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Imprinting of distal mouse chromosome 2 is associated with phenotypic anomalies *in utero*†

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Summary

Previous studies have shown that the distal region on mouse chromosome (Chr) 2 is subject to imprinting as mice with maternal duplication/paternal deficiency (MatDp.dist2) and the reciprocal (PatDp.dist2) for this region exhibit phenotypic anomalies at birth and die neonatally. We show here that imprinting effects are detectable *in utero*. Notably PatDp.dist2 embryos show an increase in wet weight compared with normal, which peaks at 16·5 d post coitum (dpc), and diminishes by birth, whereas the wet weight of placenta is slightly reduced in the latter half of gestation. Newborns have increased length of the long bones. By contrast, the wet weight of MatDp.dist2 embryos decreases during the second half of gestation. Measurements of dry weights of embryos at 16·5 dpc have indicated that there is no difference in either PatDp.dist2 or MatDp.dist2 compared with normal so that the wet weight differences are due to fluid retention in PatDp.dist2 but fluid loss in MatDp.dist2. In PatDp.dist2 embryos excess fluid is particularly prominent in the subcuticular skin layer, whereas by birth fluid is evident around the neck and tongue. At 16·5 dpc the PatDp.dist2 embryos are severely oedematous, as the average fluid content per unit dry weight per embryo was increased by 40%, whereas the MatDp.dist2 embryos are dehydrated as the average water content per unit dry weight per embryo was reduced by 6%. A preliminary conclusion is that there is neither growth enhancement in PatDp.dist2 nor growth retardation in MatDp.dist2 offspring.

1. Introduction

Genomic imprinting in mammals is the genetic mechanism whereby genes are repressed, or imprinted, depending on parental origin (Solter, 1988; Surani, 1991; Cattanach & Jones, 1994). Mice with uniparental disomies, and uniparental duplications of discrete chromosome regions, show phenotypic anomalies called imprinting effects or imprinting phenotypes (Cattanach & Jones, 1994; Cattanach & Beechey, 1997). Fourteen imprinting effects, associated with regions on six mouse autosomes, have been identified and these range from early embryonic lethalities, through fetal lethalities, to various developmental abnormalities affecting growth before and after birth, and behaviour. Each of these effects is likely to be due to one or more genes located within each region. There are currently 28 known autosomal imprinted genes, many of which are clustered within chromosome regions associated with imprinting phenotypes (for review see Beechey, 1998).

Two of the earliest discovered imprinting effects are associated with distal chromosome (Chr) 2 (Cattanach & Kirk, 1985). Mice with maternal duplication/paternal deficiency (MatDp.dist2) for the region distal to the agouti locus (*a*) recently repositioned in G-band 2G3 (E. P. Evans, personal communication), were generated by intercrossing heterozygotes for the reciprocal translocation T(2;11)30H (see Fig. 1*a*). These were described as being born with long flat-sided bodies, arched backs, an inability to suckle, being hypokinetic and dying within a few hours of birth. Newborns of the reciprocal type, paternal duplication/maternal deficiency (PatDp.dist2), were

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† This paper is dedicated to Bruce Cattanach, on the occasion of his retirement, in acknowledgement of his insight and investigative approach that have inspired our interest and experiments in imprinting.

reported as having an effectively opposite phenotype, being hyperkinetic with short square bodies, broad flat backs, and dying a few days after birth. Subsequent genetic studies showed that these imprinting phenotypes can be generated with all the tested Chr 2 translocations except T(2;16)28H, the translocation with the most distal breakpoint on Chr 2 (Cattanach, 1986; Fig. 1*a*). Thus T28H marks the distal limit of the distal Chr 2 imprinting region, the proximal limit being defined by T(2;8)2Wa (T2Wa) (Cattanach *et al*., 1992; Peters *et al*., 1994; Fig. 1*a*). The phenotypes are thought to be due to at least two genes that are imprinted in opposite directions, because studies of tertiary trisomics have shown that both Mat and PatDp.dist2 imprinting phenotypes can be rescued by the extra maternally or paternally derived copies of distal Chr 2 (Beechey & Peters, 1994). To date only one imprinted gene, *Gnas*, has been positioned to the minimal imprinting region between the breakpoints of T2Wa and T28H as shown in Fig. 1*a*, but so far *Gnas* has only been found to be maternally imprinted in the glomerular tuft of the kidney (Williamson *et al*., 1996) and thus does not immediately appear to be a good candidate for the imprinting effects found in MatDp.dist2. Another maternally imprinted gene, *Nnat*, lies distal to the T26H breakpoint but outside the minimal imprinting region (Kikyo *et al*., 1997; Williamson *et al*., 1998). Other genes judged to be candidates because they map to the region have been examined for monoallelic expression but none has been demonstrated so far (Williamson *et al*., 1995*a*). A further approach to the identification of candidate genes is through the phenotypic characterization of the imprinting effects. The imprinting phenotypes associated with distal Chr 2 had not previously been studied before birth and we describe here new observations on the phenotype *in utero* and at birth. Phenotype analysis can provide information on the consequences of the action of the responsible genes in different tissues and at different developmental stages and provide clues as to the type of genes that may be involved.

2. Materials and methods

(i) *Generation of duplication/deficient mice*

Mice and embryos with maternal duplication/paternal deficiency (MatDp.dist2) and the reciprocal (PatDp.dist2) for the region distal to the T26H breakpoint on Chr 2, were generated by the standard method of intercrossing reciprocal translocation heterozygotes (Searle & Beechey, 1978; Cattanach, 1986; Williamson *et al*., 1996). In each mating one of the parents was homozygous for brachypodism, *bp*, which lies less than 1 cM distal to the T26H breakpoint and results in shortened limbs (Grüneberg & Lee, 1973; Storm *et al*., 1994). Thus from the reciprocal crosses of T26H+/++ × T26H*bp*/+*bp* it is expected that one-third of live-born offspring will be either maternal or paternal duplication/deficient genotypes for the region distal to the T26H breakpoint, the parental origin of the *bp* young depending on the direction of the cross. These offspring will have originated from the complementation of unbalanced gametes arising from adjacent-1 disjunction. The T26H+/++ parent in the above reciprocal cross is produced from crosses to C3H/HeH and the T26H*bp*/+*bp* parent is produced from crosses to a Harwell stock, LL. It should also be noted that all Mat and PatDp.dist2 mice will be heterozygous for T26H. Mice/embryos that appear phenotypically normal from these crosses will be either homozygous or heterozygous for T26H or non-carriers; this group will hereafter be referred to as normal siblings or littermates. To increase numbers of zygotes, females were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotrophin followed 48 h later by 5 IU of human chorionic gonadotrophin. The number of days gestation were calculated by counting the day of the vaginal plug as 0·5 days post coitum (dpc).

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.

(ii) *Typing of microsatellite markers*

Although the *bp* marker was an aid to identification of Mat and PatDp.dist2 embryos at later gestational ages and birth, all offspring and embryos were classified using either *D2Mit51* or *D2Mit226* in order to identify both types of duplication genotype and normal littermates in each cross (Williamson *et al*., 1994, 1995*b*). DNA was extracted from tail tissue as described by Whitelaw *et al*. (1991). More recently we have used a modification of this method whereby after incubating tissues overnight, the samples were heated at 90–100 °C for 10 min to inactivate the proteinase K. The DNA was then precipitated with isopropanol and analysed by PCR using standard methods. Primers for *D2Mit51* were 5′-GTGAGGGGTCAATGCCA-CCA-3′ and 5′-GGCTCAGTTGTAAGCACAAG-3′ and for *D2Mit226* were 5′-TTTTTGCAACTTTGT-TAAGAATTCC-3′ and 5′-AAAACACCCTCCCA-CCCTT-3′ (Dietrich *et al*., 1994; sequence from mapbase@genome.wi.mit.edu) and were supplied by Genosys Biotechnologies. PCR was carried out in a buffer containing 1·5 mM $MgCl_2$, using an annealing temperature of 55 °C.

(iii) *Weight data*

Uterine contents were examined from 11·5 to 19·5 dpc, and embryos and placentas were weighed using an

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electronic balance. A number of females were allowed to litter to obtain weight data at birth. At 16·5 dpc dry weights of some embryos and placentas were measured after 72 h in a standard freeze-dryer. All weight comparisons of Pat and MatDp.dist2 mice and their normal sibs were assessed by Student's *t*-test on the basis of weight ratios (PatDp versus normal and MatDp versus normal) within litters as this eliminates weight differences between different litters of the same age attributable to varying litter sizes. In the prenatal studies placental weight was similarly evaluated.

(iv) *Skeletal studies*

Skeletons of six newborn non-*bp* MatDp.dist2 and four newborn non-*bp* PatDp.dist2 mice plus normal sibs were stained with alizarin red-S and cleared to allow examination of bones and skeletal structure. Bone lengths, including tibia/fibula length, femur width and length, skull width and length, and spinal column length from the axis to the first sacral vertebra were measured using a travelling microscope. Embryos and placentas were also examined histologically by standard methods. For analysis of oedema, embryos were fixed in 4% paraformaldehyde followed by short processing steps to minimize shrinkage, and longitudinal sections were stained with haematoxylin and eosin.

3. Results

(i) *Identification of Mat and PatDp.dist2 offspring*

Both MatDp.dist2 and the reciprocal PatDp.dist2 were identified by PCR using primers for microsatellite markers *D2Mit51* and *D2Mit226*. These markers lie distal to the T26H breakpoint (H. J. Miller and E. R. Dutton, personal communications; Fig. 1*a*) and give a simple sequence variation between the parental genotypes as shown for *D2Mit51* in Fig. 1*b*. Mice of two genotypes were used: T26H+/++ with a *D2Mit51* allele determining a 142 bp band (Fig. 1*b*, lane 1) and a *D2Mit226* allele determining a 124 bp band (not shown); and T26H*bp*/+*bp* with a *D2Mit51* allele determining a band of approximately 134 bp (Fig. 1*b*, lane 2) and a *D2Mit226* allele determining a band of 104 bp (not shown). Thus from the cross T26H+/++ female × T26H*bp*/+*bp* male, a MatDp.dist2 will be homozygous for the *D2Mit51* band of 142 bp (Fig. 1*b*, lane 3) and the *D2Mit226* band of 124 bp, whereas a PatDp.dist2 will be homozygous for the *D2Mit51* band of 134 bp (Fig. 1*b*, lane 4) and the *D2Mit226* band of 104 bp. Homozygosity for the *Mit* marker is diagnostic for both MatDp.dist2 and PatDp.dist2; all normal siblings are heterozygous for both *Mit* markers, as shown for *D2Mit51* in Fig. 1*b* (lane 5). In total 679 embryos from 11·5 to 19·5 dpc were typed for the microsatellite markers; of these a mean of 17·1% (116/679) PatDp.dist2 and 15·6% (106/679) MatDp.dist2 were recovered. This frequency is in good agreement with 16·67%, the expectation for complementation of distal regions (Searle *et al*., 1971).

(ii) *Oedema in PatDp.dist2*

All embryos were examined for overt abnormalities from 11·5 dpc. A clearly visible anomaly from 14·5 dpc onwards (Fig. 1*c*) was the presence of oedema in a small number of embryos, which was seen after removal of the amnion, and was observed as fluid accumulation under the skin. The oedema persisted through the remainder of gestation and was still visible just prior to birth at 19·5 dpc but was less obvious in newborns. Typing with the microsatellites described above showed that the oedematous embryos were PatDp.dist2 and histological analysis from 14·5 dpc to birth revealed the presence of fluid in the subcuticular skin layer (Fig. 1*d*). At birth oedema could be detected in muscle close to the skin, in the tongue and particularly around the neck (results not shown). In addition, a tail bend could usually be detected in the PatDp.dist2 animals at birth. No PatDp.dist2 survived beyond 4 d after birth and most died at 1–2 d, but the limited data indicate that these mice were losing weight and the oedema was diminishing.

(iii) *Wet weights of MatDp.dist2 and PatDp.dist2* in utero *and at birth*

All live embryos and their placentas were weighed from 11·5 dpc prior to removal of the tail tip for typing with the microsatellite markers. The visceral yolk sac, allantois and amnion were dissected away from the fetal material and excess fluid was wiped clear prior to measuring the weight. Only litters containing at least one PatDp.dist2 or one MatDp.dist2 were used in the wet weight analyses; consequently not all of the 679 typed embryos were used. Table 1 shows the wet weights of *bp* and non-*bp* Pat and MatDp.dist2 offspring expressed as ratios of the wet weights of their normal littermates and averaged over all litters of a given age to eliminate weight differences due to litter size effects. Depending on the direction of the cross Pat and MatDp.dist2 offspring were generated that were unmarked or were marked with brachypodism, *bp*. The data shown in Table 1 were analysed to see whether there were any wet weight differences between *bp* and non-*bp in utero* and at birth as described in the Appendix. A difference was found and the pooled ratio described in the Appendix was used to correct for weight differences between *bp*

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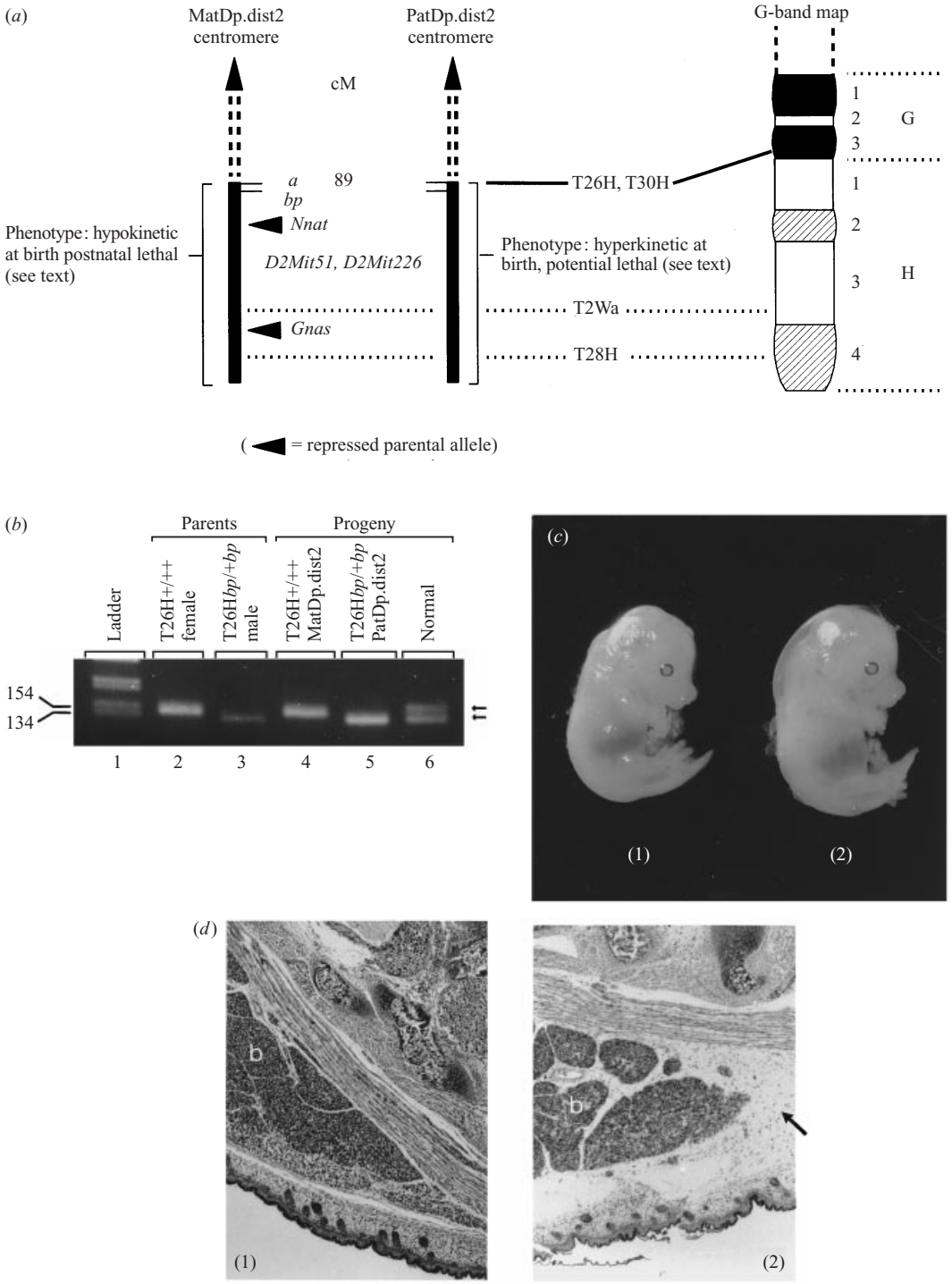

Fig. 1. Distal Chr 2 and oedema. (*a*) Diagram of Chr 2 showing the imprinting region (black), defined by the T30H and T26H breakpoints (bold) at the agouti locus (*a*), and the imprinting effects seen in Mat and PatDp.dist2 mice at birth. Approximate positions of imprinted genes, *Gnas* and *Nnat*, are shown as well as the markers brachypodism (*bp*), *D2Mit51* and *D2Mit226* that were used for classification of embryos. The T2Wa and T28H breakpoints that define the proximal and distal limits of the region are also shown. The imprinting phenotype described by Cattanach & Kirk (1985) can be generated with T26H and T2Wa but not with T28H. (*b*) PCR products of microsatellite marker *D2Mit51* for the identification of MatDp.dist2, PatDp.dist2 and normal littermates. Lane 2, T26H+/++ female parent; lane 3, T26H*bp*/+*bp* male parent; lane 4, MatDp.dist2 embryo; lane 5, PatDp.dist2 embryo; lane 6, normal littermate. (*c*) Oedema visible in PatDp.dist2 embryos at 14·5 dpc. 1, normal embryo; 2, PatDp.dist2 littermate. (*d*) Longitudinal sections of skin and underlying tissue from the nape of neck showing the position of fluid at 18·5 dpc. 1, normal littermate; 2, PatDp.dist2 embryo. The arrow shows the position of the fluid in the subcuticular skin layer. b, brown fat.

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Table 1. *Prenatal and birth wet weights of PatDp.dist2* bp *and non-*bp *and MatDp.dist2* bp *and non-*bp *expressed as ratios of the wet weights of normal littermates and averaged over all litters of each age*

Gestational age (dpc)	Wet weight ratios ± SE			
	PatDp.dist2		MatDp.dist2	
	bp (*n*)	non-*bp* (*n*)	*bp* (*n*)	non-*bp* (*n*)
11·5	–	0·959 ± 0·043 (6)	1·051 ± 0·052 (5)	1·374 ± 0·182 (1)
12·5	1·043 ± 0·052 (5)	1·053 ± 0·048 (6)	1·030 ± 0·048 (6)	1·102 ± 0·110 (1)
13·5	1·061 ± 0·056 (6)	1·100 ± 0·086 (2)	0·914 ± 0·048 (5)	0·844 ± 0·055 (4)
14·5	1·100 ± 0·057 (5)	–	–	1·011 ± 0·053 (5)
15·5	1·143 ± 0·054 (7)	1·273 ± 0·045 (11)	0·881 ± 0·028 (14)	0·970 ± 0·043 (7)
16·5	1·187 ± 0·049 (6)	1·295 ± 0·053 (8)	0·920 ± 0·035 (9)	0·959 ± 0·042 (5)
17·5	1·230 ± 0·069 (4)	1·249 ± 0·055 (8)	0·923 ± 0·033 (12)	0·971 ± 0·111 (1)
18·5	1·052 ± 0·044 (9)	1·119 ± 0·047 (8)	0·874 ± 0·034 (11)	0·991 ± 0·086 (1)
19·5	1·189 ± 0·072 (4)	1·274 ± 0·070 (4)	0·844 ± 0·048 (4)	1·015 ± 0·064 (3)
birth	1·002 ± 0·028 (21)	1·071 ± 0·043 (10)	0·857 ± 0·027 (18)	0·823 ± 0·019 (34)

n refers to the number of offspring used to calculate wet weight ratios.

Table 2. *Prenatal and birth wet weights of Pat Dp.dist2 and MatDp.dist2 expressed as ratios of the wet weight of normal littermates and averaged over all litters at each age*

Genotype/ gestational age (dpc)	No. of:			Embryos		Placentas	
	Pat or Mat.Dp	Normal sibs	Litters	Wet weight ratios ± SE	Significance of ratio (*P*)	Wet weight ratios ± SE	Significance of ratio (*P*)
Pat.Dp							
11·5	6	20	4	0·940 ± 0·043	0·18	0·969 ± 0·080	0·70[a]
12·5	11	31	8	1·046 ± 0·035	0·18	0·886 ± 0·055	0·054[a]
13·5	8	18	5	1·085 ± 0·048	0·065	0·964 ± 0·078	0·65[a]
14·5	5	9	3	1·127 ± 0·059	0·023	0·964 ± 0·091	0·70[a]
15·5	18	41	12	1·216 ± 0·034	$< 10^{-10}$	0·923 ± 0·048	0·13[a]
16·5	14	35	12	1·240 ± 0·036	$< 10^{-10}$	0·924 ± 0·049	0·14[a]
17·5	12	19	10	1·235 ± 0·043	$8{\cdot}4 \times 10^{-9}$	0·915 ± 0·069	0·24[a]
18·5	17	30	13	1·087 ± 0·032	0·0049	0·960 ± 0·055	0·48[a]
19·5	8	16	7	1·232 ± 0·050	$7{\cdot}8 \times 10^{-7}$	1·068 ± 0·080	0·38[a]
Birth	31	31	27	1·035 ± 0·024	0·13	–	
Mat.Dp							
11·5	6	16	5	1·107 ± 0·052	0·031	1·092 ± 0·094	0·31[b]
12·5	7	23	7	1·061 ± 0·045	0·17	0·997 ± 0·077	0·96[b]
13·5	9	16	7	0·892 ± 0·036	0·0055	0·983 ± 0·073	0·82[b]
14·5	5	11	3	0·986 ± 0·052	0·78	1·069 ± 0·102	0·49[b]
15·5	21	45	11	0·917 ± 0·024	0·0097	0·988 ± 0·046	0·80[b]
16·5	14	31	11	0·941 ± 0·027	0·036	0·967 ± 0·051	0·53[b]
17·5	13	21	11	0·948 ± 0·033	0·12	1·080 ± 0·091	0·36[b]
18·5	12	17	9	0·906 ± 0·032	0·0060	0·963 ± 0·062	0·55[b]
19·5	7	20	6	0·920 ± 0·039	0·049	0·891 ± 0·069	0·14[b]
Birth	52	44	39	0·829 ± 0·016	$< 10^{-10}$	–	

[a] Ratios do not differ significantly from each other overall [$F(8, 121) = 0{\cdot}573$; $P = 0{\cdot}80$] although the mean ratio of all ages, 0·944 ± 0·020, is significantly different from 1 [$t_{137} = 2{\cdot}67$; $P = 0{\cdot}0086$].
[b] Ratios do not differ significantly from each other overall [$F(8, 121) = 0{\cdot}656$, $P = 0{\cdot}73$]. The mean ratio of all ages, 0·989 ± 0·022, does not differ significantly from 1 [$t_{137} = 0{\cdot}508$; $P = 0{\cdot}61$].

and non-*bp* Pat and MatDp.dist2 embryos and newborns. The corrected wet weights are given in Table 2 and these were plotted semilogarithmically as shown in Fig. 2.

(*a*) *Wet weights of PatDp.dist2*

At the earliest age studied (11·5 dpc) the PatDp.dist2 embryos weighed less than normal littermates al-

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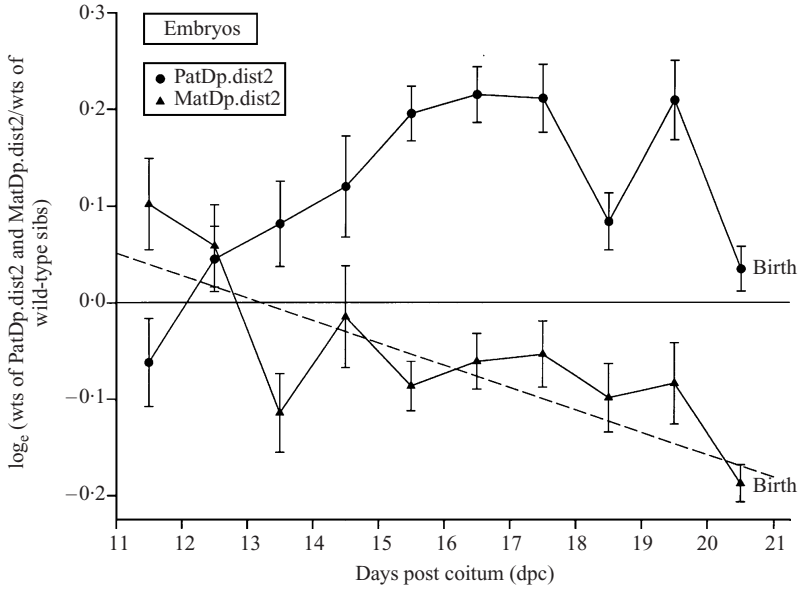

Fig. 2. Graph of the logarithms of the ratios, [wet weights of Mat and PatDp.dist2 embryos and offspring] ÷ [wet weights of wild-type siblings], against age in days. Birth is taken as day 20·5. Ratio of wet weights are averaged over all litters or uteri at each age. The weight ratio of MatDp.dist2 and PatDp.dist2 offspring compared with wild-type was always made with wild-types within the same litter.

though not significantly so (Table 2, Fig. 2). However, after 11·5 dpc the wet weight increased and was significantly more than that of their normal sibs from 14·5 dpc, peaking at 16·5 dpc. A decrease in wet weight was observed between 19·5 dpc and birth. This decrease was significant [$F(1, 192) = 13{\cdot}68$; $P = 0{\cdot}000\,28$] and since the wet weights of normal embryos increases over this period this represents a significant loss of weight in real terms.

(*b*) *Wet weights of MatDp.dist2*

At the earliest age studied (11·5 dpc), MatDp.dist2 embryos were significantly heavier than normal littermates (Table 2, Fig. 2) but then weighed significantly less than their normal littermates at 13·5 dpc ($P < 0{\cdot}05$). This difference was maintained up to 19·5 dpc, although ratios at 14·5 and 17·5 dpc were significantly less than 1. As observed with the PatDp.dist2, the wet weights of the MatDp.dist2 embryos also decreased significantly between 19·5 dpc and birth [$F(1, 192) = 4{\cdot}99$; $P = 0{\cdot}027$] and again this must represent a significant loss of weight in real terms. The MatDp.dist2 ratios across all ages were fitted, although not very satisfactorily, by a straight line [$F(8, 192) = 2{\cdot}13$; $P = 0{\cdot}035$], and despite the poor fit the slope [$(-2{\cdot}33 \pm 0{\cdot}37) \times 10^{-2}$] is very highly significant ($t_{200} = 6{\cdot}27$; $P = 2{\cdot}2 \times 10^{-9}$). Thus, for these embryos the wet weight ratios decreased significantly overall as age increased.

(iv) *MatDp.dist2 and PatDp.dist2 placental wet weights*

The placental wet weights of *bp* embryos, expressed as ratios of the placental wet weights of their non-*bp* littermates and averaged over all litters of all ages, were $0{\cdot}916 \pm 0{\cdot}044$ for PatDp.dist2 embryos and $1{\cdot}061 \pm 0{\cdot}056$ for MatDp.dist2 embryos. It should be noted that the placenta will contain tissue derived from the mother and from the conceptus. There was no significant evidence, overall, to suggest that the placental wet weights of *bp* embryos differed from those of their non-*bp* littermates. The placental weight ratios are given in Table 2 and were plotted semilogarithmically as shown in Fig. 3 and no correction factor was necessary (see Appendix). It can be seen that the placentas of PatDp.dist2 embryos weighed consistently less than those of their normal sibs, except at 19·5 dpc. Although none of these differences were individually significant and the ratios did not differ significantly overall [$F(8, 121) = 0{\cdot}573$; $P = 0{\cdot}80$], the overall ratio of $0{\cdot}944 \pm 0{\cdot}020$ was, however, significantly different from 1 ($t_{137} = 2{\cdot}67$; $P = 0{\cdot}0086$). The inability to demonstrate a significant difference on any single day is probably because the sample sizes for individual days were relatively small and so were the wet weight decreases (5–10%). The weight ratios for the placentas of MatDp.dist2 embryos did not differ significantly overall [$F(8, 121) = 0{\cdot}656$; $P = 0{\cdot}73$] and the overall ratio of $0{\cdot}989 \pm 0{\cdot}022$ was not significantly different from 1 ($t_{137} = 0{\cdot}508$; $P = 0{\cdot}61$). These data showed that the placentas of MatDp.dist2 were much the same weight, at all ages, as their normal littermates.

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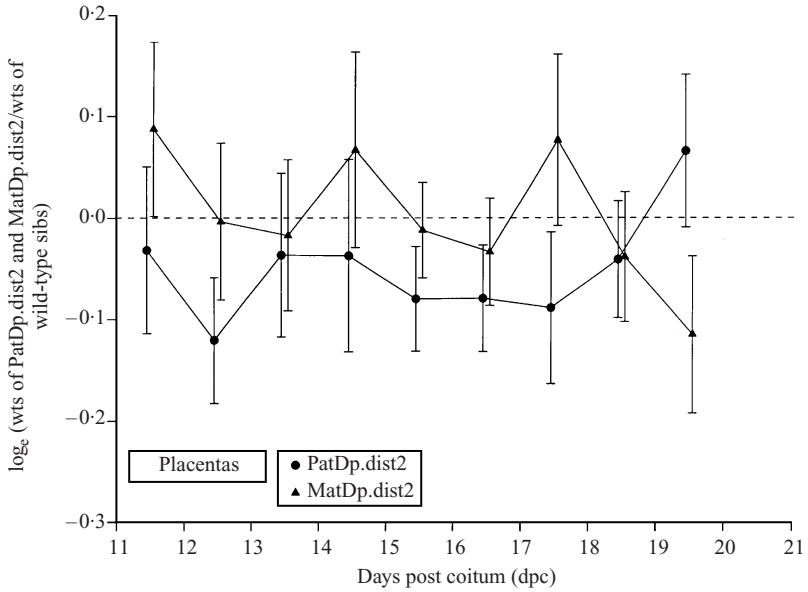

Fig. 3. Graph of the logarithms of the ratios, [wet weight of placentas from Mat and PatDp.dist2 embryos] ÷ [wet weights of wild-type siblings], against age in days. Birth is taken as day 20·5. Ratio of wet weights are averaged over all uteri at each age.

Table 3. *Dry weight ratios of Pat and MatDp.dist2 embryos and placentas at 16·5 dpc expressed as ratios of the dry weights of normal littermates and averaged over all litters*

Genotype	No. of: Pat or Mat Dp	Normal sibs	Litters	Embryos: Mean dry weight ratios ±SE	Embryos: Significance of ratio (*P*)	Placentas: Mean dry weight ratios ±SE	Placentas: Significance of ratio (*P*)
Pat.Dp							
bp	4	14	4	0·941 ± 0·051	0·29[a]	0·921 ± 0·046	0·12[b]
Non-*bp*	4	9	3	0·928 ± 0·061	0·28[a]	0·981 ± 0·059	0·75[b]
Overall				0·936 ± 0·038	0·12[a]	0·944 ± 0·036	0·16[b]
Mat.Dp							
bp	5	12	4	0·913 ± 0·055	0·16[a]	0·925 ± 0·051	0·18[b]
Non-*bp*	4	9	4	1·035 ± 0·058	0·55[a]	1·048 ± 0·054	0·38[b]
Overall				0·976 ± 0·042	0·59[a]	0·989 ± 0·039	0·79[b]

[a] and [b] Dry weight ratios do not differ significantly from each other.

(v) *Dry weights of MatDp.dist2 and PatDp.dist2 embryos and placentas at 16·5 dpc*

To ascertain the effect of oedema on embryonic weight the dry weight of nine 16·5 dpc litters containing at least one PatDp.dist2 or one MatDp.dist2 embryo were measured. The dry weights of *bp* and non-*bp* mutant embryos and placentas were expressed as ratios of the dry weights of their normal littermates and averaged over all litters as shown in Table 3. Overall there was no significant evidence to suggest that the dried *bp* Pat and MatDp.dist2 embryos and placentas differed from those of non-*bp* Pat and MatDp.dist2 [$F(2, 12) = 1{\cdot}22$; $P = 0{\cdot}33$ for embryos; $F(2, 12) = 2{\cdot}35$; $P = 0{\cdot}14$ for placentas]. Similarly there were no significant differences in dry weights of Pat and MatDp.dist2 embryos at 16·5 dpc ($t = 1{\cdot}64$; $P = 0{\cdot}12$ for PatDp.dist2 embryos; $t_{13} = 0{\cdot}557$; $P = 0{\cdot}59$ for MatDp.dist2 embryos] nor in dry weights of Pat and MatDp.dist2 placentas [$t_{13} = 1{\cdot}50$; $P = 0{\cdot}16$ for PatDp.dist2 placentas; $t_{13} = 0{\cdot}269$; $P = 0{\cdot}79$ for MatDp.dist2 placentas].

(vi) *Skeletal observations and bone measurements of PatDp.dist2 and MatDp.dist2 at birth*

Skeletal measurements of six Pat and four MatDp.dist2 mice at birth, expressed as ratios of their

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Table 4. *Mean bone and skull measurements of six Pat and four MapDp.dist2 newborns expressed as ratios of their normal littermates and averaged over all litters*

	Skeletal measurement ratios ± SE			
	PatDp.dist2	Significance (*P*)	MatDp.dist2	Significance (*P*)
Tibia/fibula length	1·087 ± 0·025	$6{\cdot}3 \times 10^{-3}$[a]	0·994 ± 0·028	0·83
Femur length	1·060 ± 0·012	$7{\cdot}8 \times 10^{-4}$[a]	0·988 ± 0·013	0·39
Skull length	1·014 ± 0·031	0·66	0·963 ± 0·036	0·35
Skull width	1·026 ± 0·027	0·36	0·984 ± 0·031	0·63
Femur thickness	1·048 ± 0·049	0·34	0·932 ± 0·054	0·26
Spinal column length	0·981 ± 0·021	0·41	0·969 ± 0·026	0·28

[a] Significant, by Student's *t*-test following a logarithmic transformation of the data.

respective normal littermates, are shown in Table 4. These showed that the tibia/fibula and femurs of PatDp.dist2 mice were significantly longer than those of their normal littermates but the skull and spinal column were normal in size, indicating that the imprinting effect is probably unique to the long bones. No obvious skeletal differences were noted between MatDp.dist2 and PatDp.dist2 and no vertebral malformations were associated with the tail bend observed in PatDp.dist2 offspring at birth (not shown).

(vii) *Histological analysis of MatDp.dist 2, PatDp.dist2 and normal littermates*

A histological examination of Mat and PatDp.dist2 at 17·5 dpc and birth revealed no overt abnormalities in the following internal organs: liver, lung, heart, kidney, brain, salivary glands, muscle, bone, bone marrow, thyroid, skin, oesophagus. No gross differences could be detected between Pat and MatDp.dist2 placentas at 12·5 and 17·5 dpc (S. Monkley, personal communication).

4. Discussion

The data presented here and their analyses show that imprinting effects can be detected *in utero* for both PatDp.dist2 and MatDp.dist2. Thus, PatDp.dist2 *in utero* show a very severe oedema in late gestation, slightly diminished placental weight, and an increase in the length of the long bones at birth, whereas MatDp.dist2 are slightly dehydrated in late gestation.

The most notable feature of the PatDp.dist2 embryos is the increase in wet weight during the latter half of gestation, which culminates at 16·5–17·5 dpc when the embryos are more than 120% of normal wet weight, and has greatly diminished by birth (Table 2, Fig. 2). The increase is attributable to excess fluid because it disappears when the dry weights of PatDp.dist2 are compared with normal; at this stage we have no evidence of overgrowth of PatDp.dist2, and a preliminary conclusion from the data is that there is no excess in cell number.

The PatDp.dist2 mice are more severely oedematous than other oedematous mouse mutants such as T^{hp} or *Igf2r* mutants, for from our wet weight (ww) and dry (dw) weight data at 16·5 dpc we have calculated the average water content per unit dry weight per embryo [i.e. the ratio (ww − dw)/dw] and have found that it was increased by 40% whereas it is under 20% for T^{hp} and *Igf2r* mutants (Babiarz *et al.*, 1988; Ludwig *et al.*, 1996). The oedema does not increase in severity after 16·5 dpc, which coincides with the time when the embryonic kidney starts to function (J. Davies, personal communication), and so the diminution of the oedema by birth is presumably due to renal clearance by the fetus. However, there is still some evidence of oedema at birth, particularly around the neck, and this may account for at least part of the original description of a square-bodied phenotype in these mice (Cattanach & Kirk, 1985; Cattanach, 1986).

Increases in the ratio of wet weight to dry weight, or the ratio of wet weight to DNA content, have been observed in mouse mutants with elevated levels of insulin-like growth factor II (IGF-II), such as mice with maternally inherited inactivating mutations of *Igf2r* (Ludwig *et al.*, 1996), maternal inheritance of the T^{hp} deletion (Johnson, 1974; Babiarz *et al.*, 1988) which includes *Igf2r*, and *Igf2* transgenes (Ward *et al.*, 1994). In addition, organomegaly has been observed in the *Igf2r* mutants, mice with *Igf2* transgenes, and double mutants carrying a deletion around *H19* and a targeted *Igf2r* allele, both maternally inherited (Ward *et al.*, 1994; Ludwig *et al.*, 1996; Eggenschwiler *et al.*, 1997), and Graham *et al.* (1998) have suggested that the increase in tissue fluid in the mouse mutants accompanies an increase in supply of nutrients to the embryo enabling increased growth. Unlike the mutants with excess IGF-II, the oedema in PatDp.dist2 does not appear to be accompanied by organo-

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megaly. There is no direct evidence for a cause of the oedema but it may be of relevance that, overall, the wet weight of the placenta was slightly diminished (by 5–10%), and even though it appeared histologically normal it may have been functionally impaired and failing to regulate water, leading to fluid accumulation within the embryo. No importance is attached to the observation that the weight decrease disappeared when the dry weight of the placenta was measured, because the sample size was small and further data are required.

The notable feature of MatDp.dist2 *in utero* is the fall in wet weight from an initial high point at the earliest age, 11·5 dpc, through the remainder of gestation. The absolute differences in wet weight are small, only of the order of 5–10%, but are highly significant, indicating a progressive and increasing loss. However, the decrease is attributable to fluid loss because it disappears when the dry weights of MatDp.dist2 are compared with normal. Thus MatDp.dist2 appear to be dehydrated, and increasing severity of dehydration may account for the increasing loss in wet weight. From our wet and dry weight data at 16·5 dpc we have calculated that the average water content per unit dry weight per embryo was reduced by 6% in MatDp.dist2. At this stage we have no evidence of growth retardation, either from the weight data or from the skeletal measurements at birth. There may even be growth enhancement at the earliest time point (11·5 dpc).

It is difficult to be precise about the time and primary site of onset of expression of the genes responsible for the imprinting phenotypes. For both MatDp.dist2 and PatDp.dist2 the genes must be switched on by the latter half of gestation, and the primary site of action is probably the embryo for MatDp.dist2, but the effects seen in the placenta and embryo of PatDp.dist2 may have evolved as a consequence of primary effects in either or may have evolved independently.

Two maternally imprinted genes, *Nnat* (Kikyo *et al*., 1997; Williamson *et al*., 1998) and *Gnas* (Williamson *et al*., 1996), lie within the duplicated region investigated in this study, but this is not the case for any paternally imprinted genes. *Nnat* is expressed in the nervous system (Wijnholds *et al*., 1995) and is therefore unlikely to be responsible for any of the new effects described in this paper. *Gnas*, the gene for the alpha subunit of the guanine-nucleotide binding protein, is maternally imprinted specifically in the glomerular tuft of the kidney, and is biallelically expressed in a wide range of tissues (Williamson *et al*., 1996). It is difficult to envisage how imprinting of *Gnas* in the MatDp.dist2 kidney glomerulus could lead to dehydration because the kidney does not function before 16·5 dpc, when the dehydration is already observable and is presumably due to earlier-acting imprinted genes. Using the same argument, it seems unlikely that a double dose of *Gnas* in the PatDp.dist2 kidney glomerulus would result in oedema observable from 14·5 dpc. It is of interest that a new phenotypic feature has been found in the PatDp.dist2 at birth, notably an increase in the length of the long bones. *Gnas* is known to play a role in ossification, as patients with the disorder Albright hereditary osteodystrophy (AHO), a syndrome with 50% deficiency of Gsα, have shortened long bones, but there is no clinical evidence of imprinting in human bone (Davies & Hughes, 1993; Wilson *et al*., 1994). Thus further candidate genes are being sought for both the MatDp.dist2 and PatDp.dist2 phenotypes, specifically those genes that map between the T2Wa and T28H translocation breakpoints that define the minimal imprinting region and may play a role in fluid regulation, placental weight and long bone length.

Appendix. Correction for wet weight differences between *bp* and non-*bp* Pat and MatDp.dist2 embryos and placentas

As the marker gene brachypodyism, *bp*, causing shortening of the limbs, was segregating among the Pat and MatDp.dist2 embryos, depending on the direction of the cross, the data (Table 1) were analysed to see whether there were any wet weight differences between *bp* and non-*bp* *in utero* and at birth.

The *bp* PatDp.dist2 embryos and newborns weighed consistently less at all ages than non-*bp* PatDp.dist2 and although none of the individual ratios was statistically significant (not shown), the mean ratio of wet weights averaged over all age groups ($0{\cdot}940 \pm 0{\cdot}021$) was highly significant [$t_{191} = 2{\cdot}72$; $P = 0{\cdot}0071$]. Thus the *bp* PatDp.dist2 were smaller than non-*bp* PatDp.dist2 by a factor that did not vary with age. The *bp* MatDp.dist2 embryos and newborns, likewise, weighed less than non-*bp* MatDp.dist2, although in this case the difference was not as consistent as with the PatDp.dist2. Similarly the ratio of wet weights did not vary significantly with age as shown for PatDp.dist2, although in this case the mean ratio of weights ($0{\cdot}962 \pm 0{\cdot}022$) was no longer significant [$t_{191} = 1{\cdot}65$; $P = 0{\cdot}10$]. The mean *bp* to non-*bp* ratio for PatDp.dist2 did not differ significantly from that of MatDp.dist2 with age [$F(1, 191) = 0{\cdot}439$; $P = 0{\cdot}51$] and the pooled ratio ($0{\cdot}951 \pm 0{\cdot}014$) was highly significant [$t_{192} = 3{\cdot}41$; $P = 0{\cdot}00080$]. Furthermore the two sets of individual ratios did not vary significantly with age [$F(15, 176) = 1{\cdot}09$; $P = 0{\cdot}37$]. The data suggest that the wet weights of *bp* Pat and MatDp.dist2 are smaller than non-*bp* Pat and MatDp.dist2, respectively, by a factor that is independent of both age and phenotype. The pooled ratio described in the Results was used to correct for

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weight differences between *bp* and non-*bp* Pat and MatDp.dist2 embryos and newborns as follows:
Let the mean weight of all PatDp.dist2 embryos (*bp* and non-*bp*), expressed as a ratio of the mean weight of their normal sibs and averaged over all litters at a given age t, be denoted by b_t, and let the corresponding quantity for all MatDp.dist2 embryos (*bp* and non-*bp*) be denoted by s_t. Since at all ages there appears to be a constant ratio k of $0{\cdot}951 \pm 0{\cdot}014$ between the weights of PatDp.dist2 *bp* embryos and their non-*bp* counterparts and also between MatDp.dist2 *bp* and MatDp.dist2 non-*bp* embryos, the weights of the separate classes of embryos, expressed as ratios of the weights of their normal sibs and averaged over all litters of age t, are:

PatDp.dist2 $bp = b_t \sqrt{k}$,

PatDp.dist2 non-$bp = b_t/\sqrt{k}$,

MatDp.dist2 $bp = s_t \sqrt{k}$,

MatDp.dist2 non- $bp = s_t/\sqrt{k}$.

Estimates of the overall mean ratios b_t *and* s_t at each age given in Table 2 were obtained by fitting these quantities to the ratios shown in Table 1, using the calculated non-*bp* to *bp* mean ratio $0{\cdot}951 \pm 0{\cdot}014$ as an estimate of k. This correction was not necessary for placentas as no differences were found between placentas of *bp* and non-*bp* Pat and MatDp.dist2 embryos (data not shown).

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