Mouse embryos with paternal duplication of an imprinted chromosome 7 region die at midgestation and lack placental spongiotrophoblast

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SUMMARY

Imprinted genomic regions have been defined by the production of mice with uniparental inheritance or duplication of homologous chromosome regions. With most of the genome investigated, paternal duplication of only distal chromosomes 7 and 12 results in the lack of offspring, and prenatal lethality is presumed. Aberrant expression of imprinted genes in these two autosomal regions is therefore strongly implicated in the perimplantation lethality of androgenetic embryos. We report that mouse embryos with paternal duplication of distal chromosome 7 (PatDup.d7) die at midgestation and lack placental spongiotrophoblast. Thus, the much earlier death of androgenotes must involve paternal duplication of other autosomal regions, acting independently of or synergistically with PatDup.d7. The phenotype observed is similar, if not identical to, that resulting from mutation of the imprinted distal chromosome 7 gene, Mash2, which in normal midgestation embryos exhibits spongiotrophoblast-specific maternally active/paternally inactive (m+/p−) allelic expression. Thus, the simplest explanation for the PatDup.d7 phenotype is p+/p− expression of this gene. We also confirm that PatDup.d7 embryos lack H19 RNA and possess excess Igf2 RNA as might be expected from the parent-specific activities of these genes in normal embryos.

Key words: uniparental duplication, imprinting, translocation, mouse, genetics, chromosome 7

INTRODUCTION

Imprinting of the mammalian genome has been demonstrated experimentally in the developmental failure of (1) mouse embryos with two maternal or two paternal genomes, termed parthenogenones (PG) and androgenotes (AG), respectively (Barton et al., 1984; McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani et al., 1984) and (2) embryos with maternal duplications and balancing paternal deficiencies of autosomal regions, or the reciprocal, termed maternal, paternal or uniparental duplication (UPD) (Lyon and Glenister, 1977; Cattanach and Kirk, 1985; Cattanach and Beechey, 1990). For the majority of the genome, UPD has no discernable consequences for development. Therefore, only a minor proportion of the genome appears to undergo developmentally significant genomic imprinting (Beechey and Cattanach, 1995a). The basis for the functional differences in homologous maternal and paternal chromosome regions appears to be parent of origin dependent gene activity. A growing number of genes which exhibit a monoallelic (+/−) mode of expression are being identified (Beechey and Cattanach, 1995a), and at least some appear to be biallelically expressed (+/+ or inactive (−/−) in mice that have inherited both alleles from one parent (Cattanach et al., 1992; Sasaki et al., 1992; Ferguson-Smith et al., 1993). Gene targeting experiments have demonstrated that the lack of functional product of some of these genes is associated with a deleterious developmental effect: Igf2, small size (DeChiara et al., 1991); H19, large size, through activation of the normally silent paternal Igf2 allele (Leighton et al. 1995); Igf2r, perinatal lethality (Lau et al., 1994; Wang et al., 1994); and murine achaete-scute homologue 2, Mash2, midgestation lethality and lack of spongiotrophoblast development (Guillemot et al., 1994, 1995).

One of the most important regions with respect to genomic imprinting is distal chromosome 7. First, offspring with maternal or paternal duplication of this region are not observed in intercrosses of reciprocal translocations involving chromosome 7. With respect to the T(7;15)9H (T9H) and T(7;11)65H (T65H) reciprocal translocations, those with maternal duplication of distal 7 (MatDup.d7) are small and die at 15.5 to 17.5 days post coitum (d.p.c.), while their paternal counterparts, PatDup.d7 embryos, are presumed to die prior to 11.5 d.p.c. (Searle and Beechey, 1990; Beechey and Cattanach, 1995b). Only one other paternal duplication, that of distal chromosome 12, is presumed to result in early embryonic lethality (Cattanach and Rasberry, 1993). These observations strongly implicate PatDup.d7 in the perimplantation death of AG embryos (Searle and Beechey, 1990) and also in the abnormal developmental effects induced by AG cells in chimeras (Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991; Mann, 1992). Second, five imprinted genes have been ascribed a distal 7 location with respect to the T9H breakpoint; H19,
Ig2, Ins2, Mash2 and small nuclear ribonucleoprotein N, Snrpn (Searle and Beechey, 1974; Leff et al. 1992; Zemel et al. 1992; Giddings et al. 1994; Guillemot et al. 1995). Only the former four might be contained in the most distal 7 region defined by the T65H breakpoint and therefore are implicated in the PatDup.d7 and MatDup.d7 lethalities (Beechey and Cattanach, 1993b). H19 and Mash2 exhibit maternally active/paternally inactive (m+/p−) allelic expression (Bartolomei et al., 1991; Guillemot et al., 1994; 1995) and Ins2, Ig2 and Snrpn exhibit m+/p+ expression (DeChiara et al., 1991; Sasaki et al., 1992; Cattanach et al., 1992; Leff et al., 1992; Giddings et al. 1994; Barr et al. 1995; Szabó and Mann, 1995). Thus, in PatDup.d7 embryos, it would be predicted that H19 and Mash2 would display p−/p−, and Ins2, Ig2 and Snrpn would display p+/p+ expression. Here, we define the phenotype of PatDup.d7 conceptuses and provide an assessment of their expression of distal 7 imprinted genes. The possible relationship between this phenotype and the expression observed is discussed.

MATERIALS AND METHODS

Mouse strains
To enable genotypes to be identified, a PstI polymorphism located on distal 7 (Silver and Buckler, 1986) was bred into the T9H translocation. The int2a probe, which recognizes the PstI RFLV, was sequenced and found to contain the polymorphic PstI site, allowing the development of a PCR-based assay (Fig. 1). T9H/+ males (Jackson Laboratory, Stock No. JR1752) with T9H/T9H, P/P inbred females (Stevens, 1970; Jackson Laboratory, Stock No. JR1752). In producing T9H/+ males (Jackson Laboratory, Stock No. JR0090) with T9H/T9H, P/P inbred males (Jackson Laboratory, Stock No. JR1752). In producing T9H/+ males (absence of the PstI site) for the intercross, the T9H chromosome was recombined with the distal 7 region of the C57BL/6J strain, in which the PstI site is absent. T9H/+ males were identified by the presence of a quadrivalent in meiotic chromosome spreads made from one surgically removed testis (Chandley, 1987).

Genotype determination
Yolk sacs were digested in 0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.5% (v/v) Tween 80, 0.5% (v/v) Nonidet P40 and 500 mg/ml proteinase K at 55°C overnight. The digest was diluted 20 fold in water, and 2 μl of this sample was used in a total reaction volume of 20 μl. Cycling conditions were 95°C, 30 seconds, 58°C, 30 seconds, 73°C, 30 seconds for 30 cycles using Taq polymerase and PCR Reaction Buffer (Boehringer Mannheim). Oligonucleotide primer sequences, upper and lower respectively, were 5′-CAAGGAGTGGTGGATGCCAAC-3′ and 5′-CAATATAGTCAGGCTCGCCTCA-3′. The amplified fragment was digested with 5 U of PstI added directly to each 20 μl reaction, then the digested product was subjected to electrophoresis as described in the legend to Fig. 1.

Northern blots
Embryos and tissues were homogenized by repeated passage through a 20-gauge needle in RNazol B (Tel-Test Inc., Friendswood, Texas, USA). The homogenates were stored at −70°C until genotypes were...
determined. Total RNA was isolated from RNAlater B according to the manufacturer’s protocol, run on denaturing formaldehyde gels and transferred with phosphate buffer to GeneScreen nylon membrane (NEN Research Products) according to the manufacturer’s protocol. Probes recognizing transcripts of Igf2, H19, Snrpn and glyceraldehyde 3-phosphate dehydrogenase, Gapd, and hybridization conditions were as described by Szabó and Mann (1994).

**In situ hybridization**
This was performed essentially as described (Jostarndt and Puntchart, 1994), using the DIG-RNA labeling, and the DIG nucleic acid detection kits (Boehringer Mannheim). The digoxigenin-labeled riboprobes were prepared from plasmid pBSMash2 kindly provided by F. Guillemot. Slides were lightly counterstained with eosin.

**RESULTS**

**PatDup.d7 embryos are lethal at midgestation**
The T9H/+ intercross used to produce PatDup.d7 and MatDup.d7 embryos is depicted in Fig. 1. In this intercross, at 9.5 d.p.c., 286 implantation sites were observed in 50 natural matings (mean of 5.7 implantations per female, scoring only those females with at least one implantation site). 91/286 (32%) of implantations observed were associated with the development of embryos, while the remainder of implantation sites contained no discernable embryonic material. This low frequency of postimplantation development indicates that most, if not all, unbalanced zygotes resulting from the intercross were lethal by the perimplantation stage. At 9.5 d.p.c. and 10.5 d.p.c., PatDup.d7 embryos were observed at a similar frequency as MatDup.d7 embryos (Table 1), demonstrating that both types of embryos were viable up to midgestation. At 9.5 d.p.c., PatDup.d7 embryos were overtly normal and indistinguishable from siblings but, by 10.5 d.p.c., were significantly retarded in size and developmental stage (Fig. 2). Thus, PatDup.d7 embryos underwent an abrupt cessation in growth and development at 9.5 d.p.c., or upon reaching approximately the 25-somite stage. This stage of onset of cessation in development was not invariant, as one PatDup.d7 embryo was observed at 10.5 d.p.c. with an overtly normal morphology expected for this age. Also, abnormal PatDup.d7 embryos at 10.5 d.p.c. often had beating hearts; therefore death did not necessarily coincide with the cessation in development. No PatDup.d7 embryos were recovered at 13.5 d.p.c. (Table 1) and, as all resorption sites contained no discernable embryonic material, they must have died and been resorbed by this stage.

**PatDup.d7 embryos lack placental spongiotrophoblast**
The distal 7 imprinted gene Mash2 exhibits m+/? expression and is required for spongiotrophoblast development; embryos homozygous for a knockout of Mash2 lack a placental spongiotrophoblast layer and die at 10.5 d.p.c. Further, this phenotype also occurs in embryos heterozygous for maternal, but not paternal inheritance of the mutation (Guillemot et al., 1994, 1995). As PatDup.d7 conceptuses would be expected to exhibit p/?? expression of Mash2, we examined the morphology of the PatDup.d7 placenta and the expression of Mash2 therein by in situ hybridization. The morphology of the PatDup.d7 placenta was similar, if not identical to, that reported for Mash2 mutants. A placental spongiotrophoblast layer was missing and the layer of mural trophoblast giant cells was thicker than normal (Fig. 3B,H). This abnormal morphology was evident in all of five 9.5 d.p.c. PatDup.d7 embryos examined. In addition, no Mash2 RNA signal was obtained by in situ hybridization of these PatDup.d7 sections (Fig. 3E). In one 9.5 d.p.c. MatDup.d7 embryo obtained in this series, no obvious difference to wild-type was observed in the morphology of any of the placental layers, or in the pattern of Mash2 expression (compare Fig. 3A,D,G with Fig. 3C,F,I respectively). Thus, at least at 9.5 d.p.c., the presumed m+/m+ expression of Mash2 in MatDup.d7 embryos has no overt abnormal effect.

**PatDup.d7 embryos possess excessive levels of Igf2 RNA and lack H19 RNA**
Northern blot analyses are shown in Fig. 4. In three PatDup.d7 embryos, the level of Igf2 RNA relative to Gapd RNA was approximately three times that of the mean WT value (values given under autoradiograms). In another five PatDup.d7 embryos, the level of Igf2 RNA relative to Gapd RNA was approximately four times that of the mean WT value (values given in legend to Fig. 4). In subjecting these eight relative values to the Exact Permutation statistical test, the relative PatDup.d7 RNA level was significantly greater than 1.0, or the WT level (P=0.008). In MatDup.d7 embryos at an equivalent stage of development, Igf2 RNA was undetectable, which is consistent with previous findings (DeChiara et al., 1991; Sasaki et al., 1992). For H19 RNA, the opposite was observed. None was detected in PatDup.d7 embryos and, in MatDup.d7 embryos, the level relative to Gapd RNA was twice that of WT as previously

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described (Ferguson-Smith et al., 1993). These results were close to expectation based on the presence of two active or inactive copies of these imprinted genes. Based on these results, it is highly unlikely that any of these embryos could have been of the unbalanced distal 7 monosomy class, which would mistakenly be classified as PatDup.d7 or MatDup.d7 on the basis of the PCR assay (legend to Fig. 1). Thus, it would appear that all unbalanced zygotes were lethal by the periimplantation stage.

**PatDup.d7 and MatDup.d7 embryos possess normal levels of Snrpn RNA**

Using interspecific hybrid backcross analysis, the *Snrpn* gene has been assigned as distal to the pink-eyed dilution, *p*, locus (Leff et al., 1992). Based on limited recombination data, the *p* locus has been assigned as distal to the T9H breakpoint (Searle and Beechey, 1974; Beechey and Cattanach, 1994). Given such a location of *Snrpn* and its ubiquitous paternal-specific pattern of expression during development (Barr et al. 1995; Szabó and Mann, 1995), we expected to observe an absence of *Snrpn* RNA in MatDup.d7 embryos and an excess of *Snrpn* RNA in PatDup.d7 embryos. However, *Snrpn* RNA levels were identical in 9.5 d.p.c. PatDup.d7, MatDup.d7 and WT embryos (Fig. 3A). Also, *Snrpn* RNA was present at normal levels in the brain of MatDup.d7 embryos at 13.5 d.p.c. (Fig. 3B). This pattern of expression with respect to the three genotypes was as expected for a gene located proximal to the translocation breakpoint. Consistent with this possibility is that no *Snrpn* RNA was detected in 4/32 (12%) of embryos classified as WT on the basis of the PCR-based assay. One of these non-expressing embryos is shown in lane 5, Fig. 3A (MatDup.p7 embryos arise at an expected frequency of 0.05 in the T9H/+ intercross; see legend to Fig. 1). This lack of *Snrpn* expression in embryos that must have possessed both parental copies of distal 7 could be explained on the basis that they possessed maternal duplication of proximal chromosome 7 (MatDup.p7) and that *Snrpn* is proximal to the T9H breakpoint (Beechey and Cattanach, 1995a). Confirmation of the location of the *Snrpn* region (syntenic to the human Prader-Willi syndrome region) relative

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**Fig. 3.** Histology of 9.5 d.p.c. placentae. (A,D,G) MatDup.d7, (B,E,H) PatDup.d7 and (C,F,I) WT placentae. (A-C) Hematoxylin- and eosin-stained sections (scale bar is 500 μm), with a higher magnification shown in (G-I) (scale bar is 100 μm). (D-F) Near adjacent sections showing hybridization of a *Mash2* probe; note absence of hybridization to the PatDup.d7 section. Color 35 mm slides taken of sections were scanned and digitized, and the areas staining blue were artificially replaced by pink using Adobe Photoshop®. Tissue layers are maternal decidua (ma), trophoblast giant cells (gi), spongiotrophoblast (sp), labyrinthine trophoblast (la) and allantoic region (al).
stage occurs rarely (Barton et al., 1984; Surani et al., 1986). It is therefore clear that PatDup.d7 cannot be the sole cause of this early inviability of AG embryos. With most of the genome now analyzed for the effects of paternal duplication, only the untested proximal portion of chromosome 18 and the distal region of chromosome 12 (Beechey and Cattnach, 1995a) are potential candidates for a single autosomal region determining perimplantation AG lethality. Otherwise, it is probable that synergistic effects of two or more autosomal regions is involved. The imprinting of the Igf2 and Igf2r genes (Barlow et al., 1991; DeChiara et al., 1991) provide an example by which such synergism could occur. Based on observations which suggest that excess IGFIIR can adversely affect development and that IGFIIR can sequester IGFIIR and reduce its concentration (Filson et al., 1993; Efstratiadis, 1994), it follows that androgenones may possess a large excess of IGFIIR resulting from the combined effect of p+/p− expression of Igf2 and p−/p− expression of Igf2r.

If the lack of placental spongiotrophoblast is the cause of the lethality of Mash2 mutants at 9.5 d.p.c. (Guillemot et al., 1994, 1995), then this defect would also explain the lethality of PatDup.d7 embryos at this same stage. As in Mash2 mutants, the simplest explanation for the lack of spongiotrophoblast development in PatDup.d7 embryos is that a lack of Mash2 expression is solely responsible. However, it is yet to be determined that PatDup.d7 embryos do not significantly express Mash2 at the stages when spongiotrophoblast would normally form. Other explanations are possible, but are not as straightforward. For example, aside from Mash2, there may exist other imprinted genes on distal 7 for which p+/p− or p+/p+ expression, either alone or in combination, could result in the phenotype. The exact role of Mash2 in the PatDup.d7 phenotype could be investigated by the introduction of a functional Mash2 transgene into PatDup.d7 embryos, and by providing the PatDup.d7 embryo-proper with functionally normal trophoblast in tetraploid diploid chimeras (Nagy et al., 1990; Guillemot et al., 1995). Circumvention of the trophoblast defect in PatDup.d7 embryos would reveal the potential for an adverse developmental effect of the aberrant expression of other imprinted genes located on distal 7. If PatDup.d7 embryos were to survive to at least 12.5 d.p.c., one possible adverse effect would be increased size: PatDup.d7×normal fetal chimeras are oversized, possibly due to excess Igf2 activity (Ferguson-Smith et al., 1991). Excess Igf2 activity appears to be the cause of increased size in H19 mutants (Leighton et al., 1995).

The possible relationship between the PatDup.d7 phenotype and lack of Mash2 activity is now the third case documented for which lack of expression of a particular imprinted gene may be responsible for the phenotype of mice with a particular UPD. With respect to the other two examples, gene knockout experiments have shown that (1) lack of IGFIIR activity (Lau et al., 1994; Wang et al., 1994) might explain the much lower than expected frequency of PatDup.p17 progeny (Lyon et al., 1972; Lyon and Glenister, 1977) and the perinatal lethality of mice which maternally inherit chromosome 17 deletions that eliminate Igf2r (Johnson, 1974, 1975; Winking and Silver, 1984; Barlow et al., 1991) and (2) lack of IGFIIR activity (DeChiara et al., 1991) might explain the small size of MatDup.d7 embryos, but not their lethality. Thus, by mimicking the −/− mode of expression of imprinted genes, knockout experiments are providing a means to determine which imprinted genes are likely to be responsible for devel-

**DISCUSSION**

The development of PatDup.d7 embryos was found to be considerably more advanced than the development seen in AG embryos. The former consistently underwent overtly normal development to 9.5 d.p.c., or approximately the 25-somite stage, whereas the latter are almost always lethal at the perimplantation stage. Development of AG embryos as far the 8-somite stage occurs rarely (Barton et al., 1984; Surani et al., 1986). It is therefore clear that PatDup.d7 cannot be the sole cause of this early inviability of AG embryos. With most of the genome now analyzed for the effects of paternal duplication, only the untested proximal portion of chromosome 18 and the distal region of chromosome 12 (Beechey and Cattnach, 1995a) are potential candidates for a single autosomal region determining perimplantation AG lethality. Otherwise, it is probable that synergistic effects of two or more autosomal regions is involved. The imprinting of the Igf2 and Igf2r genes (Barlow et al., 1991; DeChiara et al., 1991) provide an example by which such synergism could occur. Based on observations which suggest that excess IGFIIR can adversely affect development and that IGFIIR can sequester IGFIIR and reduce its concentration (Filson et al., 1993; Efstratiadis, 1994), it follows that androgenones may possess a large excess of IGFIIR resulting from the combined effect of p+/p− expression of Igf2 and p−/p− expression of Igf2r.

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**Fig. 4.** Expression of imprinted genes in PatDup.d7 and MatDup.d7 embryos. (A) Hybridizations to northern blot of total RNA extracted from 9.5 d.p.c. embryos. Each lane represents a different embryo, with approximately 6 μg of RNA loaded onto each lane. Values under the H19 and Igf2 bands represent the RNA level in that lane relative to the mean WT RNA level. Bands on autoradiograms were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale CA) and values were corrected for RNA loading based on the value obtained for Gapd RNA in the same lane. The corrected values for PatDup.d7 and MatDup.d7 embryos were then divided by the corrected mean value obtained for WT embryos. In another set of embryos (autoradiogram not shown), the corrected levels of Igf2 RNA in five PatDup.d7 embryos relative to the mean corrected level in five WT embryos were 4.3, 4.7, 4.4, 4.2 and 4.7. Again, none of these five PatDup.d7 embryos possessed detectable H19 RNA. This autoradiogram was quantified using densitometry. (B) Hybridizations to northern blot of total RNA extracted from the whole brain of 13.5 d.p.c. PatDup.d7 and WT embryos. Each lane represents a different individual. Approximately 14 μg of total RNA was loaded onto each lane.

To the T9H and Is(In7;X)1Ct breakpoints using a direct technique is required. Due to the close proximity of the Snrpn region to the breakpoints, fluorescence in situ hybridization would appear to be the method of choice.
opmental anomalies associated with UPDs. They could also help to resolve the potential consequences of the +/+ mode by introducing mutated genes into UPDs when it is suspected that +/+ expression may be deleterious. These latter experiments could help to establish if the +/− or ‘imprinted’ mode of expression per se is important in development.

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