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Two imprinted gene mutations: three phenotypes

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Genetic modifications of imprinted genes have been generated in the mouse to investigate the regulation of their expression. They show classical imprinted gene inheritances. Here we describe two imprinted gene mutations deriving from mutagenesis experiments. One is expressed only when transmitted through males. It causes a prenatal growth retardation which resembles that of the *Igf2* knockout and maps close to the locus on chromosome 7. Differences from the knockout, which include an abnormal head phenotype, homozygous lethality, and an inability to rescue a *Tme* (*Igf2r*-deficient) lethality, suggest that *Igf2* itself may not be directly affected. The second mutation maps close to the *Gnas* cluster of imprinted genes on distal chromosome 2. It gives two distinct phenotypes according to parental origin, a gross neonatal oedema with microcardia and a postnatal growth retardation. The oedema phenotype is effectively lethal and resembles that of mice with paternal partial disomy for distal chromosome 2, as well as that of mice having a maternally derived *Gnas* exon 2 knockout. However, the second growth retardation phenotype differs from that of the maternal partial disomy and the paternal knockout. A hypothesis to explain the phenotypes associated with the three genotypes based on the *Nesp*/*Nespas* sense/antisense and *Gnasxl* transcripts in the *Gnas* cluster is offered.

INTRODUCTION

Genomic imprinting in mammals specifies a germ line marking of parental alleles that results in their differential expression in the zygote. This has been most widely demonstrated in the mouse through the creation of uniparental disomies and partial disomies, which have two copies of genes within the chromosomes/regions from one parent and none from the other (1). Such studies have shown imprinting to affect genes in at least 11 regions of the mouse genome, with the imprinting anomalies or phenotypes ranging from early embryonic lethalities, through neonatal abnormalities, to postnatal growth retardations. In a number of cases, the genes responsible for the abnormalities have been identified and following genetic modification, imprinted gene inheritances, as first hypothesized by Hall (2), have been demonstrated. These include those for *Igf2* (3,4), *Rasgrf1* (5), *Gnas* (6) and *Ube3a* (7) knockouts and the targeted central chromosome 7 deletions which involve several imprinted genes (8,9). The *T* maternal effect (*Tme*) lethality, associated with the long-recognized T^{hp} mutation (10,11) which is attributable to the deletion of the maternally imprinted *Igf2r* locus (12), also shows an imprinted gene inheritance.

In humans, two inherited and imprinted forms of pseudo hypo-parathyroidism have been recognized. One, PHP-Ia, is associated with Albright's hereditary osteodystrophy (AHO) and involves the *GNAS* locus (13) on chromosome 20. The other, PHP-Ib, is not associated with AHO but maps to the same region (14). Isolated examples of familial Prader–Willi and Angelman syndromes resulting from mutations at putative imprinting control loci have also been described (15). However, up until now imprinted gene inheritances, other than those of genetic modifications of imprinted genes (3–7) and that attributable to the *Igf2r* deletion (12), have not been described in the mouse. Here we report two mouse mutations which show imprinted gene inheritances. The first mutant, called minute (*Mnt*), lies within the distal chromosome 7 imprinting region and may involve the imprinted *Igf2* locus. The phenotype resembles the *Igf2* knockout (3,4) but there are differences. The second, provisionally called oedematous-small (*Oed-Sml*), lies within the distal chromosome 2 imprinting region and uniquely shows two distinct phenotypes according to the parental route of transmission. One of the latter resembles a distal chromosome 2 imprinting effect (16).

RESULTS

Minute (*Mnt*)

Origin. The original mutant derived from one of a series of specific locus mutation experiments (17) in which spermatogonially irradiated (6 Gy X-rays) F_1 hybrid C3H/HeH × 101/H males were mated to females homozygous for seven different recessive mutations. All offspring of the cross were screened not only for mutations at the tester loci but also for new dominant mutations, many of which, with radiation, have been found to represent chromosomal changes, notably deletions and duplications (18). The *Mnt* mutation was detected in a male through its severe growth retardation (60% of normal) observed initially at birth, but this was accompanied by an abnormal domed head effect (Fig. 1). No evidence of a chromosomal change has been seen in detailed G-band analysis (E.P. Evans, personal communication).

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Table 1. Inheritance and viability of *Mnt*

Cross	No. of young	Litter size	Phenotypes at birth			*Mnt* postnatal viability[a] (%)
			Mnt live	*Mnt* dead	+	
3H1 FF × Orig M	158	7.2	45	12	99	55
3H1 FF × *Mnt* MM	471[b]	7.4	111	22	320	25
Mnt FF × 3H1 MM	320	6.3	0	0	320	–
M.cast FF × *Mnt* MM	581	4.8	265	0	329	89

3H1 = F_1(C3H/HeH × 101/H) hybrid; F, female, M, male; *M.cast.*, *M.m.castaneus*.
[a]Live at weaning relative to live at birth.
[b]Sample of data only.

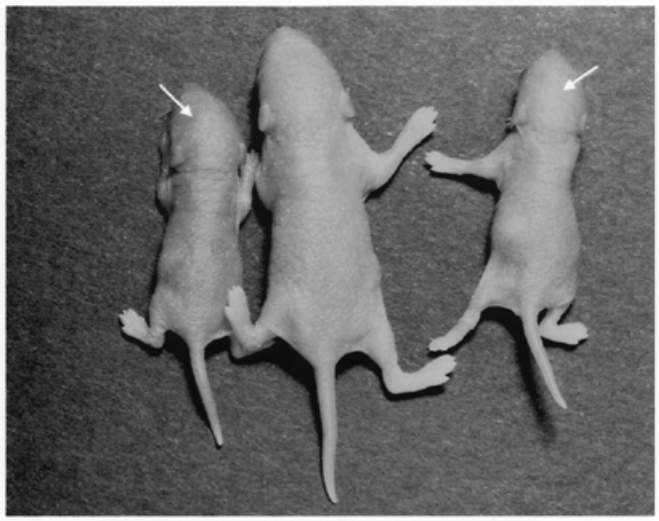

Figure 1. *Mnt* and + sib at birth. Domed heads are arrowed.

Inheritance. On crossing the original animal with normal (+) females, a proportion of the progeny (of both sexes) expressed the mutant phenotype (Table 1). A simple dominant inheritance was therefore suggested and this appeared to be confirmed on crossing affected sons with + females. Elevated levels of neonatal loss were encountered, notably in the second generation, but survival was enhanced in *Mus mus castaneus* crosses, perhaps because of the low litter size of this subspecies (Table 1). Remarkably, none of the progeny of mutant females were affected (Table 1), yet clearly the mutation was transmitted as approximately half their sons proved capable of producing *Mnt* young (Table 2). Mutant expression was therefore obtained only with transmission through males. This represents an imprinted gene inheritance as illustrated in Figure 2 and implies that the locus affected by *Mnt* is normally expressed only when transmitted through the male germ line; the maternal allele is silent.

Mutant animals recovered from the phenotypically + males that had inherited the mutation from their mothers (Mnt^M) were not detectably different from those (Mnt^P) that had inherited the mutation directly from their affected fathers (Table 2).

Further characterization of Mnt. Prenatal growth rates, as assessed by ratios of weights of mutant to + sibs within litters declined from the earliest prenatal age at which the size differences could be distinguished (13 days gestation) up until the time of birth when they approximated <50% of normal (Fig. 3). The size was significantly different from that of the sibs throughout ($P < 0.1 \times 10^{-9}$). Extrapolating the data backwards, the straight line of best fit crossed the age axis at embryonic day (E) 10.7 (data not shown), possibly defining the age at which the size difference in the fetus might first be initiated. Placental weight ratios were more affected than fetal weight ratios (see legend to Fig. 3) and the straight line of best fit crossed the age axis ~3 days earlier. This might suggest that the reduced fetal growth is at least in part mediated through the placenta.

Table 2. Breeding tests on phenotypically + male progeny of *Mnt* females

Deduced genotype	No. tested	No. of young	Litter size	No. classified at birth	
				Mnt^P	+
Mnt^M/+	37	2431	7.0	612	1689
+/+	42	1121	8.3	0	1121

Mnt^M, maternally inherited *Mnt* allele; Mnt^P, paternally inherited *Mnt* allele.

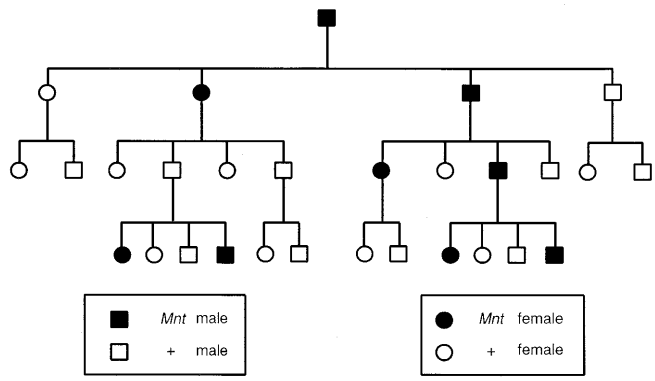

Figure 2. Diagram showing inheritance of *Mnt.* Semi-hypothetical pedigree in which the original affected male produces up to 50% affected sons and daughters in matings to + females. Affected sons breed like the father, producing up to 50% affected male and female progeny in crosses with + females. Affected females produce only + progeny in crosses with + males, but 50% of their progeny inherit *Mnt*. When transmitted into the next generation through males, the *Mnt* phenotype reappears.

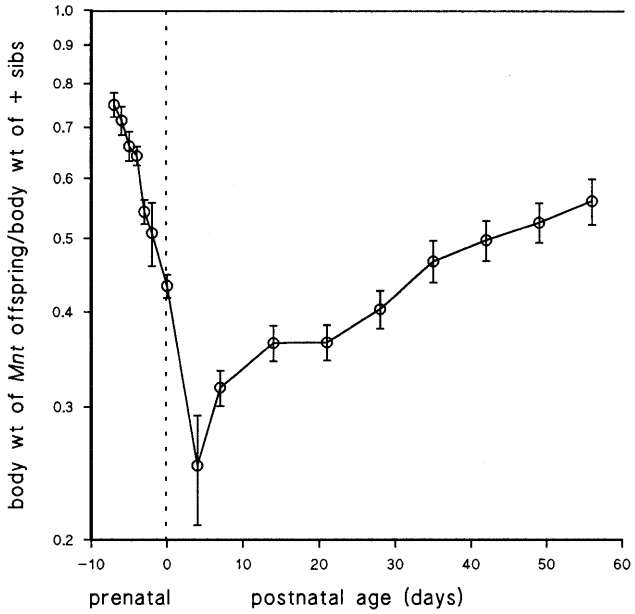

Figure 3. Growth rates of *Mnt* animals shown as weight ratios relative to their + sibs. All ratios are plotted on a logarithmic scale. Placental weight ratios (data not shown) were significantly smaller than those for embryos (t = 4.41; df = 82; P = 0.000031) and did not vary significantly with age [$F(5,77)$ = 0.874; P = 0.50].

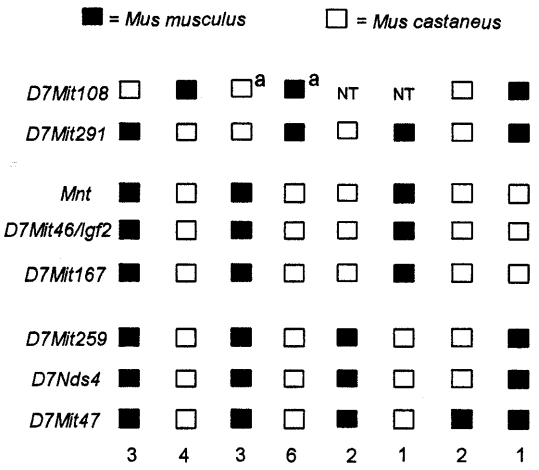

Figure 4. Mapping of *Mnt* within distal chromosome 7. Only recombinants are shown. NT, not tested with *D7Mit108*; a, one animal of each marked genotype was not tested with *D7Mit108*.

Postnatal *Mnt* growth rates suggested that the size difference declined further to reach a minimum at ~4 days of age (Fig. 3) when most inviability occurred. It seems likely that the lethality is only a consequence of difficulties that these small animals have in competing with their larger, normal sibs, as growth and survival improve when litter sizes are artificially or naturally reduced, as in *M.m.castaneus* crosses which have low litter sizes (Table 1). At later ages there appeared to be a gradual recovery or 'catch up' (Fig. 3) but this could be due to the surviving animals being the least affected. Thus, differences between the weight ratios at birth of non-survivors (beyond 4 days) and survivors to later ages were highly significant [$F(5,11)$ = 6.14; P = 0.000046]. *Mnt* adults were still ~60% of the weight of their + sibs. The domed head phenotype was most clearly evident shortly after birth (Fig. 1) but could be observed at all ages.

Mapping of Mnt. Almost all imprinted genes identified so far have been mapped within the imprinting regions defined with the use of chromosomal translocations (1). It was therefore anticipated that the *Mnt* locus would lie within one of these regions, several of which give growth retardation imprinting effects (1). On the basis that the maternal allele is normally silent and only the paternal allele is expressed, proximal chromosome 11 was initially considered the most likely site of the *Mnt* mutation but linkage tests (data not shown) with appropriate genetic markers negated this possibility. Distal chromosome 7 provided a further candidate region as a paternally inherited knockout of one of the known imprinted genes in this region, *Igf2*, also bringing about a growth retardation (3,4) that closely resembles that of *Mnt*. This gene therefore became the most likely candidate gene.

Close linkage with *Mit* markers located within the distal chromosome 7 imprinting region (19) established that this was the location of the mutation. Thus, with *D7Mit108* and the more distal *D7Mit47*, which span the imprinted *Igf2* locus and lie 12 units apart, 13 and 2 recombinants, respectively, were detected among 100 backcross progeny, suggesting that *Mnt* maps between these loci and therefore close to *Igf2*. This possibility was confirmed in linkage tests with *D7Mit291*, which lies between *D7Mit108* and *Igf2*. Thus, using a further 96 backcross animals, two recombinants with the phenotype were detected and a further four were found with *D7Mit47*. Finally, when all 22 recombinants were tested with other markers in the region (*D7Mit167, D7Mit259, D7Nds4* and specifically *D7Mit46*, which primes up part of the *Igf2* gene itself), cosegregation with *D7Mit46* and *D7Mit 167* was demonstrated (Fig. 4). *Mnt* therefore involves, or lies very close to, *Igf2* within the distal chromosome 7 imprinting region. All *Mit* markers selected could be studied. No evidence of a deletion on the *Mnt* chromosome was therefore indicated.

Fate of Mnt homozygotes. Investigations into the fate of the *Mnt* homozygotes were initiated before the map positions were known and did not provide a clear conclusion. The litter size of *Mnt* × *Mnt* intercrosses were notably low (mean = 3.9, n = 78), suggesting that the homozygote dies prenatally. On the other hand, openings of pregnant females from intercross matings failed to demonstrate a novel or lethal class at 17.5 days gestation, pre- and post-implantation losses approximating normal levels (2.4 and 9.2%, respectively). Notably, litter size was within the normal range at this time (mean = 6.5, n = 35) and *Mnt* and + embryos were detected in near-equal frequencies (124 and 102, respectively). It therefore seemed that at this stage of development *Mnt* homozygotes are viable and indistinguishable from heterozygotes. On this basis, the *Mnt* classes would comprise Mnt^{P}/Mnt^{M} and Mnt^{P}/+ and the + classes Mnt^{M}/+ and +/+.

Confirmation that the homozygote indeed survived until at least shortly before birth was finally obtained by opening

Table 3. Inheritance and viability of *Oed* and *Sml* phenotypes

Cross	No. of young born	Litter size at birth	No. classified at birth		No. classified at 5–7 days		Viability at classification (%)
			Oed	+	*Sml*	+	
Orig. *Sml* F × 3H1 MM	73	6.7	17	56	–	–	*Oed* 0.30
Oed FF × 3H1 MM	77	3.9	18	59	–	–	*Oed* 0.31
Sml FF × 3H1 MM	615	5.1	213	402	–	–	*Oed* 0.53
Sml MM × 3H1 FF	1498	7.4	–	–	454	799	*Sml* 0.57

M, male; F, female.

females from *Mnt* F_1 *M.m castaneus* intercrosses and screening fetuses for the closely linked *Mit46(Igf2)* and/or *Mit167* markers. At 16–17 days gestation, 21 Mnt^M/Mnt^P, 28 Mnt^P, 21 Mnt^M and 18 + fetuses were identified. Seven of the homozygotes had recently died, and from the failure to find any of this class at birth it may be concluded that *Mnt* is homozygous lethal and that the loss of this class occurs late in gestation to around the time of birth. The homozygotes did not show any features that could enable them to be distinguished from heterozygotes. While the abnormal *Mnt* head phenotype provided the first indication that the mutant and the *Igf2* knockout (3,4) were not identical, the homozygous inviability illustrated a second key difference.

Effect of Mnt on brachyury (T) deletion lethality. The combination of a paternally inherited *Igf2* knockout, in which the gene is not expressed, with maternally inherited brachyury deletions, which encompass the *Igf2r* receptor locus, allows partial rescue of the fetuses with the *T* maternal effect (*Tme*) lethality (20). The lethality, which typically occurs late in gestation (10,11), has been attributed to an excess of the *Igf2* ligand in the absence of the receptor and the rescue being achieved by the deficit of *Igf2* (20). To determine whether *Mnt* could similarly effect such a rescue, females heterozygous for the brachyury mutation, T^{37H}, which causes the typical, variable *Tme* lethality when maternally inherited (B.M. Cattanach, unpublished data), were crossed with *Mnt* males. Some T^{37H} survival to birth was found in control crosses of such females with + males (five dead T^{37H} among 92 + young born), as occasionally found with the T^{hp} deletion (10,11) on the same genetic background (B.M. Cattanach, unpublished data). However, when mated with *Mnt* males, no T^{37H} young were recovered among 61 offspring born to T^{37H} mothers, although *Mnt* young were obtained in the expected frequency. No suggestion of a rescue was therefore identified.

Further investigation of T^{37H} × *Mnt* crosses at 17.5 days gestation indicated a high incidence of late deaths and early (small mole) losses and, perhaps as a result, the segregation of both *T* and *Mnt* were skewed. However, there was a clear shortage of both *T* and *T Mnt* offspring relative to their + and *Mnt* sibs (19:29 and 27:43, respectively), again showing no indication of rescue. Similar results were obtained with a small sample of equivalent crosses using T^{hp} females (3:4 and 5:8, respectively). Weight ratios, however, indicated that the T^{37H} fetuses were significantly heavier (due to oedema or overgrowth) than their + sibs (1.274 ± 0.041; $t_{33} = 7.53$, $P = 8.1 \times 10^{-9}$), but this was not found when *T Mnt* and *Mnt* fetuses were compared (1.039 ± 0.03; $t_{35} = 0.437$, $P = 0.20$). The latter finding might suggest that some element of the *T* maternal effect might be modified by the presence of *Mnt*, but it may just be more difficult to validly assess weight differences in these already severely compromised fetuses. The overall inability of *Mnt* to rescue the *Tme* lethality suggests a third difference from the *Igf2* knockout (3,4).

Oedematous-small (*Oed-Sml*)

Origin. As with *Mnt*, the *Oed-Sml* mutation derived from a specific locus mutation experiment and was detected because of a growth retardation evident at birth and later ages. In this case the mutagen used was ethylnitrosourea (ENU) (250 mg/kg body wt; B.M. Cattanach, unpublished data) and the original variant animal was female. As ENU is known to cause mutations by AT→GC base pair transitions or AT→TA transversions (21), *Oed-Sml* was not expected to be a chromosomal mutation. No evidence of a chromosomal change has been seen in detailed G-band analysis (E.P. Evans, personal communication).

Inheritance. On being subjected to breeding tests with a + male, the variant female did not produce growth-retarded young like herself. Instead, a new variant phenotype appeared and clearly had a genetic basis as near-identical examples (n = 17) were found in 11 litters (Table 3). The novel phenotype principally comprised a gross oedema, hereafter denoted *Oed*. None of the affected young lived for more than a few days.

Ovary transplantation from neonates was used to try and achieve survival of the mutant stock. This yielded further affected animals, but the risk of losing the mutation remained high. Only the survival of a single *Oed* male assured the future of the mutation and this had to be accomplished using *in vitro* fertilization and embryo transfer, the male seemingly being unable to breed normally. Litters were produced by 16 normal recipient females and, on classification, these yielded a further unexpected finding. Of the 116 progeny born, none showed the *Oed* phenotype but, within 5–7 days of birth, approximately half became obviously growth retarded, like the original variant female. Subsequent studies established that when females showing this growth-retarded or small phenotype (denoted *Sml*) were mated with + males, the *Oed* phenotype reappeared among their offspring. On the other hand, when their *Sml* male sibs, or surviving *Oed* males, were crossed with + females, the *Sml* phenotype was produced, appearing in about half the offspring. Data establishing the inheritance of the double, parental source-dependent phenotypes are

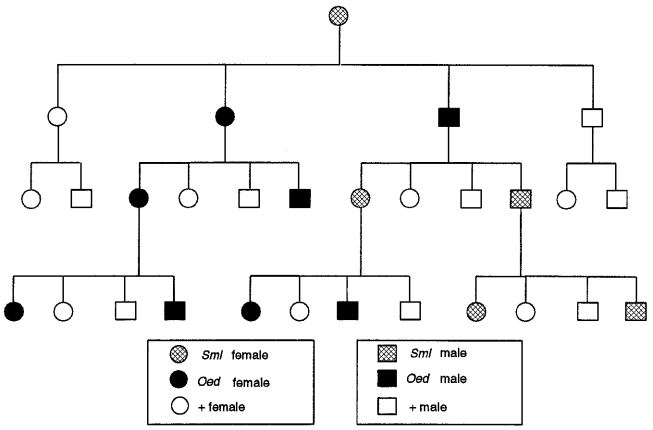

Figure 5. Diagram showing inheritance of *Oed-Sml*. Semi-hypothetical pedigree in which the original *Sml* female produced up to 50% *Oed* progeny of both sexes in crosses with a + male. Surviving *Oed* females breed like their *Sml* mother, producing up to 50% mutant progeny which are *Oed*. *Oed* males, on the other hand, produce a proportion of mutant progeny all of which are *Sml* like the original female. *Sml* females breed like *Oed* females and produce a proportion of mutant young all of which are *Oed*. *Sml* males produce a proportion of mutant young all of which are *Sml*. + animals produce only + progeny.

presented in Table 3 and illustrated diagrammatically in Figure 5. The *Oed-Sml* mutation therefore presents a second type of imprinting inheritance.

Further characterization of the Oed phenotype. The *Oed* phenotype was readily detectable at birth but the recovered frequency of affected young at this age was only half that expected (Table 3). Prenatal studies indicated an elevated level of resorption sites (9.9%) and dying *Oed* fetuses (15.3%) at 17.5–19.5 days gestation, accounting for the shortage.

At birth, *Oed* animals resembled mice with the oedematous imprinting phenotype caused by paternal duplication/maternal deficiency (partial disomy) for distal chromosome 2 (PatDp.dist2) (13). However, in addition, brown fat accumulations could be seen through the skin, localized at the scapular region on the back (Fig. 6A) and around the throat. The oedema, as seen at birth [36% difference between wet and dry weights (Fig. 6B)], was similar to that of the PatDp genotype at its peak at 16 days gestation (40% difference) (22), but was notably more evident before birth (56% difference). With both genetic conditions the oedema appears to decline just before and also subsequent to birth (Fig. 7A) and the few adult *Oed* survivors (obtained only following crosses with *M.m.castaneus*) were not overtly oedematous. However, in sharp contrast to the PatDp class, *Oed* neonates showed no indication of the hyperkinetic behaviour that characterizes the PatDp class (16).

The ongoing loss of *Oed* young after birth was accompanied by a decline in the weight ratios (clearly observable beyond the loss of oedema). This reached minimum values over the second and third weeks and then, among the few that survived longer, appeared to rise (Fig. 7A). In view of the small numbers of survivors, the significance of the rise is not clear.

Pathological examination of those *Oed* young that survived for several days after birth showed inflammatory reactions in the larynx and pharynx and, in the lungs, there was a haemorraghagic reaction with an infiltration of blood cells. Perhaps

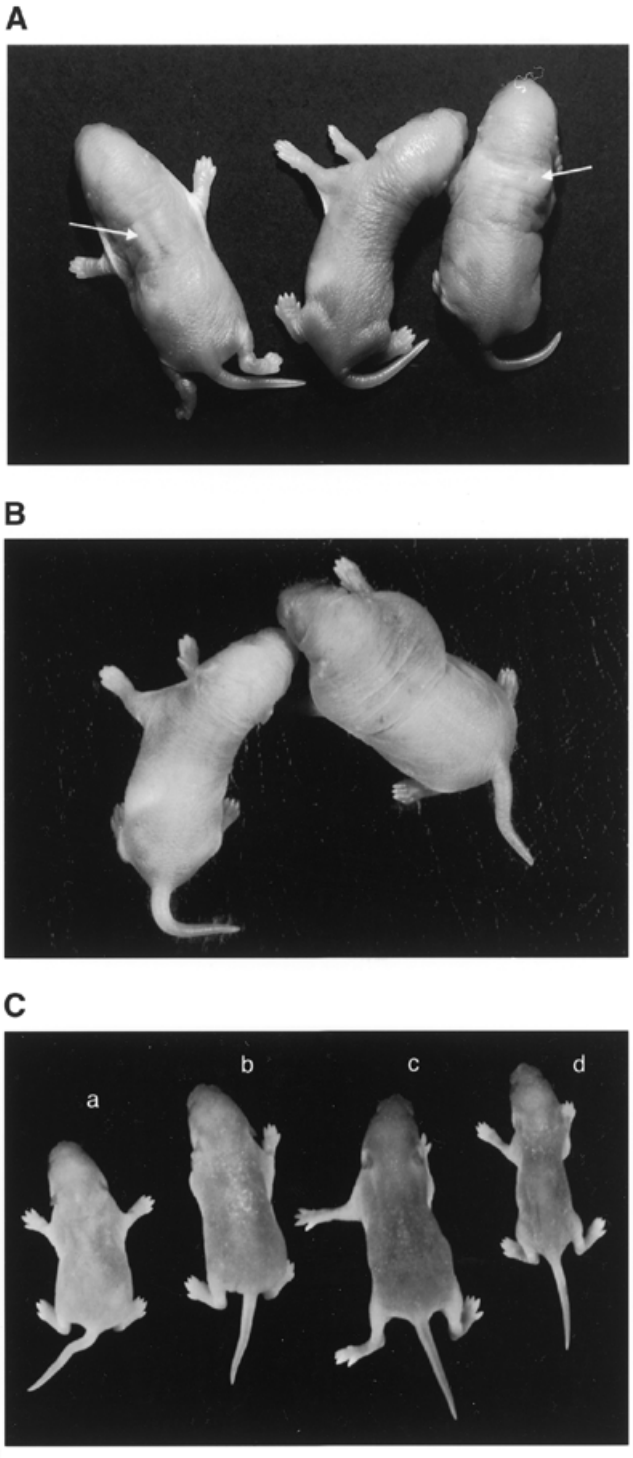

Figure 6. (**A**) *Oed* mice showing brown fat at scapular regions and around base of neck (arrowed), with + sib. (**B**) Grossly oedematous *Oed* mouse at birth with + sib. (**C**) Declining *Oed* mice (a and b) at 5 days of age, + sib (c) and growth-retarded *Sml* sib (d) deriving from an *Sml* × *Sml* intercross.

consistent with the latter finding was the observation of breathing difficulties in the few animals that survived to such ages. The aetiology of these effects was not clear; they may be only secondary consequences of a primary defect. Of more significance was the finding that heart size was severely

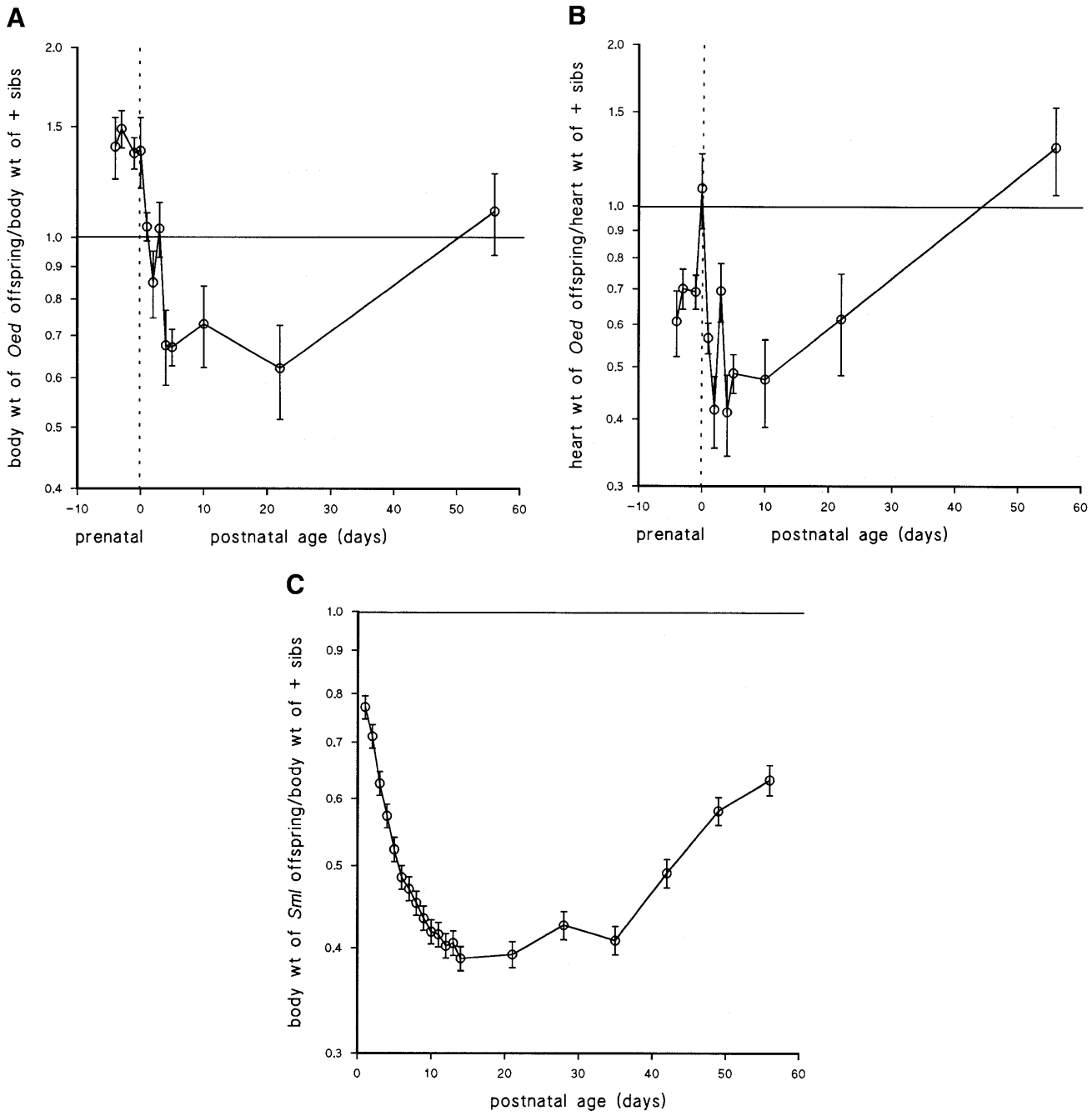

Figure 7. (**A**) Pre- and postnatal body weight ratios of *Oed* mice relative to their + sibs. Placental weight ratios were only marginally greater than 1 (mean ratio = 1.084 ± 0.047; $t = 1.86$; $df = 13$; $P = 0.085$). (**B**) Heart weight ratios of *Oed* mice relative to their + sibs. (**C**) Postnatal body weight ratios of *Sml* mice relative to their + sibs. All ratios are plotted on a logarithmic scale.

reduced, typically being less than half that of normal. This was evident from 16 days gestation to 10 days after birth during which time heart weight ratios declined further (Fig. 7B). However, two surviving adults had normal heart sizes. The early effect appeared that of a heart-specific growth retardation as no other abnormality within the heart was detected. Other organs appeared normal.

Further anomalies noted in *Oed* mice that survived beyond 2 weeks of age included sparse, wiry coats that became dense and rough in adults. Adult *Oed* animals also looked shorter bodied than their sibs, but body weights were not notably affected. Similar effects have been noted with those PatDp.dist2 mice that survive to adulthood (B.M. Cattanach, unpublished data). *Oed* mice of both sexes can be fertile.

Further characterization of the Sml phenotype. Weight ratios based on whole litters indicated a statistical difference [$P < 0.1 \times 10^{-8}$] from one over the ages tested [birth to 8 weeks (Fig. 7C)].

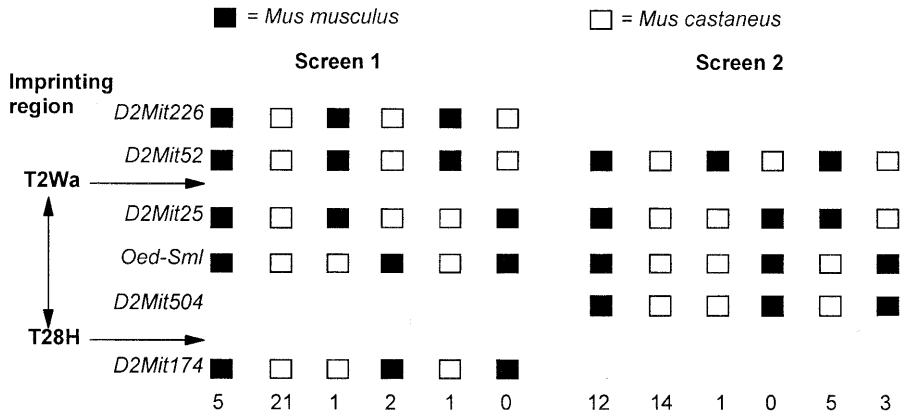

Figure 8. Mapping of *Oed-Sml* relative to *Mit* markers and the breakpoints of translocations defining the imprinting region.

Some *Sml* individuals were visibly growth retarded shortly after birth but classification became reliable only at ~5–7 days. Weight ratios were minimum at 10–14 days after birth. Subsequently there was some indication of recovery, but comparisons of surviving and non-surviving *Sml* mice at earlier ages showed that the survivors were marginally bigger [$F(14,121) = 1.64$; $P = 0.07$]. The apparent recovery may therefore be attributable to survivors being the least affected. Losses of the *Sml* class were substantial with most occurring around the ages when weight ratios were at their lowest. The size difference appeared to be a whole animal effect as weight ratios of their organs (heart, kidney, liver), as a proportion of body weight, did not differ from 1 (data not shown).

Mapping of the Oed-Sml mutation. The similarity between the *Oed* and PatDp.dist2 phenotypes suggested that the *Oed-Sml* locus might lie within the distal chromosome 2 imprinting region. Linkage back-crosses using *Sml* males and *Mit* markers which lie close to the region (*D2Mit226* and *D2Mit52* proximal and *D2Mit174* distal) or within it (*D2Mit25* and *D2Mit504*) (19, and J. Peters, unpublished data) confirmed that the mutation lies distally on chromosome 2. Thus, in the first screen of 30 animals there was complete linkage with *D2Mit174*, although there was a remarkably high recombination frequency (3/30, 10%) with *D2Mit25* (Fig. 8) considering the distal location of these markers on the chromosome. In a second screen with 35 further animals, complete linkage was found with *D2Mit504*, but a high recombination frequency (8/35, 22.8%) was again found with *D2Mit25* (Fig. 8). The high recombination frequencies are puzzling but the data nevertheless establish that the *Oed-Sml* mutation lies distally in chromosome 2 and probably at the distal end of the imprinting region. As such it becomes the candidate locus for at least the oedema component of the PatDp.dist2 imprinting phenotype.

Fate of the Oed-Sml homozygote. Intercrosses of phenotypically *Sml* animals generated 310 young among which, at birth, there were 54 *Oed* and 256 +. Of 224 + progeny that were reclassified at 5–7 days of age, 92 proved to be *Sml* and 132 were +. Twenty of the + class were test-mated and all proved to be genotypically +. A novel phenotype that might identify the homozygote was not therefore evident, and the proportions of *Sml* and *Oed* classes in relation to + were in accord with expectation (Table 3). Prenatal lethality of the homozygote was thus suggested. Consistent with this, the mean litter size of intercrosses was notably low (4.13/female, $n = 75$), especially in comparison with that of outcrosses of *Sml* females (5.13/female) (Table 3) in which the low viability *Oed* class is twice as numerous.

Openings of females derived from *Sml M.m.castaneus* F_1 intercrosses at 17–19 days gestation again failed to identify a novel class that could represent the homozygote. On typing 90 fetuses using the two closely linked *Mit* markers, *D2Mit25* and *D2Mit174*, 13 were recognized as *Oed*, 46 were identified as heterozygotes and 31 were deduced to be +. None were homozygous for the *M.m.musculus* alleles, indicating that the homozygote must die early in development. Post-implantation losses were marginally higher than that in *Sml* female × + male crosses (28 cf. 22%). Given that the frequency of *Oed* fetuses in the intercrosses should be only half that in the outcrosses, it would seem likely that much of the post-implantation loss in intercross matings is attributable to lethality of the homozygotes.

DISCUSSION

Mnt provides a classic example of an imprinted gene inheritance as postulated by Hall (2). Thus, in some crosses a clear dominant inheritance is evident and yet in others the phenotype disappears as though inherited as a recessive. The parental origin of the mutation provides the explanation. Expression is only seen with transmission through males; when maternally transmitted the phenotype is wild-type. The only other example in the mouse is seen with brachyury (*T*) mutations that are associated with deletions that encompass the paternally imprinted/repressed *Igf2r* locus (12). Described long before imprinting in mammals was recognized (10,11) these give the *Tme* lethality when transmitted though females; a functional maternal *Igf2r* copy is required (12). Knockouts of imprinted genes have in more recent times also shown imprinted gene inheritances (3–7), as have targeted deletions (8,9). The *Oed-Sml* mutation provides a variation on the theme. However, rather than giving a mutant phenotype when transmitted through one sex and wild-type or normal when transmitted

through the other, two different mutant phenotypes are produced according to parental route of transmission. The double phenotype therefore resembles the seemingly 'opposite' phenotypes of distal chromosome 2 and proximal chromosome 11 reciprocal uniparental duplications/partial disomies (16).

Consistent with their imprinting inheritances, both *Mnt* and *Oed-Sml* map within known imprinting regions of mouse chromosomes (1). *Mnt* maps close to *Igf2* within the distal chromosome 7 imprinting domain (1,19), and *Oed-Sml* maps within the distal chromosome 2 imprinting domain in the vicinity of the *Gnas* imprinted gene cluster (22–24).

In the case of *Mnt*, it is possible that the mutation involves the imprinted *Igf2* locus itself. Thus, recombinants with *D7Mit46*, which is intrinsic to *Igf2*, have not yet been found, and the growth retardation phenotype closely resembles that of the *Igf2* knockout (3,4). Furthermore, the time in development when the growth retardation is first seen accords with that observed with the *Igf2* knockout (25). Placental weight growth retardation was seen with *Mnt* fetuses and this has also been found with the knockout (25). The knockout data show the growth effects to appear within the 24 h immediately prior to E13.5 and remain almost constant thereafter (25), whereas with *Mnt* both the fetal and placental growth ratios showed a significant decrease with increasing age (slopes = –0.0832 ± 0.0077 and –0.0570 ± 0.0149, respectively). Whether this represents a real difference between the mutant and the knockout is not clear. Extrapolation of the *Mnt* data backwards showed that the lines of best fit to cross the age axis at ~E10.7 and ~E14.0, respectively, providing estimates of when the fetal and placental growth differences might first be initiated. The approach assumes linearity of growth, which in view of the knockout data (25) may not be valid.

In contrast, at least two and possibly three observations clearly distinguish the mutant from the knockout. The first is the head phenotype. This has not been described with *Igf2* knockout mice (3,4). The *Mnt* mutation, however, occurred following parental radiation exposure. It is therefore likely to represent a chromosomal change. A deletion could therefore account for the compound growth retardation and head anomaly phenotype, just as the T^{hp} and many other brachyury deletions bring about the *Tme* lethality attributable to the absence of *Igf2r* (12), as well as the tail effect (10,11).

The second difference between *Mnt* and the *Igf2* knockout is that the mutant is lethal in the homozygous condition. Here again, chromosomal deletion could be responsible as most deletions are homozygous lethal, suggesting that genes within them are haplo-insufficient (18).

The third and less firmly established difference between the mutant and the knockout is that *Mnt* appears to be unable to rescue a *Tme* lethality as does the knockout (20). Genetic background is unlikely to be responsible for the difference as the brachyury deletion mutant used (T^{37H}) is not distinguishable from that employed with the knockout (T^{hp}) on the same genetic background; with both, affected mice also occasionally survive to birth without rescue. A failure to effect *Tme* rescue suggests that at least some level of *Igf2* expression remains in *Mnt* mice. If verified, this would rule out deletion of the *Igf2* locus itself as the basis for the mutation and would suggest that either some other paternally expressed locus in the region is responsible for the growth effects, or that it is the regulation of *Igf2* expression that is affected. Ongoing *Igf2* expression studies and detailed analysis of the region are providing evidence that the mutation is complex. The findings will be the subject of a further paper.

In seeking interpretation of the double *Oed-Sml* phenotype, it may be noted that similar parent of origin-dependent phenotypes are brought about by uniparental duplications/partial disomy of the distal chromosome 2 imprinting region (16) and also with a knockout of exon 2 of *Gnas* (6). Comparison of the three phenomena suggests that the effects of all three conditions can be interpreted in terms of an absence of functional maternal/paternal alleles of distal chromosome 2 loci.

With absence of a functional maternal allele, oedema is found with all three genotypes. Microcardia is characteristically seen with the *Oed-Sml* mutation and we have since detected it in lower degree in paternal duplication (PatDp) mice derived in studies using the T30H translocation (weight ratio = 0.796 ± 0.050; $P = 0.0024$ compared with 0.542 ± 0.03 for *Oed* mice). Microcardia has not, however, been reported with the maternally inherited knockout (6). Postnatal viability is poor to zero with all three classes. On the other hand, no trace of the abnormal hyperkinetic behaviour seen with the PatDp genotype (16) is found in *Oed* mice, whereas some possibly different form of 'ataxia' has been described with the maternally inherited knockout (6).

In the reciprocal situation with functional paternal allele absence, clear similarities are seen for two of the three genotypes. With the MatDp (16) and the paternally derived knockout (6), neonates show thin flat-sided bodies and typically fail to suckle well if at all. They are hypokinetic. The only recognized difference between the two phenotypes is that the maternal duplication (MatDp) class invariably dies within a few hours, whereas a significant number of the knockouts survive despite the feeding difficulties.

The *Sml* phenotype obtained with paternally *Oed-Sml* transmission differs considerably. They do not have detectably abnormal body shapes, nor the hypokinetic behaviour with near-absent suckling ability. Generally they are normal at birth although there appears to be some early postnatal inviability that is detectable by a shortage in their numbers at 5–7 days of age. They cannot be reliably identified earlier and the only recognized effect is the growth retardation. The paternally inherited knockout is also reported to show growth retardation, but it is possible that this is due to the suckling difficulties. Overall, the *Sml* phenotype differs considerably from the hypokinetic MatDp and paternally derived knockout classes but, like the homozygous knockout (6), the *Oed-Sml* homozygote is an embryonic lethal.

It has recently been shown (6) that the loss of exon 2 of *Gnas* disrupts expression of the neighbouring imprinted genes, *Nesp* and *Gnasxl*. Both genes could therefore have roles in producing the phenotypes brought about in the uniparental duplication (16) and in the knockout (6) mice. For the *Oed-Sml* mutation, the expectation is that it represents a point mutation, but it also gives two parent of origin-dependent phenotypes. However, taking everything together, a hypothesis can be constructed that can explain the clearly established findings (Fig. 9).

If *Nesp* and the newly identified imprinted gene *Nespas*, which runs antisense to *Nesp* (23), are taken as candidate loci for the *Oed-Sml* mutation, a single point mutation could disrupt

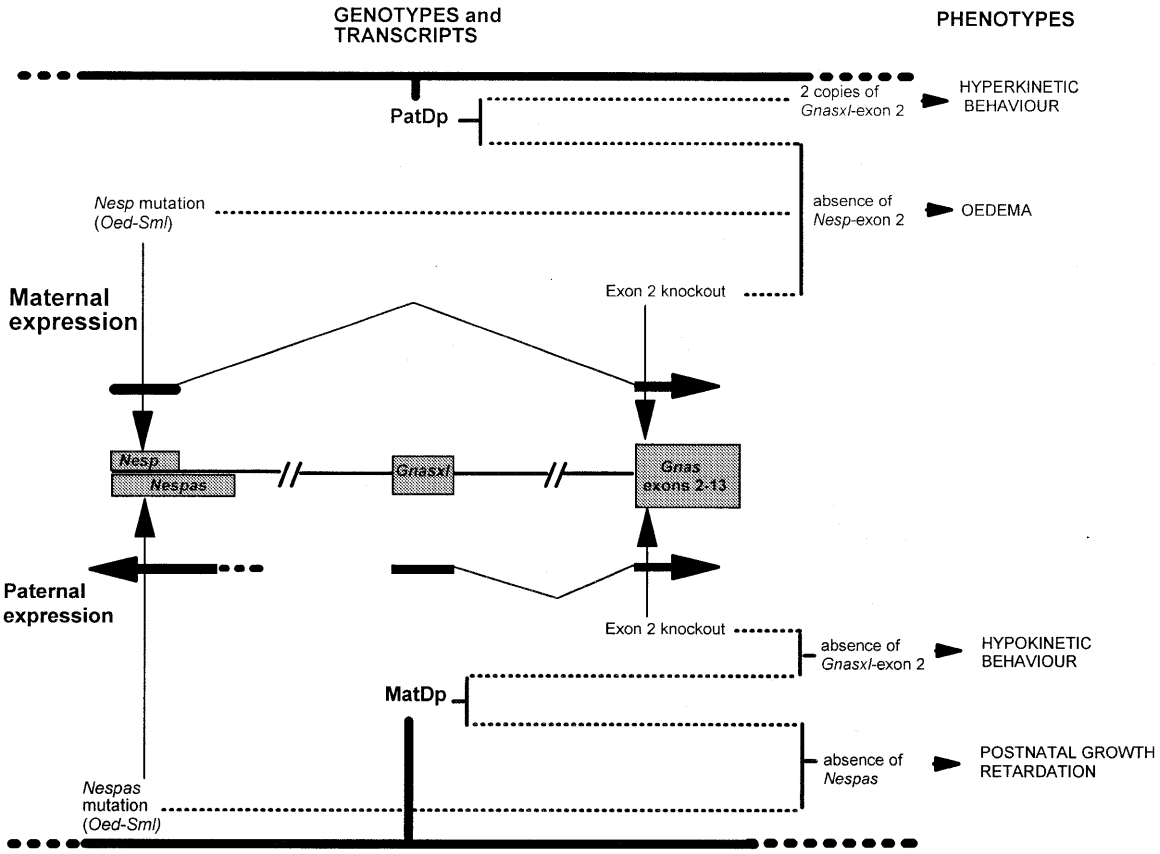

Figure 9. Hypothesis to account for the phenotypes produced by the *Oed-Sml,* the *Gnas* exon 2 knockout, and MatDp.dist2 and PatDp.dist2 phenotypes (maternal and paternal duplication of distal chromosome 2, respectively) based on *Nesp/Nespas* being the loci mutated in *Oed-Sml*. Developed from Figure 4 of Wroe *et al.* (24). Blocks show, from left to right, exon-specific *Nesp*, *Gnasxl* and *Gnas.* The maternally expressed *Nesp* transcript is alternatively spliced with exon 2 of *Gnas*, as is the paternally expressed transcript of *Gnasxl.* The directions of transcription are shown with arrows.

expression of both to give two phenotypes that are parent of origin dependent. The absence of a maternal *Nesp* transcript in *Oed* mice, in PatDp and in maternally inherited knockout mice, could then account for the oedema and the absence of the paternal *Nespas* transcript could cause the *Sml* growth retardation phenotype (Fig. 9). The *Sml* phenotype would also be expected in MatDp mice as these animals can have no *Nespas* transcript, but these hypokinetic non-suckling mice die before this effect can be detected. In contrast, no such growth retardation would be expected with the paternally inherited knockout, as the *Nespas* transcript would be expressed normally. The hypothesis could be equally well applied to any gene within the region which has sense and anti-sense transcripts and which is disrupted by *Gnas* exon 2 knockouts.

If *Gnasxl* is taken as the candidate locus for the behavioural effects the transcript, which splices onto exon 2 of *Gnas*, would be disrupted in paternally inherited knockout mice. The absence of this transcript could then account for the hypokinetic second phenotype of the knockout and MatDp animals. On this basis, the hyperkinetic behaviour of the PatDp class, which is absent in *Oed* mice, could be explained by the presence of two expressed copies of *Gnasxl* (Fig. 9). The hypothesis ignores the ambiguous growth retardation suggested for the maternally inherited knockout and also the possibility that the late onset 'ataxia' described for the paternally inherited knockout (6) might correspond to the neonatal hyperkinetic behaviour of PatDp animals (16). Further studies with knockouts of the *Nesp*, *Gnasxl* and other genes in the region should elucidate the genetic complexities of this imprinting domain in the mouse. Further such studies in the mouse may provide a better understanding of imprinting in the homologous human chromosome domain.

Appraisal of the imprinting effects attributable to genes within the imprinting regions has suggested from the first that imprinted genes have important roles in development and commonly affect growth (26). Whether or not *Mnt* affects *Igf2,* the prenatal growth effects of the mutation accords with this observation. In the case of *Oed-Sml*, two such growth effects are evident. The first, seen in *Oed* mice, is a growth retardation of the heart. It might be expected that this organ will be one site of the responsible mutant gene expression. The second, seen in *Sml* mice, is the postnatal growth retardation, which no doubt is growth hormone-dependent.

It seems remarkable that all three phenotypes described, *Mnt*, *Oed* and *Sml*, show similarities with imprinting effects associated with other chromosomal regions. Thus, the prenatal growth reduction of the paternally inherited *Mnt* (and the *Igf2* knockout) closely resembles the prenatal growth effect caused by an absence of a paternal copy of proximal chromosome 11 (27). An oedema resembling that caused by the maternally

inherited *Oed-Sml* mutation, with congenital heart abnormality (although with enlargement rather than reduction), also occurs with absence of a maternal copy of *Igf2r* (20,27). Postnatal growth retardation effects that are similar to that illustrated with the paternally inherited Oed-*Sml* mutation occur with both a paternally transmitted knockout of the imprinted *Rasgrf1* locus on chromosome 9 (5) and with an absence of a maternal copy of central chromosome 7 (with mouse AS) (28). This duplication of phenotypes is also seen with some other chromosome regions. Thus, suckling problems are seen with absences of a paternal copies of both distal chromosome 2 (16) and central chromosome 7 (mouse PWS) (28) and neurological abnormalities are seen with absence of maternal copies of both distal chromosome 2 (16) and central chromosome 7 (mouse AS) (28). More detailed characterization of these imprinting effects are needed to establish how well these similarities really correspond. Most of them fit with the 'conflict hypothesis' of Moore and Haig (29), but elucidation of this seeming phenotype duplication may help to resolve further the intriguing puzzle of the role of imprinting in mammalian development.

MATERIALS AND METHODS

Breeding and experimental procedures

The specific locus tests from which the mutants derived utilized F_1 hybrid males deriving from the cross of the C3H/HeH and 101/H inbred strains. The tester females used were PT random breeds which are homozygous for seven different recessive mutations: non-agouti (*a*), brown (*b*), pink-eyed dilution (*p*), chinchilla (c^{ch}), short-ear (*se*), dilute (*d*) and piebald spotting (*s*). All genetic analyses of the two mutants, other than mapping, was carried out in crosses using the F_1 hybrid, or with animals of an equivalent but mixed genetic background.

Growth of the mutants relative to their + sibs was made on the basis of weight ratios to allow for variation between litters and was assessed by Student's *t*-tests. Wet and dry weights of neonates were similarly investigated. Dry weights were measured after freeze-drying chopped-up carcasses for 30 h. The brachyury mutation, T^{37H}, used in crosses with *Mnt*, derived from the same series of specific locus mutation radiation experiments as *Mnt* (17). Like the well-studied T^{hp} allele, T^{37H} represents a chromsome 17 deletion (E.P. Evans, personal communication) and shows a *Tme* lethality when transmitted through females. Affected young display an oedema and overgrowth with occasional omphalocoele or hare-lip (B.M. Cattanach, unpublished data) and most die prior to birth. Mice with PatDp.dist2 used for comparison with *Oed-Sml* mutants were generated using the T(2;11)30H translocation.

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Mapping procedures

Mnt and *Oed-Sml* mapping was carried by crossing mutant males with *M.m.castaneus* females, backcrossing to C3H/HeH and typing the backcross progeny for *M.musculus*–*M.m.castaneus* polymorphisms at *Mit* marker loci in the candidate regions. DNA was extracted from tail tissue using standard procedures. For PCR analysis, 50 ng of DNA was added to a final volume of 25 µl of PCR master mix (Advanced Biotechnologies, Epsom, UK), including 2.5 mM $MgCl_2$ and 0.4 µM of relevant primers. PCR was performed at a 55°C annealing temperature and with 35 cycles. Products were run out on 3% agarose gels with TBE buffer.

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