was $7{\cdot}56 \pm 0{\cdot}30$ ($t = 6{\cdot}73$, d.f. = 125; $p = 6 \times 10^{-10}$). A further sign of

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Table 6. *Numbers of fetuses of various phenotypes in pregnant females in Dbf/+ × Dbf/+ and control crosses*

Cross	Age	Normal	Wide feet	Wide feet and split face	Other	Dead fetuses	Moles
Dbf/+ × Dbf/+	$12\frac{1}{2}$	23	26	20[a]	2[b]	2[c]	15
	$13\frac{1}{2}$	11	25[d]	10[e]	–	–	5
	$14\frac{1}{2}$	9	10	2	–	2[f]	–
	$15\frac{1}{2}$–19	14	10[d]	–	–	7[f]	8
3H1 × *Dbf/+*	$12\frac{1}{2}$–$13\frac{1}{2}$	67	44[e]	1	–	6[g]	15

[a] Four with cranioschisis also.
[b] One with cranioschisis and wide face but normal feet; 1 retarded normal.
[c] One dead at 11 days; 1 with widely split head and face.
[d] Two with cranioschisis also.
[e] One with cranioschisis also.
[f] Two with wide feet and split face.
[g] Three at 11–12 days with wide feet, 3 at 10 days.

impaired fertility of *Dbf/+* females came from matings with *Dbf/+* males. When a sample of 13 known fertile *Dbf/+* males were crossed with 3H1 females, 24 of 31 females showed copulation plugs and became pregnant. However, of 46 *Dbf/+* females placed with the same males only 18 became pregnant. The difference between the two groups of females is statistically significant ($\chi^2 = 9{\cdot}46, p = 0{\cdot}0021$). In most cases the sterile females showed no plugs, but in a few females repeated plugs were found which did not lead to pregnancy.

Some studies were made of the cause of this impaired fertility. In young *Dbf/+* females the age of vaginal opening was delayed. In normal sibs the vagina opened at 5–6 weeks, whereas in *Dbf/+* females vaginal opening did not occur until 7–8 weeks. (The tests of fertility of *Dbf/+* females were all made on animals over 8 weeks old with open vaginas). Vaginal smears were studied over a period of 12 days in 6 *Dbf/+* females and normal sibs. The normal females all showed typical cyclic changes in the smears, with cycles ranging from 4 to 10 days, and at least one period of oestrus during the 12 days. By contrast, the *Dbf/+* females showed no normal cycles, and none exhibited an oestrous smear during the time of observation. These results suggested that *Dbf/+* females might lack gonadotrophic hormones or a response to them. In an attempt to test whether the response to gonadotrophins was normal, females were treated with hormone doses intended to induce superovulation. Females were injected intraperitoneally with 5 IU pregnant mare's serum (PMS), followed by 5 IU human chorionic gonadotrophin (hCG) 44–48 h later. They were then caged with males overnight, and inspected for copulation plugs the next morning. Six control 3H1 females were mated to known fertile *Dbf/+* males and 4 became pregnant, and of 12 *Dbf/+* females treated and mated to 3H1 males 7 became pregnant. These results are taken as evidence that the *Dbf/+* females did respond to the gonadotrophic hormones. However, of 7 *Dbf/+* females treated and mated with known fertile *Dbf/+* males only one became pregnant. Thus, the cause of the reduced fertility of *Dbf/+ × Dbf/+* crosses is not clear.

(c) *Embryonic development*

In order to study the development of heterozygotes, and if possible to recognize homozygotes, pregnant females were dissected at various stages from $9\frac{1}{2}$ days gestation onwards. In addition to crosses of *Dbf/+ × Dbf/+* there were some control crosses of 3H1 × *Dbf/+*, in which homozygous *Dbf/Dbf* fetuses would not occur.

Affected *Dbf/+* fetuses were first recognizable at $11\frac{1}{2}$ days gestation, by the presence of abnormally wide fore limb buds. Fetuses with abnormal limb buds were seen both in *Dbf/+ × Dbf/+* crosses, and in the control 3H1 × *Dbf/+* crosses, and were taken to be heterozygous *Dbf/+*. Not all litters could be scored reliably for *Dbf/+* at $11\frac{1}{2}$ days, presumably due to some variation among litters in the exact stage of development reached. The abnormality was clearer in $12\frac{1}{2}$-day fetuses when the footpads of both the fore and hind feet were abnormally broad (Fig. 5). In $12\frac{1}{2}$-day fetuses from *Dbf/+ × Dbf/+* crosses, a new abnormality was detectable. Some fetuses with broad limb buds had a 'split face' due to the maxillae failing to grow forward to meet in the midline. At $13\frac{1}{2}$ days similar fetuses with split face were again seen. In addition, the heads of these fetuses tended to bulge in the frontal region, and a bleb of clear fluid could be seen in the midline (Fig. 5). Fetuses with split face were seen only in *Dbf/+ × Dbf/+* crosses, and not in the control 3H1 × *Dbf/+* crosses. The only exception was a single animal scored as having a mild split face at 13 days. Thus, putatively the fetuses with split face are the *Dbf/Dbf* homozygotes. The total numbers of normal fetuses, fetuses with wide limb buds, and fetuses with both wide limb buds and split face at $12\frac{1}{2}$ and $13\frac{1}{2}$ days were 34:51:30 (Table 6). This is in reasonably good agreement with an expected 1:2:1 ratio ($\chi^2 = 1{\cdot}75$, d.f. = 1, $p = 0{\cdot}19$). Some putative

Dbf/*Dbf* fetuses were also found alive at $14\frac{1}{2}$ days gestation, but 2 others were dead. A new abnormality seen in them at this stage was oedema. In addition, these fetuses appeared retarded, at about the $13\frac{1}{2}$-day stage. Among fetuses of $15\frac{1}{2}$–$19\frac{1}{2}$ days no live *Dbf*/*Dbf* animals were found, 2 were found dead at about the $14\frac{1}{2}$-day stage (Fig. 5), and there were other dead fetuses too decomposed to be classified. It was therefore concluded that *Dbf*/*Dbf* homozygotes die at about $13\frac{1}{2}$–$14\frac{1}{2}$ days gestation.

In a few fetuses, other abnormalities were noticed. Four animals of 12 days gestation or older with broad feet and split face from *Dbf*/+ × *Dbf*/+ crosses showed cranioschisis, as also did another 4 animals with broad feet but normal face (Fig. 5). These latter fetuses may have been *Dbf*/+, and a single similar animal with broad feet and cranioschisis was seen among offspring from 3H1 × *Dbf*/+ crosses, which could not have included homozygotes. Thus, it appears that cranioschisis may appear in either genetically *Dbf*/*Dbf* or *Dbf*/+ animals. One animal from a *Dbf*/+ × *Dbf*/+ cross was scored as having split face and cranioschisis but normal limb buds, and the genotype of this animal is not clear. Open neural tubes were also seen in a small proportion of younger fetuses of $9\frac{1}{2}$ and $10\frac{1}{2}$ days, including one from a 3H1 × *Dbf*/+ cross. Some fetuses of these ages were also scored as retarded, and at 12 days the number of small moles in *Dbf*/+ × *Dbf*/+ crosses was rather high. Thus, it is possible that some affected *Dbf* animals die about half way through gestation. In the control 3H1 × *Dbf*/+ crosses at $12\frac{1}{2}$ days the number of small moles was also rather high, but this may be due to hormone-induced superovulation in some of the females, leading to crowding of implantation sites.

Thus, it appears that most homozygous *Dbf*/*Dbf* die at $13\frac{1}{2}$–$14\frac{1}{2}$ days gestation, probably due to oedema, with split face and some also with cranioschisis. A small proportion of *Dbf*/+ also appear to have cranioschisis, and death of these might partly account for the observed shortage of *Dbf*/+ at birth. In addition, some heterozygotes and homozygotes may die at around $8\frac{1}{2}$–$10\frac{1}{2}$ days gestation.

2. Discussion

In the doublefoot mutant major effects on development of the limbs and head are combined with those on growth and reproduction. The abnormally thick tail and swollen ventral surfaces of the feet of heterozygotes, together with the oedema seen in homozygotes shortly before their death, suggest the possibility of circulatory disturbances also. At present it is not clear whether the many effects seen are due to abnormal gene expression in various organs or whether the defects in head pattern may lead to maldevelopment of the hypothalamus or pituitary and hence to the effects on growth, reproduction and circulation.

The phenotype is distinct from that of any known mutants, and from its map position the mutant gene is at a new locus. Thus, the question arises what the underlying gene could be. The most striking effect is that in the limbs that has given the mutant its name. Recently there have been considerable advances in knowledge of the interplay of genes in the development of limb pattern (Johnson *et al.*, 1994; Tickle, 1995; Cohn & Tickle, 1996). This development is controlled by the action of genes in the apical ectodermal ridge (AER) at the distal tip of the limb bud, and in the zone of polarizing activity (ZPA) in the mesenchyme at the posterior margin of the distal part of the bud. The AER is thought to control development along the proximo-distal axis, whereas the antero-posterior pattern is directed by the ZPA. The genes involved include fibroblast growth factors, bone morphogenetic proteins, homeobox genes, and the sonic hedgehog gene. The sonic hedgehog gene, *Shh*, is expressed in the ZPA (Riddle *et al.*, 1993). In the AER fibroblast growth factors are expressed: *Fgf*2 (Savage *et al.*, 1993) and *Fgf*8 (Mahmood *et al.*, 1995) throughout the ridge, and *Fgf*4 (Johnson *et al.*, 1994) in the posterior region. *Fgf*4 and *Shh* form a feedback loop (Laufer *et al.*, 1994; Niswander *et al.*, 1994), together with the gene *Wnt7a*, which controls dorso-ventral limb pattern (Parr & McMahon, 1995; Yang & Niswander, 1995). These genes control the expression of downstream genes, such as bone morphogenetic protein and homeobox genes. In particular the *Hoxd* genes have been suggested to regulate antero-posterior patterning (Johnson *et al.*, 1994). *Shh* has been shown to induce expression of *Bmp2* in mesenchyme (Laufer *et al.*, 1994), and the activity of *Hoxd* genes is thought to be controlled by cooperation among signals from the genes *Shh*, *Fgf4* or *Fgf8* and *Wnt7a* working along the different limb axes (Tickle, 1995). Mutations or changes in expression in any of this complex system of genes might be expected to result in limb abnormalities, and indeed evidence of this is already available. Ectopic expression of *Shh* at the anterior margin of the limb bud can induce extra digits (Riddle *et al.*, 1993). Similarly, *Hoxb8* is normally expressed in the posterior fore limb mesenchyme. When its domain of expression was extended more anteriorly the digit pattern of the fore limb was duplicated (Charité *et al.*, 1994). In certain polydactylous mutants abnormal patterns of gene expression have been found. In the talpid[3] chick mutant there are numerous extra similar digits, i.e. there appears to be a loss of the normal antero-posterior pattern. Expression of *Fgf4*, normally seen in the posterior part of the AER, in the talpid mutant extends throughout the AER. In addition *Bmp2* and *Bmp7*, which again are normally expressed posteriorly, are expressed uniformly across the limb bud (Francis-West *et al.*, 1995). A different situation is seen in the mouse mutant *Rim4* studied by Masuya *et al.* (1995). In this mutant the polydactyly is suggested to be due to mirror image duplication of the

antero-posterior pattern, rather than loss of the pattern as in the talpid chick. In *Rim4/Rim4* homozygotes Masuya *et al.* found ectopic expression of *Shh*, *Fgf4* and *Hoxd11* at the anterior margin of the limb bud, in addition to the normal posterior expression. Studying two other mutants – hemimelic extra toes, *Hx*, and extra-toes, *Xt* – both of which result in preaxial polydactyly, these authors have shown that in both *Hx* heterozygotes and *Xt* homozygotes *Shh* and *Fgf4* were expressed at the anterior margin of the limb bud in a proportion of the embryos. Since the *Xt* phenotype is known to result from a mutation in the transcription factor *Gli3* (Vortkamp *et al.*, 1992), this gene may act to regulate the expression of *Shh*, *Fgf4* or other genes involved in antero-posterior limb patterning.

In the case of *Dbf*, none of the genes known to take part in development of limb pattern maps in the relevant region of chromosome 1. Thus *Dbf*, like *Xt*, may involve a mutation in a gene which somehow regulates the complex interplay of the genes concerned. A gene located very close to *Dbf* on chromosome 1 is the paired-box gene *Pax3* (Goulding *et al.*, 1991; Fleming *et al.*, 1996). *Pax3* is known to have a major role in pattern formation in development, primarily in the central nervous system. The phenotype of null mutations in *Pax3* is that of the splotch, *Sp*, mutations in mouse (Epstein *et al.*, 1991), and Waardenburg's syndrome (*WSI*, *WSII* and *WSIII*) in man (Hoth *et al.*, 1993; Tassabheji *et al.*, 1993). These both include minor white spotting in heterozygotes, and *WSIII* (Klein–Waardenburg syndrome) also involves limb defects but not polydactyly (Sheffer & Zlotogora, 1992). Splotch, when homozygous, results in severe neural tube defects, but not limb defects. Thus, the *Pax3* null phenotype is very different from that of *Dbf*. However, on the basis of its striking effect in the heterozygote, *Dbf* is more likely to be a gain-of-function rather than a loss-of-function mutation. There is evidence that the phenotypes of gain-of-function and loss-of-function mutations in the same gene may be very different. In man, mutations in fibroblast growth factor receptors result in various clinical syndromes affecting the limbs. In particular, mutations in *FGFR3* are found in types of dwarfism due to shortening of the long bones, including achondroplasia, thanatophoric dwarfism, and hypochondroplasia (Yamaguchi & Rossant, 1995). However, two groups of workers (Colvin *et al.*, 1996; Deng *et al.*, 1996) have shown that null mutations in *Fgfr3* in the mouse lead to a converse phenotype with skeletal overgrowth. This led Deng *et al.*, to postulate that *Fgfr3* normally functions to restrict cartilage growth, and that the mutations underlying the human dwarfisms are gain-of-function mutations in *FGFR3*. In the case of *Dbf*, it is known that *Pax3* is normally expressed in limb mesenchyme of 10–12 day fetuses (Goulding *et al.*, 1991) and the possibility that *Dbf* involves a gain-of-function mutation in *Pax3* was considered. However, the mapping results, showing three crossovers between the *Dbf* phenotype and the *Pax3* locus, rule out *Pax3* as a candidate gene for the *Dbf* phenotype.

The authors are very grateful to K. Whitehill and S. Morse for sperm counts, to A. Ford and K. Glover for photography and to D. Papworth for statistical tests. This work was partly supported by EU contract no. CHRX-CT93-0181.

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