

Does monozygotic twinning occur in mice?

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Summary

Published reports suggest that the incidence of monozygotic twinning in women is increased after hormonally induced ovulation. Since some statistical evidence exists to indicate that monozygotic twinning may also occur in mice, we attempted to devise a mouse system in which the incidence of such twinning could be compared after spontaneous versus hormonally induced ovulation, in order to analyse the developmental basis of such an effect. We used phenotypic identity in litters segregating for ten genetic loci (not all independent) to indicate possible twin pairs. DNA fingerprinting using three human minisatellite probes was then performed blind on these pairs and on sibling controls. From a total of over 2000 mice born, 40 apparently identical pairs were identified, on which DNA finger-printing was successfully carried out on 35 pairs. All proved to be derived from different zygotes. We conclude that monozygotic twin pairs are either extremely rare in the stock of mice that we studied, or have such reduced viability that their chance of surviving to weaning is low.

INTRODUCTION

In women, the incidence of monozygotic (uniovular, 'identical') twinning at birth is estimated to be about 3.5 twin pairs per thousand deliveries, and is remarkably constant in all parts of the world and in all ethnic groups (Bulmer, 1970). This is in marked contrast to the incidence of dizygotic twinning, which varies widely.

Diagnosis of the zygosity of twin pairs has traditionally been done on the basis of sex, physical appearance, blood group and serum protein determinations, and placentation. Twin pairs with separate placentae (dichorionic) may be either dizygotic or monozygotic, but all monozygotic (whether di- or monoamniotic) pairs may be regarded as monozygotic. In Europe, about 80% of twin pairs have dichorionic placentation, and about 1 in 7 of those will be monozygotic.

Evidence is accumulating that the incidence of monozygotic twinning in women may be higher when ovulation is hormonally induced. At the time when no

more than 900 births had occurred worldwide following induced ovulation, *in vitro* fertilisation (IVF) and embryo transfer, nine sets of IVF monozygotic twins had been reported, significantly more than expectation (Edwards, Mettler and Walters, 1986).

A survey of multiple births in Belgium, after hormonally induced ovulation not involving IVF, found that the frequency of monozygotic twinning (1.2%) was again significantly higher ($P < 0.001$) than the expected frequency among spontaneous twins and triplets (Derom *et al.*, 1987). After induced ovulation 13% of twin sets were monozygotic, and 22% of the triplet sets contained monozygotic twins. The frequency of monozygotic twinning was significantly higher in the triplets than in the twins (2.6% v. 1.0%; $P < 0.001$), suggesting a relationship between the extent of stimulation of ovulation and the probability of embryo splitting.

In theory, monozygotic twinning could arise in various different ways, e.g. by embryo splitting during cleavage or at the blastocyst stage, leading to dichorionic placentation, or by formation of two inner cell masses or two primitive streaks, leading to monozygotic twinning. Derom *et al.* (1987) suggest that hardening of the zona pellucida in oocytes induced to ovulate by hormone treatment may lead to herniation and splitting at the blastocyst stage. More of the monozygotic twins were dichorionic after

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induced than after spontaneous ovulation (54% v. 37%), but this difference was not significant.

Because experimental investigations of the mechanism of monozygotic twinning would be hard to carry out in the human, we decided to look at monozygotic twinning in mice, after both spontaneous and induced ovulation.

There is equivocal evidence for monozygotic twinning in mice based on statistical probability arguments, i.e. what is the likelihood of two phenotypically identical individuals occurring in the same litter of a mating segregating for several loci? Castle *et al.* (1936) claimed to have detected a monozygotic twin pair: two mice of a very rare crossover class, identical also in two colour factors and sex, in a single litter. Green (1934) claimed a large excess of genetic identities in litters from a species cross. Stevens (1937), however, questioned the significance of both these observations, and found no evidence of monozygotic twinning in data from a seven-fold back-cross experiment of Fisher and Mather (1936). On the other hand Wallace and Williams (1965), using nine independent chromosome markers and sex, estimated that the frequency of monozygotic births was about 1%.

In the present study our diagnosis of zygosity is based on the more secure foundation of DNA fingerprinting (Hill and Jeffreys, 1985; Motomura *et al.*, 1987). We have used phenotypic identity in litters segregating for multiple genetic loci, not to indicate zygosity but only as a means to reduce the number of mice on which DNA finger-printing had to be performed.

The most widely used human minisatellite multi-locus probes 33·6 and 33·15 (Jeffreys *et al.*, 1985a) have been shown to cross-hybridise also to multiple variable loci in the mouse genome. The complexity of banding patterns shows similar characteristics in terms of the number of loci revealed and their autosomal location. However, while the DNA profiles show substantial differences between inbred strains, the patterns are remarkably stable – though not absolutely identical – among members of a particular inbred strain (Jeffreys *et al.*, 1987). This allows the assignment of a distinct DNA ‘signature’ for several strains analysed, so that genetic contamination between strains can be detected (Signer, 1989).

MATERIALS AND METHODS

Mice

Colony-bred females of the multiple recessive stock described in McLaren and Bowman (1969) were mated to males of the C3H/BiMcl inbred strain. Female progeny, all multiply heterozygous, were then back-crossed to males of the same multiple recessive stock, which was homozygous for agouti black tan belly (*a*^t), chinchilla (*c*^{ch}), brown (*b*), pink-eye (*p*), dilute (*d*), short-ear (*se*), waved-2 (*wa*-2), vestigial tail (*vt*), and the *a* isozyme of glucose phosphate isomerase

(*Gpi*-1^a). Eye colour (*pp* mice have pink eyes), hair type (*wa*-2 *wa*-2 have curly whiskers) and tail length (short in *vt vt*) could be scored at birth; ear size (*se se*) was scored at about 15 days of age; coat colour was identified and genotype for the relevant loci was deduced at weaning.

The mice were then killed. If there were any apparently identical mice in the litter, their spleens were kept for glucose phosphate isomerase electrophoresis (carried out as described in Buehr and McLaren, 1985) and samples of their tails were processed immediately for DNA extraction, or stored at –70 °C. Mice that died before weaning were not included in the study. The colony was maintained on a 12 hour light/12 hour dark cycle, at a temperature of around 21 °C.

DNA fingerprinting

For the mouse strains analysed, three human minisatellite probes were applied: 33·15 (DNA template and RNA transcript), MS51 (Armour *et al.*, 1989) and *p*lg3 (Wong *et al.*, 1986). From probe 33·15 both DNA template and RNA transcripts were labelled and used for hybridisation in order to test whether, in mice as has been reported for other species (Carter *et al.*, 1989; Signer and Jeffreys, 1992), more polymorphic DNA fragments are revealed by RNA transcripts than by DNA template. Although probes MS51 and *p*lg3 are locus-specific in humans, they produce complex banding patterns in mice and some other animal species under low stringency conditions (Signer and Jeffreys, 1993). They were used as additional probes instead of probe 33·6 which had been described as hybridising to loci of extreme length instability in both germline and somatic cells (Kelly *et al.*, 1989). Since somatic instability for two of such loci (Ms6-hm and Hm-2, Kelly *et al.*, 1989) was found to occur in the first few cell divisions of the zygote, their co-detection in the DNA profiles could hamper accurate zygosity diagnosis, in that monozygotic twins would not be detected as such if somatic mutation events occurred after splitting of the cell mass. Since it was not known whether probes 33·15, MS51 and *p*lg3 cross-hybridise to somatically unstable loci, Ms6-hm and Hm-2 were also applied to some of the blots.

DNA was extracted from pieces of tail using a protocol modified from Hogan *et al.* (1986), and posted to Leicester in solution (approximately 40 μ g DNA in Tris-EDTA buffer). Occasionally samples of tail were sent to Leicester in 70% ethanol and DNA was extracted there.

DNA was digested by *Alu*I, *Hae*III or *Hinf*I, which produced the most informative profiles in an initial experiment. After digestion, loading buffer was added and the DNA concentration was determined by fluorimetry to allow for equal amounts (5 μ g) to be loaded for each sample. The DNA digests were then run in a 0·8% agarose gel (30 cm long) in 1x Tris-

Borate-EDTA buffer at 60 V until the bromphenol blue dye had reached the end. A 1 kb ladder marker (Gibco BRL) was loaded on the side tracks to indicate molecular weight sizes. After blotting of the DNA fragments onto a nylon membrane (Hybond NFP, Amersham), they were fixed by UV cross linking. The probes were labelled using [α^{32} P] dCTP for DNA templates (Feinberg & Vogelstein, 1983) or [α^{32} P] CTP for RNA transcripts (Carter *et al.*, 1989) and subsequently hybridised to the membranes in 0.5M Na phosphate (pH 7.2), 7% SDS, 1 mM EDTA at 65 °C overnight in the absence of any competitor DNA. Post-hybridisation washes were done twice for 10 min in 2x SSC, 0.1% SDS at 65 °C. After a final rinse in 2x SSC at room temperature X-ray films were exposed to the blots at -70 °C in the presence of intensifier screens for 1 to 4 days. The DNA banding patterns were pairwise compared manually and differences in presence or absence of bands and intensity were recorded. The mouse samples were received and analysed in batches (two per blot) each typically comprised of twelve individuals from three different litters. One batch normally consisted of three sets of phenotypically identical siblings (possibly monozygotic twins) and two of their litter mates as controls. Further controls involved unrelated individuals from the progenitor strains and also from five other inbred strains (C57BL/6J, C57BL/10J, A/J, BALB/c and DBA/2J).

Induced ovulation

When ovulation is hormonally induced in mice, the number of eggs shed increases (up to a point) with the dose of pregnant mare's serum, but the number of liveborn young is inversely proportional to the number of implants (McLaren and Michie, 1959a) and preweaning mortality is increased.

After some preliminary trials, a dose of 1 i.u. pregnant mare's serum (Folliculin, Organon) was selected as yielding the highest live birth rate. Female progeny from the multiple recessive x C3H cross, 6–10 weeks of age, received an intraperitoneal injection of 1 i.u. pregnant mare's serum at 5 pm on Day 1. On Day 3 they received an injection of 1 i.u. human chorionic gonadotrophin at 12 noon and were then paired with stud males from the multiple recessive stock, and examined for copulation plugs the following morning.

RESULTS

From spontaneous ovulation, 171 surviving litters contained 1456 young at birth, 1386 at weaning. From induced ovulation, 83 surviving litters contained 681 young at birth, 610 at weaning. Not all the 8 loci (9 if sex is included) segregated independently. *vt* and *wa-2* are both on chromosome 11, about 30 cM apart; *p* and *c* are on chromosome 7, about 14 cM apart; *d*

and *se* are so closely linked on chromosome 9 that we only encountered a single cross-over (or revertant), a *se/se d/+* female with behavioural abnormalities who died after her first litter, but whose genotype was nonetheless confirmed. *vt/vt* and *wa-2/wa-2* mice suffer prenatal mortality leading to a significant deficiency of the mutant phenotype at birth; other genes gave good 1:1 ratios. Not all genotypes could be distinguished phenotypically: in particular *a/a* and *b/b* could not be reliably distinguished from *A/a* and *B/b* on a *p/p c^{ch}/c^{ch}* background, nor could *a/a* be distinguished from *A/a* on a *b/b d/d p/p* background.

In practice, we were able to distinguish some 25 colour phenotypes. Taking into account *vt*, *wa-2* and sex, this gave some 200 different possible phenotypes, but of course some were much rarer than others. In view of the complications of linkage, embryonic mortality and possible mis-classification, and since we were only using phenotypic resemblance to reduce the number of candidate mice for DNA typing, we did not attempt to apply the sophisticated statistical methods of Wallace and Williams (1965) to calculate the likelihood of finding two similar individuals in a single litter by chance alone.

The 171 litters from spontaneous ovulation yielded 50 apparently identical pairs. One died before GPI-1 typing could be carried out; 20 pairs differed in GPI-1 type; the remaining 29 pairs were either both GPI-1A or both GPI-1AB. DNA from 27 pairs (54 mice), along with 43 sibling controls, was successfully fingerprinted: no identical pairs were found. Examples are shown in figure 1 for probe 33-15 (RNA) and figure 2 for probe MS51.

In the smaller series from induced ovulation, the 83 litters yielded 17 apparently identical pairs. Of the 16 pairs on which GPI-1 typing was carried out, 5 differed and 11 resembled one another. DNA fingerprinting was carried out on 8 pairs (16 mice) and 12 sibling controls; again no identical pairs were found. Some of the results by probe 33-15 (DNA) are shown in figure 3.

All samples were classified 'blind', i.e. their origin was not known to the DNA fingerprinter. In three instances a DNA sample from one member of a putative 'twin pair' was deliberately replicated and the replicates were classified by DNA fingerprinting as 'identical'. In contrast to the colony-bred multiple recessive progenitor strain in which extensive length variation in the DNA fragments was found among individuals, samples from different individuals of the inbred C3H progenitor strain showed only minor variations (data not shown). Such variations are to be expected, on account of minisatellite mutational events occurring spontaneously in the genome, and cannot be abolished by inbreeding.

The DNA patterns produced by probes 33-15, MS51 and *p*lg3 revealed extensive polymorphisms, especially when applying *AluI* for restriction digestion. Even though the probes overlapped in their DNA

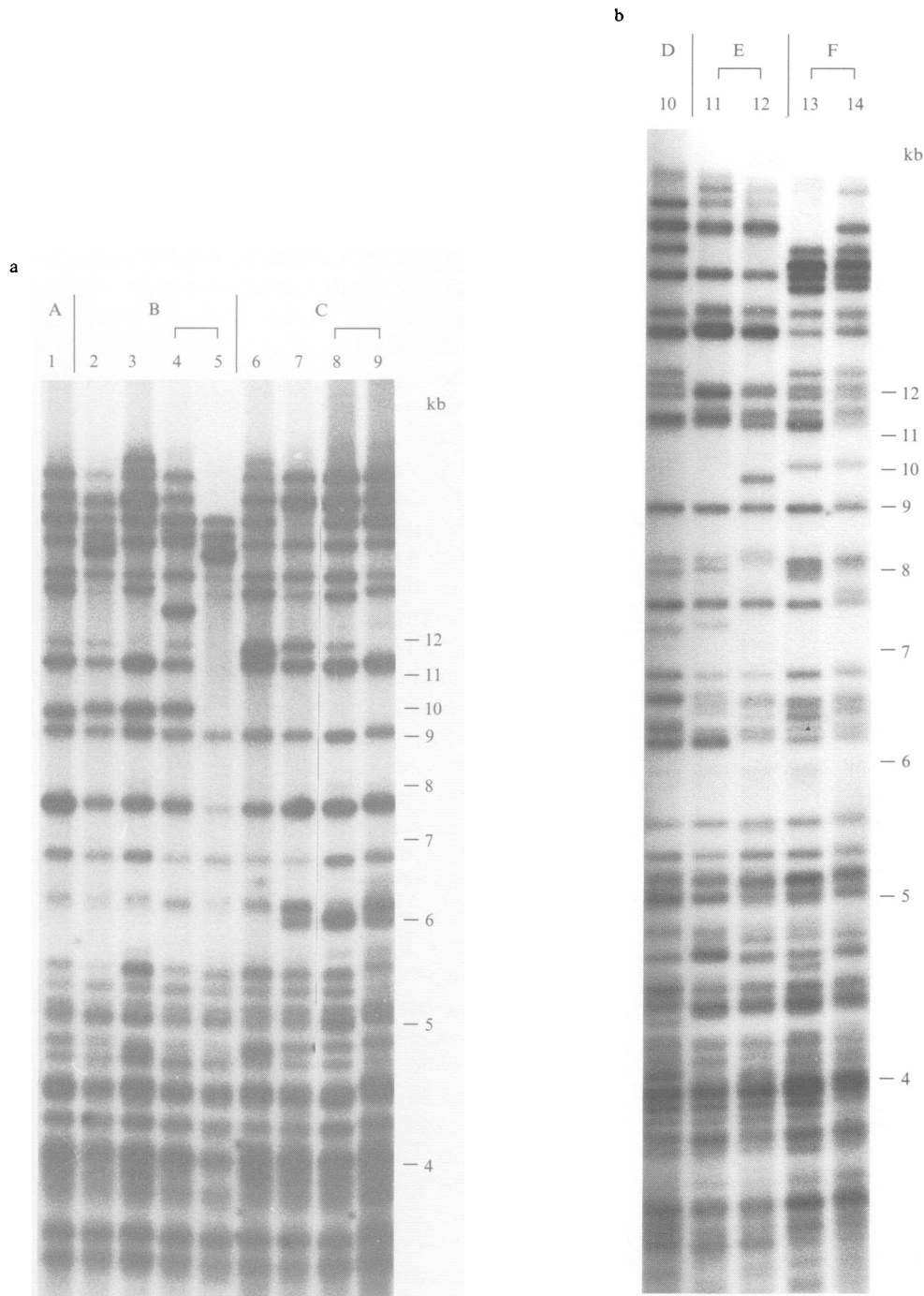


Figure 1. DNA fingerprints of mouse candidate twins and sibling controls using probe 33·15 (RNA) and restriction endonuclease *Hae*III (in *a*) and *Alu*I (in *b*). Individuals from six different litters (A to F; spontaneous ovulation) are shown. Possibly identical twins based on phenotypic markers are indicated by brackets. Like their sibling controls, all twin partners have distinct DNA profiles indicating dizygosity. Molecular weight sizes are indicated on the right.

banding patterns, by cross-hybridising to several loci they also detected unique DNA fragments. By combining the results polymorphic information could be increased. With respect to individual probes, the RNA transcripts of 33·15 revealed more variable DNA fragments than either its DNA template, MS51 or p λ g3. Nevertheless each probe gave individual-specific and reproducible DNA profiles, allowing unambiguous distinction between individuals within all phenotypically similar twin sets. A separate study on

segregation analysis in twenty-six offspring derived from a mating between two different mouse strains has shown that each probe hybridised to multiple polymorphic loci (unpublished results); but since in the present study the parents were not available for DNA analysis, we could not determine the number of independent polymorphic loci.

With respect to the DNA results at the hypermutable loci, the locus at Ms6-hm was detected by all three probes, while that at Hm-2 was also detected by

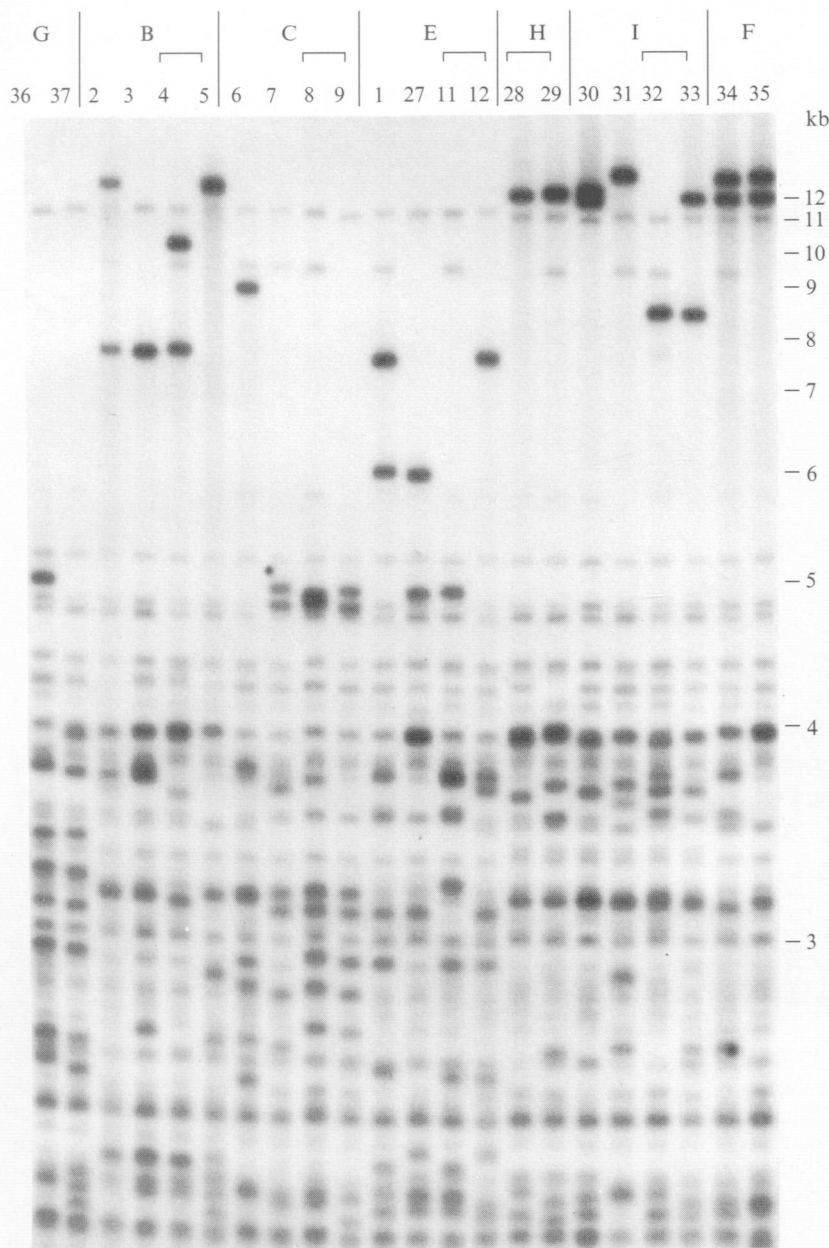


Figure 2. DNA fingerprints produced by probe MS51 on *AluI* digested DNA from seven different litters (B, C, E to I; spontaneous ovulation). Although this probe reveals fewer polymorphic DNA fragments than probe 33-15, it confirms the results on non-identity of all candidate twins (such as for sets 4 and 5, 8 and 9, 11 and 12 which are also shown in figure 1). Results from blots like this were used for statistical analysis.

pλg3 (the results from this probe were therefore ignored). In general however such bands did not account for the differences observed between a given pair. More often they either appeared identical between the two individuals or resided in parts of the DNA profiles which were not considered, due to insufficient resolution. Nonetheless, in order to address the possibility that the DNA patterns might have involved other unstable loci, new blots containing all candidate twins and some of their control siblings were produced, with comparison facilitated by loading the individual samples of each twin and control pair adjacent to each other. For this experiment, only *AluI* digests were used, probed by 33-15 (RNA). The differences (D_{xy}) in the DNA fingerprints within all 35

candidate twin pairs and within 139 dizygotic sibling control pairs were then calculated as:

$$D_{xy} = \frac{2n_{xy}}{n_x + n_y}$$

where $2n_{xy}$ is the number of differences found between a given set of two individuals x and y , and n_x and n_y are the total numbers of bands scored in individual x and y , respectively. Should all differences found in the candidate twins merely have been due to somatically unstable loci (i.e. identical twins might carry length mutations at these loci which occurred after the separation of the cell mass), then we would expect significantly fewer differences in the DNA profiles between candidate twins than between sibling controls.

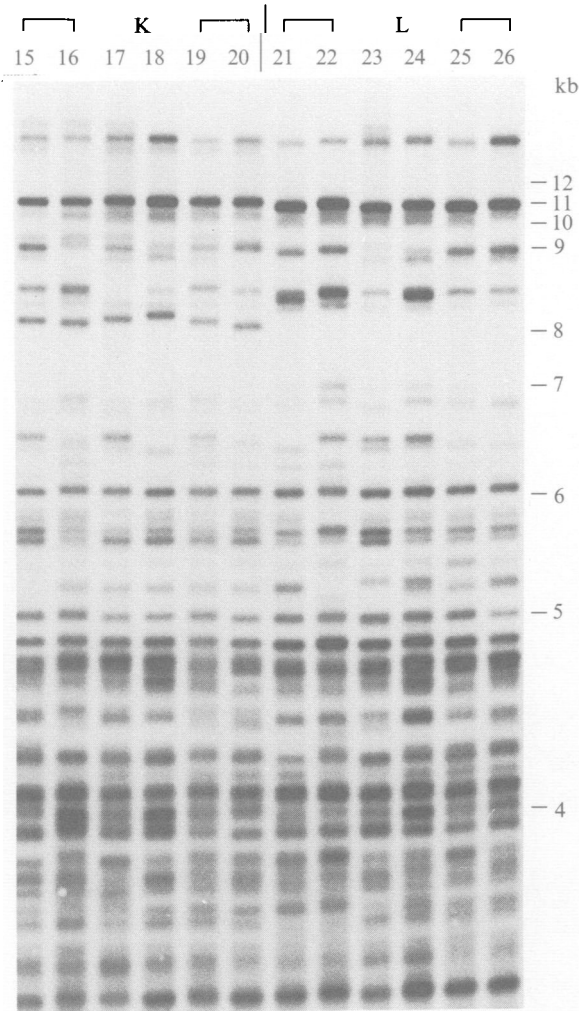


Figure 3. DNA fingerprints produced by 33-15 (DNA) on *AluI* DNA digests of two litters derived from hormonally superovulated females. Note that the DNA template of this probe reveals fewer informative fragments than RNA transcripts. Nevertheless all four sets of candidate twins are clearly distinguishable in their DNA profiles indicating dizygosity.

The results are illustrated as two superimposed histograms in figure 4. Since chi-square statistic and median test did not show any significant difference between the two data sets, we are confident that our conclusion on all mice sampled being derived from different zygotes remains valid.

DISCUSSION

In our main series, 1386 mice survived to weaning. We were unable to carry out DNA finger-printing on 3 of the 30 pairs that appeared identical on other criteria. Since these 3 pairs could have included a monozygotic twin pair, the effective number of mice screened should be reduced by 10%, to 1247. If the frequency of monozygotic births was as high as 1%, as estimated by Wallace and Williams (1965), it is very unlikely ($P < 0.001$) that we would have failed to detect a single monozygotic pair.

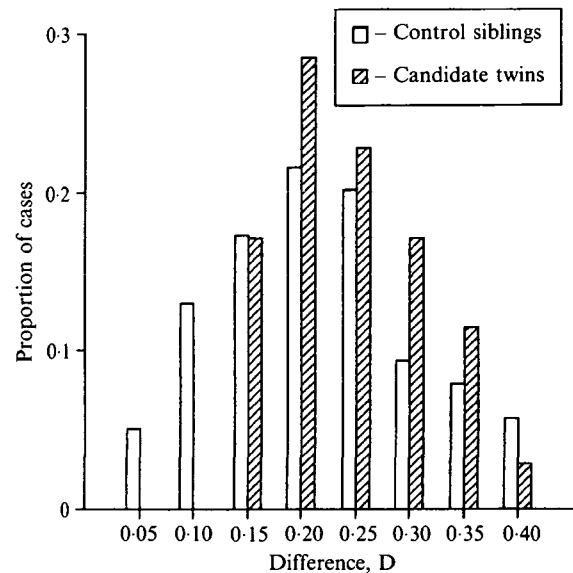


Figure 4. Histograms showing frequencies of D values obtained in pairs of candidate twins and sibling controls, respectively (χ^2 on median test = 0.38, df = 1; $P < 0.05$). For further details see text.

The possibility that the minisatellite probes used under low stringency conditions could have cross-hybridised to some somatically unstable locus or loci not yet identified was ruled out by segregation analysis of the banding patterns produced by the probes. In human twin zygosity testing, no consideration has yet been given to the incidence of somatic mutations, and the possible limitation of typing systems that they impose.

It is of course possible that different mouse stocks differ in their monozygotic twinning rate. It is also possible that monozygotic twinning occurred in our mice, but in no instance did both members of a pair survive to weaning. When two mouse blastocysts implant close enough together in the uterus for placental apposition ('fusion') to occur, fetal weight is reduced and runting or death may ensue, because both implants are supplied by a single branch of the uterine artery (McLaren and Michie, 1959b, 1960). Any monozygotic splitting that occurred at the blastocyst stage or later would be subject to this effect. Even if both members of the pair survived to term, their birth weights would be reduced and they might die before weaning. Pre-weaning mortality rate in our main series was 4.8%.

When mouse embryos were experimentally split during the preimplantation period to give half embryos, fetal weight at the end of gestation was significantly reduced (Tsunoda and McLaren, 1983).

In each of the dichorial, presumed monozygotic, pairs of embryos reported by Bateman (1960), one member of the pair was small, and unlikely to survive because its position was too antimesometrial for normal placentation to have occurred. Similarly, Cole (1965) mentions observing some possible monozygotic twin pairs at 9–10 days *post coitum*, but never later.

Bodemann (1935) described two egg cylinders which were attached to the same ectoplacental cone within a single yolk sac. These must have arisen by splitting of a single inner cell mass. To our knowledge, no other presumed monozygotic twin pairs have been reported, other than those due to experimental manipulation (Tarkowski, 1965) or genetic abnormality (Gluecksohn-Schoenheimer, 1949).

In pigs, another species with large litter sizes, DNA fingerprinting has been performed either shortly before or after weaning in 13 families with a total of 121 offspring (unpublished observation). Again, no pairs of identical siblings were found. Unless monozygotic twins in multiparous species escape detection by dying early, it may be that with multiple ovulation as an efficient means to produce many offspring, monozygotic twinning is an unnecessary luxury.

In our induced-ovulation series, only 8 pairs out of 12 possible monozygotic twin pairs were tested by DNA finger-printing: the effective number screened should therefore be estimated at 407 rather than 610. No monozygotic twin pairs were found. Prewaning mortality was higher and litter size at weaning was slightly lower than in the main series (7.35 versus 8.11). A reduction in litter size would decrease the number of apparently identical pairs but should not in itself affect the proportion of monozygotic twin pairs since each egg shed would have a given probability of splitting irrespective of how many others there were in the litter. However, any prenatal or preweaning mortality would be likely to bear more heavily on monozygotic twins than on their litter mates, so the proportion of monozygotic twin pairs detected would be reduced. Although our failure to detect any monozygotic twin pairs cannot rule out the possibility that hormonal induction of ovulation increases the rate of monozygotic twinning, our original aim of developing a system to analyse the causes of any such increase is clearly impractical.

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