Identification of germ-line chimaeras by polymerase chain reaction and isoenzyme analysis of mouse spermatozoa

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In this study a rapid, simple and inexpensive procedure is described which allows potential germ-line male mice to be identified with confidence. Spermatozoa recovered by uterine washing following mating with normal female mice was analysed in two ways. First, the patterns of expression of the different isoforms of glucose phosphate isomerase were determined. Since the glucose phosphate isomerase isoforms expressed in embryo stem (ES) cell lines are frequently different from those associated with the host blastocyst, it is possible to determine the proportion of spermatozoa produced by an individual animal that was of ES cell or host-blastocyst origin. Second, DNA of spermatozoa was subjected to polymerase chain reaction (PCR) analysis using primers with specificity for the targeted mutation in the ES cells. The PCR analysis was particularly valuable in identifying germ cell chimaeras in which the contribution of ES-derived spermatozoa was significantly less than that specified by the host blastocyst.

Introduction

Mouse embryonic stem cells (ES cells) derived from the inner cell mass of day 3.5 blastocysts (Evans and Kaufman, 1981; Martin, 1981) can be cultured indefinitely on feeder cells or in growth medium supplemented with leukaemia inhibitory factor (LIF) (Williams *et al.*, 1988). The ability to propagate ES cells *in vitro* without compromising their developmental potential provides an opportunity for creating targeted mutations by homologous recombination (for review see Capecchi, 1989). Modified ES cells generated in this way can be microinjected into mouse blastocysts, which in turn can be transferred into recipient mice to generate germline chimaeras. Through interbreeding of heterozygous offspring, animals homozygous for the desired mutation can be produced. Mice with dominant or recessive mutations have been successfully generated in a number of laboratories (for reviews see Capecchi, 1989; Joyner, 1991; Koller and Smithies, 1992).

In most experiments involving the introduction of genetically modified ES cells into blastocysts, the long-term goal is the production of animals homozygous for the desired mutation; it is therefore usual to use animals displaying some level of coat chimaerism for subsequent breeding programmes. In some instances, the coat may appear to be wholly derived from the introduced ES cells, while in other cases the contribution may amount to a few patches of isolated hairs on a background specified by the choice of host blastocyst. Obviously, the composition of subsequent litters sired by any male chimaera is dictated solely by the germ cells that may originate from the introduced ES cells, cells of the host blastocyst or both. One of

the principal influences on germ-line transmission is the sex of the host blastocyst (Zijlstra *et al.*, 1989), whereas coat colour composition is determined by the random integration of ES cells into the mesoderm of the host blastocyst (Koller *et al.*, 1989). Thus, coat chimaerism serves only to identify animals in part derived from the introduced ES cells and the degree of chimaerism is, at best, a rough guide to their germ-line potential.

When the outcome of a particular experiment involving injection of ES cells into blastocysts is a collection of low level coat chimaeras, it is often difficult to decide which animals to use as founder members of a breeding programme. In the event that only a small proportion of germ cells are derived from the introduced ES cells, it is quite possible that all the offspring in a particular litter would be of host blastocyst origin. In some instances male chimaeras transmitted the ES cell genotype to fewer than 3% of their offspring (Zijlstra *et al.*, 1989; DeChiara *et al.*, 1990; Grusby *et al.*, 1991). The number of chimaeras chosen to serve as foundation members of a breeding programme is dictated by factors such as the availability of resources. These considerations would undoubtedly profit from some knowledge of the potential that any particular animal has for germ-line transmission.

In the present study we outline a scheme for assessing the likelihood that any particular chimaeric animal will transmit the genetic modification through the germ line. The procedure involves glucose phosphate isomerase isoenzyme and PCR analysis of spermatozoa derived from individual chimaeric animals. By judicious choice of oligonucleotide primers, PCR analysis provides a sensitive measure of whether the germ cells of a particular male chimaera are in part derived from the genetically modified ES cells. Isoenzymic analysis takes advantage of electrophoretic differences between isomers encoded by

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the glucose phosphate isomerase *Is* gene, in tissues originating from the host blastocyst and introduced ES cells (Bradley *et al.*, 1987). The ratio of these isoforms provides a rational basis for establishing breeding programmes using particular chimaeric animals which have the greatest potential for transmitting the genetic trait through the germ line.

Materials and Methods

Cell cultures

E14 ES cells (Handyside *et al.*, 1989) were grown on feeder layers of irradiated or mitomycin C-treated STO Neo^r mouse fibroblasts in Dulbecco's modified Eagle medium with sodium pyruvate and high glucose (Irvine Scientific, Santa Ana, CA) containing 15% heat-inactivated fetal bovine serum (Cytosystems, Castle Hill), non-essential amino acids (Flow Laboratories, Seven Hills), nucleosides, 10^{-4} mol β -mercaptoethanol l^{-1} (Sigma, St Louis, MO), 10^3 U murine LIF ml^{-1} (Esgro; Amrad, Melbourne) plus 60 μg benzyl penicillin ml^{-1} (sodium salt, CSL, Parkville) and 100 μg streptomycin sulfate ml^{-1} (CSL, Parkville). All culture surfaces were treated with gelatin (Robertson, 1987). Incubation was at 37°C in a humidified atmosphere of 10% CO₂. Cells were tested mycoplasma free using the Gen-probe Rapid Detection System and by Hoechst 33258 staining (Chen, 1977).

Generation of chimaeric mice

Approximately 15 ES cells were injected into blastocysts and late morulae flushed from superovulated and naturally mated C57BL/6 mice (4–5 weeks old). After injection, embryos were cultured for 4–20 h in HTF medium (Quinn *et al.*, 1982) or in M16 medium (Hogan *et al.*, 1986). Twelve to twenty embryos with visible blastocoels were transferred to the uteri of pseudo-pregnant random-bred Swiss and F₁ (C57BL/6 $\times$ CBA/CaH) recipients (8–12 weeks old) at 2.5 days *post coitum*, as described by Bradley (1987). Pups were born 17–18 days after transfer. Heavily chimaeric pups were identified 2–3 days later.

Experiments were conducted in accordance with the Royal Melbourne Hospital Campus Animal Ethics Committee (Parkville, Australia). Mice were purchased from Animal Resources Centre (Willetton). Mice were housed in standard shoebox mouse cages and were fed with feeder cubes (Barastoc ARM diet, Pakenham) and were given water *ad libitum*.

Collection of spermatozoa

Fertile male mice were kept on a 16 h light (16:00–08:00 h): 8 h dark cycle. At 08:00 h, female mice (6–10 weeks old) were given an i.p. injection of 2.5 i.u. human chorionic gonadotrophin (hCG; Chorulon; Intervet, Lang Cove) and were immediately housed with male mice. Female mice were examined for vaginal plugs every 2 h. Once a plug was observed, female mice were killed by cervical dislocation and their reproductive tracts removed. Spermatozoa were flushed from each uterine horn using a 2 ml disposable syringe attached to a 27-gauge needle.

Spermatozoa samples for glucose phosphate isomerase analysis were collected in 0.2–0.5 ml distilled water, whereas samples for PCR were collected in 1–2 ml PBS. Samples were

Fig. 1. Glucose phosphate isomerase-1s (Gpi-1s) analysis of tissues from (a) male 129/SvHe and (b) C57BL/6 mice. Markers (lane 1) are a mixture of blood from 129/SvHe and C57BL/6 mice. Blood (lane 2), spermatozoa from the epididymis (lane 3) and spermatozoa from uterine flushing of both 129/SvHe (lane 4) and C57BL/6 (lane 5) females as well as testis (lane 6) are shown. The direction of the electrophoretic field (+/–) and the position of the Gpi-1s^{aa} (marked aa) and Gpi-1s^{bb} (marked bb) isoforms are indicated.

examined by microscope for the presence of motile spermatozoa. Particular care was taken with the collection of samples for glucose phosphate isomerase analysis to avoid contamination with maternal blood.

Glucose phosphate isomerase analysis

All samples for glucose phosphate isomerase analysis were lysed in distilled water and kept frozen. Glucose phosphate isomerase analysis was carried out using Titian III Zip Zone cellulose acetate plates (Helena Laboratories, Beaumont, TX) as described by Bradley (1987).

Estimation of the percentage of ES cell contribution in blood and spermatozoa was done on glucose phosphate isomerase plates using Chromscan 3 (Joyce Loebel, Vickers Instruments Inc., Malden MA) at 530 nm and with 0.3 mm aperture width.

PCR analysis

PCR conditions were established on cells that had been transfected with a vector containing both primer binding sites. Cells were washed with phosphate-buffered saline (PBS) and then lysed in a solution of 0.5% SDS, 0.1 mol NaCl l^{-1} , 0.01 mol EDTA l^{-1} , 0.1 mol Tris-HCl l^{-1} pH 7.5 and 0.5 mg pronase ml^{-1} . Sperm samples that had been collected in PBS were spun at 5000 r.p.m. for 3 min using Biofuge A (Heraeus Christ; Foss Electric Australia Pty. Ltd, Melbourne); the supernatant was removed and the cell pellet resuspended in lysis buffer. All samples were incubated at 55°C for 2 h. After a phenol and a chloroform extraction, the DNA was precipitated in isopropanol, and resuspended in Tris-EDTA buffer at about 250 ng μl^{-1} . PCR was carried out in a final volume of 20 μl containing 0.067 mol Tris-HCl l^{-1} , 0.00166 mol (NH₄)₂SO₄ l^{-1} , 0.45%

Fig. 2. The five E14-C57BL/6 chimaeras used for Gpi analysis and test breeding.

Triton X-100, 20 $\mu\text{g ml}^{-1}$, 0.002 mol $\text{MgCl}_2 \text{ l}^{-1}$, 250 $\mu\text{mol l}^{-1}$ of each dNTP; 10 ng of each primer and 1 U of Taq polymerase. Thirty-five temperature cycles were used as follows: 95°C, 50 s; 63°C, 50 s; 72°C, 70 s, after an initial denaturation step at 95°C for 150 s.

The following synthetic oligonucleotide primers were used to identify the rearranged transforming growth factor α (TGF- α) locus. 5'-sense primer is taken from the sequence of a genomic fragment adjacent to the targeting vector and is: 5'-dGACTAGCCTGGGCTACACAGTG-3' (Mann *et al.*, 1993). 3'-antisense primer is derived from the neomycin gene (Tn5, nucleotides 2264-2285, Colbere-Garapin *et al.*, 1981) and is 5'-dCCGCTTCCTCGTGCTTTACGGT-3'.

Five microlitres of the reaction mix was resolved on a 1% agarose gel and stained with ethidium bromide, prior to photography.

Results

Isoenzymic analysis of spermatozoa

We exploited the fact that glucose phosphate isomerase-1s is expressed in spermatozoa (Brock, 1977) as the basis for assessing whether the spermatozoa of individual chimaeric animals was derived from cells of the host blastocyst or from ES cells

Fig. 3. Glucose phosphate isomerase-1s (Gpi-1s) analysis of tissues from E14-C57BL/6 chimaeras 1–5. Markers (lane 1) are a mixture of blood from 129SvHe and C57BL/6 mice. Extracts of blood (lane 2), epididymis-derived spermatozoa (lane 3) and testis (lane 5) were harvested after mice were killed. Spermatozoa were collected by uterine flushing of a C57BL/6 female (lane 4). The direction of the electrophoretic field (+/–) and the position of the Gpi-1s^{aa} (marked aa) and Gpi-1s^{bb} (marked bb) isoforms are indicated.

introduced into blastocysts by microinjection. We reasoned that since the ES cells used for gene targeting are usually derived from a different mouse strain to that of the host blastocyst, it might be possible to assess the origin of the spermatozoa by taking advantage of the fact that different strains of mice express different isomers of glucose phosphate isomerase (Green, 1989).

We first set out to establish the patterns of glucose phosphate isomerase expression in spermatozoa from 129/SvHe (Pease *et al.*, 1990) and C57BL/6 mice. The choice of these strains simulates the combination of ES cells (E14, Handyside *et al.*, 1989) and the host blastocyst (C57BL/6) that are routinely used for gene targeting exercises in our laboratory. Sperm samples from 129/SvHe and C57BL/6 male mice were recovered by uterine

Table 1. Germ-line transmission of E14-derived male mouse chimaeras

Chimaera	Estimated percentage of ES cell contribution by coat colour	Number of agouti versus total offspring
1	5% ^{c^h} , 95%A	*0/23
2	10% ^{c^h} , 90%A	*17/17
3	25% ^{c^h} , 75%A	17/17
4	25% ^{c^h} , 75%A	13/13
5	25%A, 75%B	*4/20

Coat colours, ^{c^h}: chinchilla; A: agouti; B: black. Black hair indicates absence of contribution of ES cells. Agouti indicates that F₁ mice are heterozygous for E14 genetic material (Koller *et al.*, 1989).

*E14-1 embryos, one dead E14-2 pup and one dead E14-5 pup were analysed by glucose phosphate isomerase.

washing following mating with hCG-treated 129/SvHe and C57BL/6 females. Spermatozoa derived from 129/SvHe (Fig. 1a) and C57BL/6 (Fig. 1b) mice express the Gpi-1s^{aa} and Gpi-1s^{bb} isoforms, respectively, which are clearly distinguished by their different electrophoretic mobilities. For both 129/SvHe and C57BL/6 males, only the respective Gpi-1s^{aa} and Gpi-1s^{bb} isoforms are present indicating that the method of spermatozoa collection had not resulted in significant contamination by tissues of the female mice to which individual males had been mated. The patterns of expression of isoforms of glucose phosphate isomerase (Fig. 1) detected in spermatozoa recovered from the epididymides, testes and blood of 129/SvHe (Fig. 1a) and C57BL/6 (Fig. 1b) male mice was the same as that detected in spermatozoa recovered from uterine washing.

Analysis of glucose phosphate isomerase of chimaeric mice

A series of chimaeric animals were generated following injection of E14 ES cells into C57BL/6 blastocysts, to establish whether typing glucose phosphate isomerase isomers in spermatozoa might provide a basis for predicting the genetic composition of offspring. E14 cells, derived from strain 129/Ola (A^w^{c^h}) mice, carry mutations at three loci, agouti (A), albino (c) and pink-eyed (p) that are known to affect coat colour. The 129/Ola mice have pale cream coat colour and pink eyes (Koller *et al.*, 1989). C57BL/6–E14 chimaeras are readily identified 7 days after birth by lighter patches of fur on a black background. Of the numerous animals we have generated in this way, five male chimaeras were selected for detailed analysis on the basis of the extent and range of coat chimaerism displayed by each animal (Fig. 2). These animals were typical of those routinely generated in our laboratory using this combination of ES cell and host blastocyst. Each chimaera was mated with a C57BL/6 female; spermatozoa were recovered by uterine washing and the pattern of glucose phosphate isomerase isoforms was determined (Fig. 3). In the case of chimaera E14-1, the main isoform was Gpi-1s^{bb}, indicating that the ejaculated spermatozoa were predominantly of C57BL/6 origin. By contrast, spermatozoa recovered from E14-2 and E14-3 displayed the Gpi-1s^{aa} isoform, suggesting that the spermatozoa of these animals was of

Fig. 4. (a) Schematic representation of disrupted transforming growth factor α (TGF- α) allele. Predicted structure of disrupted TGF- α locus. Arrowheads indicate the position of the diagnostic oligonucleotide primers. The 5'-sense primer sequence lies 5' to the *Eco*R1 site that marked the limit of homology in the targeting vector. The 3'-antisense primer sequence lies at the 3' end of the neo coding sequence. Filled box represents the diagnostic 1.35 kb PCR band. PGKNeo:neomycin phosphotransferase coding sequence, under control of murine phosphoglycerol kinase regulatory elements; E:*Eco*R1; B2:*Bgl*II. (b) PCR analysis of DNA from ES cell lines. DNA from targeted cells, C12, and from parental ES cells, E14, mixed in various ratios then subjected to PCR analysis using diagnostic primers. An unexplained 'control' band is seen in all PCR using these primers and murine DNA. A diagnostic band is present when the targeted DNA is present in a 1:100 dilution. (c) PCR analysis of DNA from chimaeric mouse spermatozoa. PCR on DNA from control ES cell lines and from spermatozoa samples of four chimaeras, A, T, G and B, with and without germ-line potential.

Table 2. Characteristics of transforming growth factor α (TGF- α) mouse chimaeras

Chimaera	Estimated percentage of ES cell contribution			Number of agouti versus total offspring
	Coat colour	Blood (%)	Spermatozoa (%)	
A	25% c ^h , 75% A	51	99	*44/44
T	15% c ^h , 85% A	38	35	19/58
G	25% A, 75% B	30	25	*15/57
B	10% c ^h , 90% W	NP	NP	0/32

Coat colours, c^h: chinchilla; A: agouti; B: black; W: white; NP: not possible, as mouse B was the result of injection of E14 ES cells into BALB/c blastocysts, both of which express the Gpi-Is aa isoform (see Table 4).

*One dead A pup and two dead G pups were analysed by glucose phosphate isomerase.

129/Ola origin. In the case of E14-4 the glucose phosphate isomerase isoform present in spermatozoa was predominantly Gpi-Is^{aa}, although some Gpi-Is^{bb} was present. Finally, spermatozoa from E14-5 expressed both Gpi-Is^{aa} and Gpi-Is^{bb} isoforms at a ratio of about 1:4.

Once spermatozoa from chimaeras had been analysed and a breeding programme from each animal had been completed, mice were killed. Spermatozoa were recovered from the epi-

didymides and simultaneously samples of blood and testis were collected for glucose phosphate isomerase analysis. For most of the chimaeric animals, (for example E14-2, E14-3, E14-4 and E14-5), the patterns of expression of glucose phosphate isomerase isoforms in blood and epididymides roughly recapitulated that observed in spermatozoa isolated from the same animal following mating. In chimaera E14-1, the contribution of Gpi-Is^{aa} was somewhat higher in blood than in spermatozoa or testis.

Table 3. Predicted number of offspring to secure germline transmission in mice

Chimaerism of spermatozoa (%)	Number of offspring needed to be analysed	
	$P = 0.95$	$P = 0.99$
1	299	459
2	149	228
5	59	90
10	29	44
15	19	29
20	14	21
30	9	13
50	5	7

For various degrees of chimaerism (c), the number of offspring that must be generated (n) to produce at least one ES cell-derived mouse at a confidence level (p) of 95% or 99% was calculated using the following equation:

$$1 - \left(\frac{100 - c}{100} \right)^n < p$$

Mating data from chimaeric animals

When the Gpi-isoform profiles were established (Fig. 3) and the degree of coat chimaerism (Fig. 2) of the E14-1-5 mice estimated, the frequency of agouti pups sired by individual chimaeras was determined after mating for approximately 3 months. All of the pups sired by E14-2, -3 and -4 were agouti indicating that the spermatozoa of these animals were mainly, or wholly, derived from the E14 ES cells (Table 1). By contrast, none of the pups generated from matings using E14-1, and 20% of the offspring sired by E14-5 was agouti.

PCR analysis of spermatozoa derived DNA

Although glucose phosphate isomerase analysis of spermatozoa of male chimaeras in principle provides the basis for predicting the composition of subsequent litters, its general utility is restricted by its sensitivity. When the outcome of a particular series of blastocyst transfers is the generation of a collection of poor chimaeras, it is possible that the proportion of ES cell-derived spermatozoa would be so low that it would be undetectable by glucose phosphate isomerase analysis. In these circumstances, it is important to be able to distinguish animals with some potential to transmit the ES cell-associated trait from those whose spermatozoa are wholly of host blastocyst origin. To this end, we used the polymerase chain reaction (Mullis *et al.*, 1986; Saiki *et al.*, 1988) for analysis of sperm DNA (Ficsor *et al.*, 1990) using a pair of oligonucleotide primers with specificity for the modification engineered in the original targeted ES cell line.

The feasibility of using a PCR based screening procedure was assessed by analysing spermatozoa DNA from four male chimaeric animals (designated A, B, G and T) derived from

blastocysts injected with E14 ES cells carrying a disruption of the TGF- α locus (Mann *et al.*, 1993). In the context of the present report, the significance of studying these mice is the opportunity afforded by a modified TGF- α locus of using unique oligonucleotide primer binding sites in conjunction with PCR to monitor the origin of the spermatozoa of those animals. The primers used to screen sperm DNA were the same as those originally used to identify ES cells in which a TGF- α targeting vector had recombined with the TGF- α locus by homologous recombination (Fig. 4a). One of the primers (3'-antisense) corresponds to sequences of the neomycin gene, and the other lies in the TGF- α locus but outside of the targeting vector (5'-sense). Use of DNA from the targeted ES cell line (C12) in a PCR reaction resulted in the generation of a 1.35 kb product from these primers, which is diagnostic for the disrupted TGF- α locus (Fig. 4b); no corresponding band was generated using DNA from normal E14 cells. To determine the sensitivity of the PCR assay, we set up a reconstruction by mixing DNA from the original targeted ES cell line and normal ES cell DNA. The presence of the disrupted TGF- α locus can be detected when the ratio of targeted ES:normal ES cell DNA is 1:100 (Fig. 4b).

DNA was subjected to PCR analysis using the diagnostic primers to determine whether spermatozoa from animals A, G, T and B included the TGF- α disruption. Sperm DNA from mice A, G and T but not mouse B contained the diagnostic 1.35 kb fragment (Fig. 4c). Spermatozoa samples positive by PCR were also subjected to glucose phosphate isomerase analysis and the relative proportion of Gpi-1s^{aa} and Gpi-1s^{bb} isoforms in blood and spermatozoa from these animals was determined densitometrically (Table 2). The three animals (A, T and G) showing evidence of a disrupted TGF- α locus were subsequently introduced into a breeding programme to determine the relationship between the relative proportion of glucose phosphate isomerase isoforms and transmission of the ES cell genotype. All of the offspring of mouse A were agouti, indicating transmission of ES cell-derived genetic material (Table 2). As predicted from glucose phosphate isomerase analysis of spermatozoa, about 35% of the offspring of mouse T and 25% of the offspring of mouse G were agouti.

Discussion

Transgenic mouse projects based on embryonic stem cells tend to be characterized by many blastocyst injections which, in turn, lead to the generation of many litters. Mice, which are wholly, or in part, derived from genetically modified ES cells are identified on the basis of coat chimaerism. While the extent of coat chimaerism is often used as an indirect guide to germ-line potential, it does not indicate whether the primitive gonads of a particular animal have been colonized by ES cells, leading to germ-line transmission. This is exemplified in the case of two animals generated in the present study following injection of normal E14 ES cells into C57BL/6 blastocysts. On the basis of coat chimaerism we might have expected that E14-1, with no obvious black hair, would have significant ES cell contribution to the germ tissues and likewise, that E14-5, with mainly black hair, was unlikely to have germ-line potential. In neither case was the extent of coat chimaerism a valuable guide to germ-line potential, based on the offspring sired by each of these animals.

Table 4. Glucose phosphate isomerase (Gpi-Is) isoforms of ES cell lines and mouse blastocysts

Parental ES cell		Host blastocyst	
Cell line	Gpi-Is isoform	Mouse strain	Gpi-Is isoform
ES-D3	aa (Gossler <i>et al.</i> , 1986)	C57BL/6	bb (Green, 1989)
E14	aa (Hooper <i>et al.</i> , 1987)	ICR	bb (Boulter <i>et al.</i> , 1991)
MBL5	aa*	PO	bb (Boulter <i>et al.</i> , 1991)
CCE	cc (Schwartzberg <i>et al.</i> , 1989)	MFI	bb (Beddington and Robertson, 1989)
CC1	cc (Bradley <i>et al.</i> , 1984)	CD1	bb (Schwartzberg <i>et al.</i> , 1989)
CP1	aa (Bradley <i>et al.</i> , 1984)	CFLP	bb (Bradley <i>et al.</i> , 1984)
		BALB/c	aa (Green, 1989)

*Authors' unpublished observation.

Clearly, some knowledge of the potential for germ-line transmission would be of considerable value to enable rational allocation of resources.

In the present study, glucose phosphate isomerase analysis was used to determine whether spermatozoa produced by animals displaying coat chimaerism is of 129/Ola or C57BL/6 origin or both. As predicted, the litters sired by E14-2 and E14-3 were all agouti, reflecting the fact that only the Gpi-Is^{aa} isoform was detected in spermatozoa derived from these animals. Because these chimaeras had 100% transmission, they were probably derived from injected female host blastocysts (Zijlstra *et al.*, 1989). In the case of E14-4, all of the offspring were agouti, reflecting the predominance of the Gpi-Is^{aa} isoform, although a relatively small proportion of Gpi-Is^{bb} isoform was clearly detected in spermatozoa from this animal. Assuming that ES cell and host blastocyst-derived spermatozoa were equally capable of fertilization, it is likely that some C57BL/6 derived animals would have been generated had E14-4 been used as the founder of a larger collection of offspring.

In the case of E14-1, glucose phosphate isomerase analysis indicated that most of the ejaculated spermatozoa were of C57BL/6 origin, whereas spermatozoa from E14-5 were a mixture of C57BL/6 and 129/Ola. The composition of litters sired by these two mice clearly reflects the patterns of glucose phosphate isomerase isoform distribution in spermatozoa and is at odds with predictions based solely on the extent of coat chimaerism or glucose phosphate isomerase isoform distribution in blood.

On the basis of its simplicity and reliability, we now routinely carry out glucose phosphate isomerase isoform analysis on spermatozoa from any male animal arising from gene targeting exercises before establishing a breeding programme. In addition to indicating whether any particular animal has germ-line potential, the procedure is also semi-quantitative. Thus, on the basis of the ratio of different glucose phosphate isomerase isoforms, it is possible to predict mathematically the number of offspring that would need to be sired by an individual germ-line mosaic mouse to be confident of securing at least one ES-cell derived pup. For example, germ-line transmission would be expected in the first litter where the father was a 50% germ-cell mosaic. Where the contribution of ES cell-derived spermatozoa was only 5 or 10% it would be necessary to generate 59 and 29

offspring, respectively, to be certain of securing germ-line transmission at a confidence level of 95%. A basic assumption underlying this approach is that spermatozoa from chimaeric testes are produced from both groups of germ cells at a fixed ratio and that all spermatozoa are equally likely to fertilize an ovum. A longitudinal study comparing germ-line contribution in sperm samples with breeding data would be of interest; however, this was not possible because the majority of chimaeras were killed after breeding for approximately 3 months.

However, the usefulness of glucose phosphate isomerase isoform analysis of spermatozoa is limited by its sensitivity. On the basis of reconstruction experiments, we are confident that we can observe the Gpi-Is^{bb} isoform where the proportion of Gpi-Is^{bb} isoform is greater than 5% (data not shown). However, there have been reports of males that transmit the predetermined mutation at a very low frequency; from less than 1% to 3% (Zijlstra *et al.*, 1989; DeChiara *et al.*, 1990; Grusby *et al.*, 1991). Presumably, these low level germ-line chimaeras would have been missed by glucose phosphate isomerase analysis of spermatozoa, as detection of the ES cell associated glucose phosphate isomerase isoform would fall below the sensitivity of the assay.

So that low level germ-cell chimaeras are not discarded solely on the basis of glucose phosphate isomerase analysis, we routinely subject an additional spermatozoa aliquot to PCR analysis. This is conveniently carried out using a pair of oligonucleotides with specificity for the mutation installed in the ES cells. Although the sensitivity of this analysis depends, to some extent, on the nature of any particular pair of oligonucleotides, in practice there should be little difficulty in identifying one part 129-derived spermatozoa in a background of one hundred parts C57BL/6 spermatozoa. Where the level of germ cell chimaerism falls below 1:100, the number of matings required to stand any chance of securing germ-line transmission would be prohibitive.

In summary, we believe that a combination of glucose phosphate isomerase and PCR analysis of spermatozoa from chimaeric animals has the potential to identify animals on which to base breeding programmes. While the animals that form the basis of the present study where derived following injection of E14 cells into C57BL/6 blastocysts, the technique can also be applied to other ES cell-blastocyst combinations which express the Gpi-Is^{aa} (or Gpi-Is^{cc}) and Gpi-Is^{bb} isoforms, respectively.

Of the combinations we have surveyed, only BALB/c blastocysts express the Gpi-1s^{aa} isoform and thus glucose phosphate isomerase analysis of spermatozoa cannot be used when BALB/c blastocysts are used in conjunction with ES cells expressing the Gpi-1s^{aa} isoform.

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