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XY female mice resulting from a heritable mutation in the primary testis-determining gene, *Tdy*

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Summary

Chimeric mice constructed with XY embryonic stem (ES) cells that had been multiply infected with a retroviral vector were used in a genetic screen to look for mutations affecting the sex determination pathway in mice. From a small number of chimeras screened one was identified that gave rise to a low proportion of XY females amongst his offspring. Analysis of the segregating patterns of retroviral insertions demonstrated that the mutation was found in a subset of the offspring derived from one originally infected ES cell. However, the mutation appeared to have occurred subsequent to the infection. Some of the XY females proved to be fertile, and the mutant phenotype was found to segregate exclusively with the Y chromosome. Analysis of the offspring also confirmed the absence of any retroviral insertion that could be correlated with the mutation.

Further characterisation of the Y chromosome carrying the mutation by karyotypic analysis, and by Southern blotting with a range of Y-specific DNA probes suggested that there has been no gross deletion or rearrangement of the Y carrying the mutation. There also appeared to be no loss of Y-specific gene functions apart from that of testis determination. Moreover, the mutation is complemented by Sxr', the minimum portion of the mouse Y known to carry *Tdy*. From the phenotype and deduced location of the mutation, we conclude that it is within the *Tdy* locus. This is the first such mutation to be described in mice.

Key words: sex determination, Y chromosome, *Tdy*, embryonic stem cells, chimeras, retroviral vectors, insertional mutagenesis, X:Y pairing.

Introduction

The hierarchy of genes involved in sex determination and differentiation in *Drosophila* and *Caenorhabditis elegans* has been well established largely due to the availability of a wide range of mutations. These have led to the identification and molecular cloning of many of the gene products and to some understanding of how they function (reviewed Hodgkin, 1987; 1989; Meyer, 1988; Baker, 1989). However, little is known about the primary mechanism in higher eukaryotes and a major limitation has been the paucity of mutations that affect sex determination (McLaren, 1988*a*).

In mammals, it is known that the activity of a gene on the Y chromosome is responsible for determining the primary sex of the developing embryo (Jacobs and Strong, 1959; Ford *et al.* 1959; Welshons and Russell, 1959). This gene is thought to act within the supporting cell precursors of the genital ridge, and triggers their differentiation along the Sertoli cell pathway (Burgoyne *et al.* 1988). In the absence of the Y chromosome, the bipotential gonad follows the 'default' ovarian pathway, which has been postulated to involve a set of ovary-determining genes (Eicher and Washburn, 1986). Spontaneous translocations and deletions of the mammalian Y chromosome have provided an invaluable resource for mapping and localizing the testis-determining gene (referred to as *Tdy* in mouse and *TDF* in humans). Some of the best documented and most informative examples of these are the sex reversed (Sxr) mutation in mice (Cattanach *et al.* 1971; Singh and Jones, 1982; McLaren *et al.* 1988) and a range of spontaneous XX phenotypic males in humans (Vergnaud *et al.* 1986; Page, 1986; Affara *et al.* 1987; Palmer *et al.* 1989). These do not represent specific mutations *per se* but are chromosomal abnormalities in which translocated Y-chromosome sequences determine differentiation of testes in an otherwise chromosomally normal female background. There are also many cases of human XY females. Some of these are due to chromosomal abnormalities; for example, where the region carrying *TDF* has been lost due to abnormal X:Y interchange at

meiosis (Ferguson-Smith *et al.* 1987; Weissenbach *et al.* 1987). Others may represent mutations within *TDF*. However, this has been difficult to prove because XY female humans are invariably sterile, and there are clear examples of XY female mammals, including humans, where the mutations appear to be in downstream genes not on the Y (Kent *et al.* 1986; Fredga, 1988; Eicher, 1988; Scherer *et al.* 1989).

We wished to isolate new mutations affecting the sex-determination pathway in mice. Mutations in *Tdy* itself, or in 'downstream' responder genes, would presumably result in a breakdown of the normal differentiation events to give either complete or partial phenotypic sex reversal. However, it may be difficult to detect such mutations for at least two reasons. First, they may affect the differentiation of the reproductive system of the carrier animals making them infertile. Second, it will be difficult to transmit mutations affecting sex determination because both the gonadal environment and the sex-chromosome complement determine whether a germ cell can proceed through meiosis to form functional gametes (McLaren, 1988*b*). Thus XX germ cells in testes fail to undergo early stages of spermatogenesis (see Burgoyne *et al.* 1986) and XY germ cells, while able to form oocytes, often degenerate before puberty (Taketo-Hosotani *et al.* 1989).

With these considerations in mind, we decided to make use of chimeric male mice that had been constructed using an XY embryonic stem (ES) cell line that had been multiply infected in culture with the MPSV.mos^{-1}neo replication defective retroviral vector. Previous analyses of such animals had shown them to transmit the proviral vector sequences, integrated as single copy events at many different chromosomal locations, to their F_1 progeny (Robertson *et al.* 1986). These animals offer several attractive advantages. First, in the event that a single contributing XY ES cell carries a mutation affecting testis determination, the germ cell descendants of this cell are placed in the correct gonadal setting of the testis and can contribute to the functional sperm. It should therefore be possible to screen for mutations affecting testis determination simply by looking amongst the offspring of the chimeras for the presence of XY females. Second, the proviral vector sequences carried in the ES cells act as a non-invasive constitutive marking system and allow the component cells of the germ line to be distinguished. So, by correlating the mutation with a particular segregating pattern of insertions, it is possible to ascertain which of the set of offspring arose from the mutated ES cell. A final advantage is that as the proviral sequences may insert and disrupt endogenous cellular genes, any mutations detected in the progeny may be attributable to the integration of a single specific proviral sequence and thus may allow the cloning and identification of the mutated gene.

Our primary screening identified a single founder germ line chimera, which sired phenotypically female F_1 progeny lacking paternally inherited X-chromosome markers. These females were shown to have a karyotypically normal XY-chromosome complement. We report here our initial characterisation of the mutation, which clearly maps to the testis-determining region of the Y chromosome.

Materials and methods

Mouse strains

The generation of chimeric mice from retrovirally infected ES cells has been described elsewhere (Robertson *et al.* 1986). For the initial screen, germ line chimeric males were mated either with females of the CA strain, an outbred MF1-derived line homozygous for the *Pgk-1*^{a} allele, females heterozygous for the *blotchy* mutation (from a C3H-based random bred colony) or females of the inbred 129/Sv//Ev strain. Male mice carrying the RIII strain Y-del chromosome, on an outbred MF1 background, were kindly provided by Dr Paul Burgoyne. These are referred to in the text as 'small y'. X/Y Sxr and X/Y Sxr' mice were from stocks also maintained at the MRC Mammalian Development Unit.

For PGK isozyme assays, peripheral blood samples were diluted approx 1:1 in heparinised (50 μg ml^{-1}) PBS, and stored frozen if required. Samples were electrophoresed on cellulose acetate plates and PGK activity scored according to the protocol described by Bücher *et al.* (1980).

Chromosome analysis

Adult animals were karyotyped from PHA-stimulated peripheral blood lymphocyte cultures, according to standard protocols, or from spleen biopsies. For the latter, small pieces of freshly collected spleen tissue were minced in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10 % newborn calf serum and 0.02 μg ml^{-1} colcemid, incubated at 37°C for 30 min and mitotic spreads prepared by standard methods. Newborn and juvenile animals were analyzed from primary fibroblast cultures obtained from tail tip or ear tissue biopsies. Briefly, the tissues were minced in DMEM, supplemented with 10 % newborn calf serum (selected batches) and antibiotics. Tissue fragments were transferred to tissue culture dishes, immobilised under sterile glass coverslips and allowed to proliferate for 72 h. After this time, colcemid was added to the cultures (0.02 μg ml^{-1}: 60 min incubation). The cells were collected following trypsinization and mitotic spreads prepared as normal. Chromosome preparations were either stained directly with Giemsa or G-banded using conventional protocols (e.g. see Robertson, 1987). For karyotyping, representative spreads were photographed and karyograms prepared according to standard procedures.

Analysis of ovarian tissues

Ovaries and associated reproductive tracts were dissected from freshly killed females, washed in saline and fixed in Bouins fixative. The tissues were dehydrated and embedded in paraffin wax using conventional protocols. 6 μm sections were collected, hydrated and stained with haematoxylin and eosin.

DNA probes

The 2(8) probe was provided by Dr K. W. Jones and is a 545 bp *Pst*I–*Bam*HI fragment of a *D. melanogaster* Bkm-related sequence, subcloned in M13 mp9 (Singh *et al.* 1984). The pY353/B (Bishop *et al.* 1985) and pSx1 (Roberts *et al.* 1988) clones were both provided by Dr C. E. Bishop. These contain 1.5 kb and 1.8 kb *Eco*RI inserts in puc9 and bluescript, respectively. Isolated fragments were used as probes. The *neo* probe was the 1.8 kb *Hin*dIII–*Eco*RI fragment from

pSV2*neo*. The LTR probe was the 600 bp *Rsa*I fragment from pMU3 containing the U3 region of the Mo-MuLV LTR (Reik *et al.* 1985).

Southern hybridisations

Southern hybridisation analysis was performed using total genomic DNA prepared by standard protocols (Lovell-Badge, 1987). DNA was digested with restriction endonucleases, under conditions recommended by the manufacturers, fractionated on 0.8 % agarose gels and transferred onto nylon membranes, either GeneScreen plus (DuPont) or Hybond N (Amersham). The 2(8) probe was an M13 single-stranded DNA clone, and was labelled by primer extension (Hu and Messing, 1982). All other probes were labelled by random priming (Feinberg and Vogelstein, 1983). After hybridisation, filters were washed at high stringency (0.1×SSC, 0.1 % SDS at 65°C for 30 min), and exposed to Fuji RX-100 X-ray film for 1–6 days.

Results

Screening for mutations perturbing sex determination

The production of germ line chimeras using ES cells carrying multiple copies of the MPSV.mos^{-1}neo retroviral vector has been described elsewhere (Robertson *et al.* 1986). Briefly, the ES cells (from the CCE cell line) were infected by repeated exposure to viral supernatant until they carried an average of 12 independent proviral integrations per cell. Chimeras were made by injection of 12–15 individually selected ES cells into host blastocysts. For the analysis described here, male germ line chimeras were chosen that transmitted ES cell markers to 100 % of offspring. These are likely to have resulted from phenotypic sex conversion of female host blastocysts to males by virtue of the contribution of the XY ES cells to the somatic portion of the gonad (reviewed Robertson and Bradley, 1986). The lack of offspring from the host component, due to the failure of XX cells to undergo spermatogenesis, simplifies the analysis. A detailed study of the patterns of proviral insertions segregating in the F_1 progeny showed that the germ line of the chimeras was typically mosaic, with 1 to 4 ES cells contributing to the functional germ cell pool. Any screen would therefore test between 12 and 48 random insertions per chimera.

We chose to use a simple genetic screen specifically designed to detect XY phenotypically female offspring. This involved mating the chimeras to females carrying distinct X-chromosome markers. Two systems were used. In the first of these, founder males were mated to females homozygous for the X-linked *Pgk*-1^a allele, and F_1 females were genotyped by a simple cellulose acetate electrophoretic assay of a blood sample. The CCE ES cell line is derived from a 129 inbred line and carries the *Pgk*-1^b allele, so normal XX female progeny should type as PGK-1A/B, while any anomalous females will be PGK-1A only. The second test system used females carrying the X-linked coat colour marker *blotchy* (*Mo*^{blo}, an allele at the Mottled locus). The *blotchy* allele produces a characteristic lightening of the coat hairs in hemizygous males or homozygous females, and a distinctive mottling in the coat of heterozygotes. For this screen, we used heterozygous females (*blo*/+) as homozygotes (*blo*/*blo*) showed reduced fertility and viability. From the matings with chimeras, normal XX female offspring would be either wild type or *blo*/+ while anomalous females would be either of a *blotchy* or wild-type phenotype (in a 1:1 ratio).

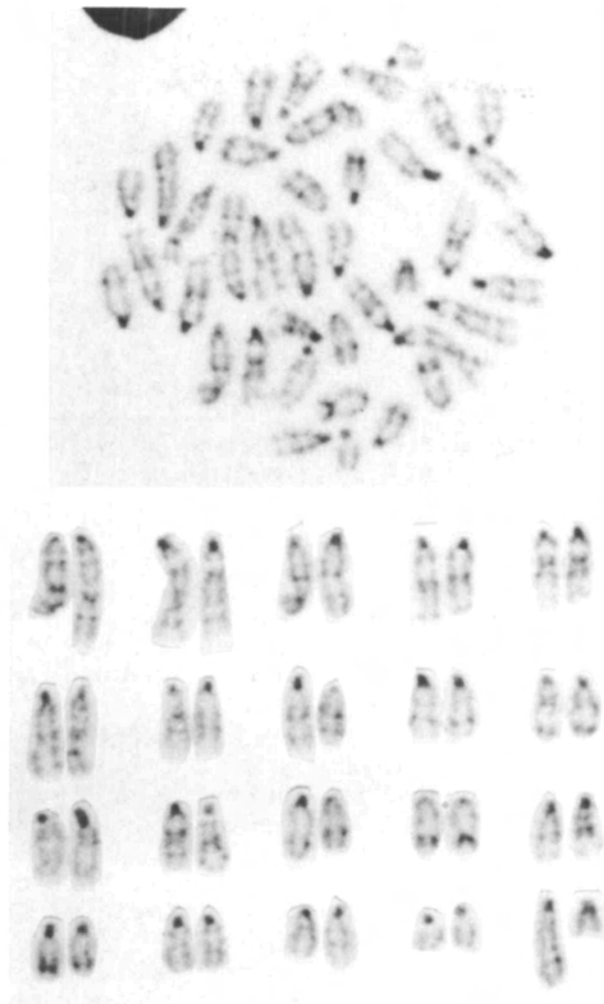


Fig. 1. Karyogram prepared from female L24. Mitotic spreads were prepared from blood cultures and analyzed by G-banding. The lower part of the figure shows the chromosomes arranged in a standard manner, with chromosome 1 at top left and chromosomes X and Y at bottom right. All spreads that could be analyzed carried a morphologically normal Y chromosome, often clearly showing the small short arm.

In the initial screen, three independent germ line chimeras were mated to *Pgk*-1$^{a/a}$ females. From the chimera designated male AL 430, two out of the six female progeny in the first litter lacked the paternally derived *Pgk*-1^b allele. In view of this result, male AL 430 was mated to successive females and a large number of progeny tested by either the PGK or coat colour assay. These data are summarized in Table 1, and demonstrate that male AL 430 sired female F_1 progeny of an inappropriate phenotype at a frequency of about 3–4 %. The offspring from three further germ line

Table 1. *Breeding data from male AL430*

Progeny recorded				Number and genotypes of progeny analysed					
					♀			♂	
(1) AL430×*Pgk*-1[a/a] ♀									
Litters	No.	♀	♂	*Pgk*-1:	a/b	a		a	
12	108	64	44		51	8*		35	
(2) AL430×*Mo*[blo]/+♀									
Litters	No.	♀	♂	*blo*/+	+/+	*blo*	+	*blo*	+
18	175	101	74	47	44	5*	1#	36	38
(3) AL430×129/Sv/Ev ♀									
Litters	No.	♀	♂	Y DNA:	−ve	+ve		−ve	+ve
nr	73	44	29		28	1		0	15

Total offspring: 356 (209 ♀, 147 ♂)
Total number analysed: 309 (185♀, 124♂)

Number of anomalous females: 15*
Number of XY females: 13

*One female from each of these two screens subsequently proved to lack Y-chromosome sequences.
# Shown retrospectively to carry Y chromosome sequences by Southern blotting. (This is animal L57.)
nr=not recorded.

chimeras were screened subsequently, but AL430 remains the only one to have given anomalous female progeny.

While the screen was designed to detect XY females, there are other explanations for the detection of phenotypic females that lack paternal X-chromosome markers. The simplest explanation is that the animals are XO, arising by meiotic non-disjunction during spermatogenesis. Depending on strain, the spontaneous occurrence of such animals ranges from 0.2 to 1 % of females (Russell, 1976). However, there are a number of other alternatives such as (1) XY:XO mosaicism where, by chance, most of the genital ridge was derived from the XO component, (2) a mutation causing non-random X-inactivation, or (3) more specific mutations affecting *Pgk* or *blotchy* gene expression.

To establish that these anomalous animals were indeed XY females, a detailed karyotypic analysis was performed on four of them as well as on a few phenotypically normal male and female sibs. Metaphase chromosome spreads were prepared from PHA-stimulated peripheral lymphocyte cultures, from cells from spleen biopsies or from primary fibroblast cultures obtained from tail and ear tissue biopsies. The four candidate females all proved to have 40 chromosomes in all metaphase spreads scored (minimum of 15), and complementary G-banding analysis verified an XY chromosome constitution. A representative karyogram from animal L24 is shown in Fig. 1. The Y chromosome appears morphologically normal, including the small short arm where recent *in situ* hybridisation and genetic studies have placed the testis-determining region (Roberts *et al.* 1988; McLaren *et al.* 1988). Control males and female sibs had normal XY and XX karyotypes, respectively.

Animals L24 and L12, both from the blotchy screen, were autopsied at 4 and 9 weeks of age, respectively. At a gross morphological level, both animals had normal ovaries and associated female reproductive tracts. There was no overt hermaphroditism. The ovaries were fixed and processed for histological analysis. Representative sections from the ovaries of both XY females and from a control sib of about 9 weeks of age are shown in Fig. 2. The ovaries from L24 appeared qualitatively normal, although there were fewer oocytes than usual for this stage (Paul Burgoyne, personal communication) and some atretic follicles. The ovaries from L12 had many corpora lutea, and atretic follicles were common. Some apparently normal oocytes were present but substantially fewer than in the control sib.

The finding that the anomalous F_1 females were chromosomally XY allowed us to use Y-chromosome-specific probes to screen additional, phenotypically wild-type, female progeny from the matings of male AL430 to blotchy heterozygotes, and to perform a retrospective analysis of material obtained from previous matings of this male with genetically unmarked 129 females. Genomic DNA samples were analysed by Southern analysis with two probes. The clone pY353/B was isolated from a Y-chromosome-enriched mouse genomic DNA library (Bishop *et al.* 1985) and detects a moderately repetitive sequence present mainly on the long arm of the Y. The probe 2(8) hybridizes in a male-specific manner to GATA/GACA repeat sequences, equivalent to the Bkm satellite (Singh *et al.* 1984), present at high copy number in the Sxr translocation, and thus present in the Y-chromosome short arm. This analysis detected a further 2 XY females and enabled us to confirm the presence of Y sequences in 11 of the initial panel of 13 candidate XY females. Fig. 3 shows the Southern analysis of a sample of F_1 progeny.

In summary, a total of 13 XY females were obtained from 185 phenotypic female progeny that we could test. These results are given in Table 1. Two further candidate females were eliminated from further analysis as they lacked Y-chromosomal sequences. One of these karyotyped as being XO while the other may have been an XO/XX mosaic as she gave both PGK-1A and PGK-

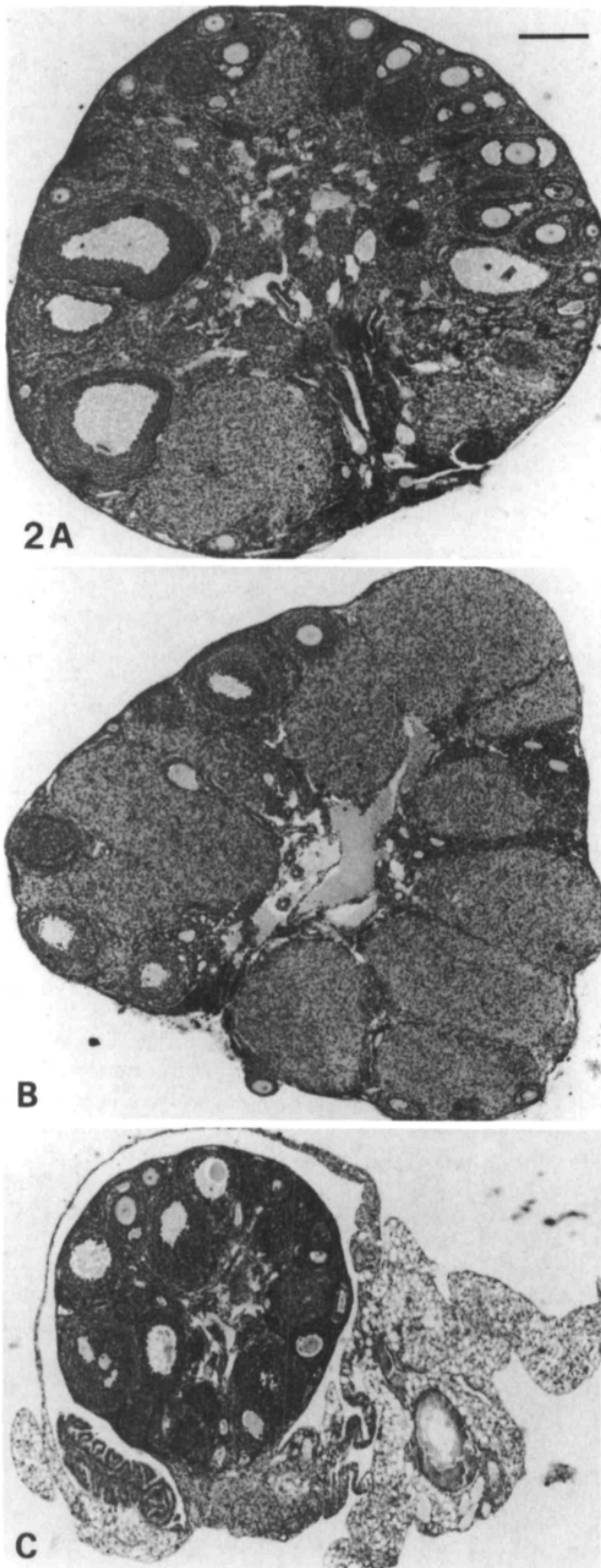


Fig. 2. Histological analysis of ovaries from XY females and XX female littermates. (**A**) Normal XX female (9 weeks *post partum* (*pp*.)); (**B**) XY female L12 (9 weeks *pp*.); (**C**) XY female L24 (4 weeks *pp*.). Bar=250 μm.

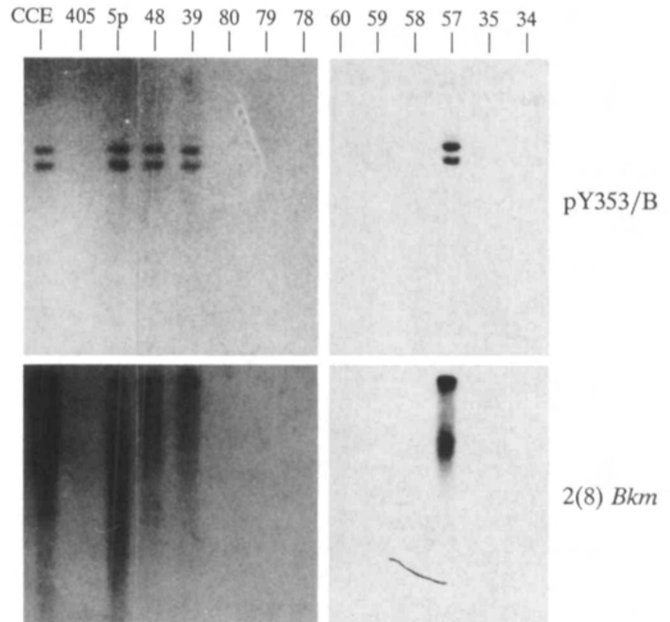


Fig. 3. Southern analysis of DNA samples from phenotypically female progeny from male AL430 using Y-chromosome specific probes. (**A**) *Hin*dIII digested DNA samples probed with the 'long arm' sequence pY353/B. The doublet, at about 9.4 kb, is characteristic of the *M. musculus musculus* Y chromosome. (**B**) *Hae*III digested DNA samples probed with the 2(8) *Bkm* probe. This detects repetitive sequences of high MW from the Y 'short arm'. (The bottom part of the autoradiograph, cut away for simplicity, shows, as expected, hybridisation in all tracks.) CCE: control DNA from the XY ES cell line, CCE. The other samples are all from offspring of AL430. 405 known XX female. 5p (from PGK screen), L48 and L39 (from *blotchy* screen) were candidate XY females. The remainder were all wild-type females from the *blotchy* screen. Animal L57 clearly carried a Y chromosome.

1B male offspring. It may also be noted from Table 1 that the sex ratio of the offspring of AL430 appears to be distorted in favour of females. This would have to stem from AL430 as the distortion is evident with all three types of partner used. However, after taking into account the number of females expected to have been XY (15) amongst the total number born, the proportion of XX compared to XY offspring (194:162) is not statistically significant ($\chi^2_{(1)}=2.9$, $P>0.05$).

Analysis of the proviral inserts transmitted by chimera AL 430

The frequency with which XY females were found amongst the offspring of AL 430 (13/309) suggested that the mutation was carried by a single class of germ cell, which contributes at a low frequency (approx. 8 %) to the functional sperm population. To characterise this further, we examined the segregating patterns of proviral sequences carried in 150 successive F_1 progeny including both male and female sibs. This allowed us to determine the total number of ES cells contributing to the germ line and the relative representation of each germ cell type to the viable sperm. An example of a

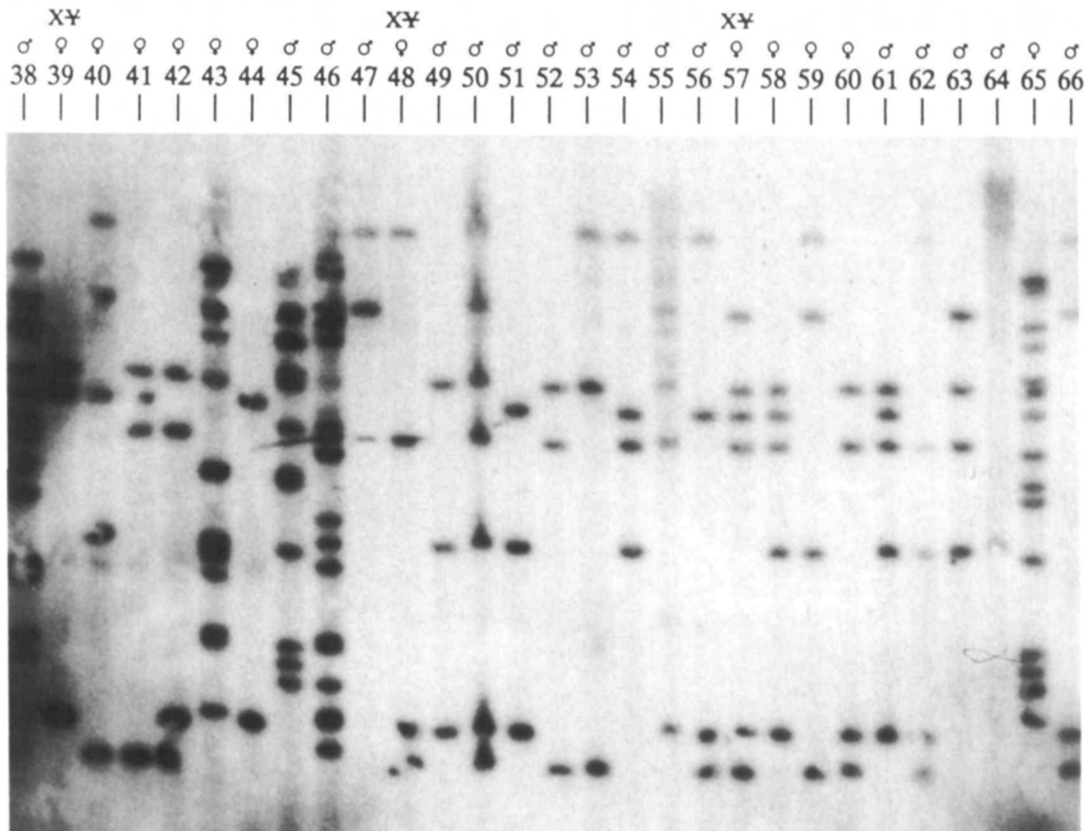


Fig. 4. Analysis of the retroviral inserts carried in a random sample of the progeny derived from male AL430. DNA samples were digested with *Bam*HI and hybridised with a probe from the *neo* gene. As the MPSVmos^{-1}*neo* retroviral vector contains a unique *Bam*HI site, each band represents a single proviral sequence inserted into the genome of the animals. There are clearly two categories of progeny which either carry a high proviral copy number (from type I germ cells, see for example animal numbers 43, 46 and 65) and those which have inherited a subset of a set of 8 proviruses (derived from type II germ cells). While normal males and females are derived from both germ cell types, the class of XY females are derived only from the type II germ cell type (see females 39, 48 and 57).

typical progeny screen, where each band represents a single proviral insertion, is given in Fig. 4. The data clearly show that the germline of male AL430 consisted of derivatives of just two separately infected ES cells. The type I germ cell carried a high copy number of proviral sequences (>20) and progeny derived from this germ cell type constituted approximately 15 % of the live-born progeny. The type II germ cell carried 8 unique proviral insertion sites and derivatives from this germ cell type constituted the remaining 85 % of the progeny.

Analysis of the proviruses carried by the XY females showed them all to be derived from the more common type II germ cells (see lanes corresponding to animals L39, L48 and L57 in Fig. 4). This result was surprising as the low frequency with which XY females appeared in the offspring had led us to believe that the mutation had to be present in an estimated 6–8 % of the viable sperm population. Furthermore, many apparently normal phenotypic males showed the type II pattern of proviruses (see Fig. 4). This could be explained if an insertion had caused a semidominant mutation affecting testis determination only in a minority of XY individuals, or if there was a true autosomal dominant that had a secondary effect on the viability of the sperm such that it was transmitted only at a low frequency. However, there was no obvious correlation between a specific retroviral insertion site (or combination of insertion sites) and the XY female phenotype. This result strongly suggests that the phenotype results from some other type of mutational event. Furthermore, this mutation must have occurred at some point during establishment of the functional germ line of AL 430 as it affected only a subset of germ cells derived from the original type II germ cell progenitor. Further evidence in support of this explanation is presented below.

Transmission of the mutation: the XY females are fertile

The histological appearance of the ovaries of the L12 and L24 XY females suggested that sufficient numbers of normal oocytes may persist after puberty for the animals to be at least transiently fertile. Initially three 6 week old XY females were caged with males of the 129 strain and checked for vaginal plugs on a daily basis. Two of these females, L39 and L48, were *blotchy* hemizygotes, whilst the third, L57, also derived from the *blotchy* screen, was wild type in coat colour and had

been identified by screening with Y DNA probes. L39 and L48 occasionally showed signs of oestrus and plugged intermittently but failed to become noticeably pregnant. However, L57 proved to be fertile. Her first litter of three were found dead shortly after birth. One was mutilated and could not be sexed, the remaining two were female. One of the latter was successfully karyotyped from a primary fibroblast culture and found to be XY. It was therefore clear that the XY female phenotype could be inherited. To determine whether the mutation segregated with the Y chromosome, L57 was subsequently mated to a male from an outbred mouse strain 'small y' (see Materials and methods). Males of this strain have a very small Y chromosome (referred to here with a lower case 'y'), that lacks approximately two-thirds of the long arm but which appears to be functionally normal (Paul Burgoyne, unpublished data). This y can readily be distinguished cytologically from the 129-derived Y chromosome carried by the XY females. L57 had a litter of 5 liveborn pups, which were all successfully karyotyped from tail tip cultures. Of these, two (57.4 and 57.5) were Xy males with the small y from the father, two (57.7 and 57.8) were XY females that inherited the normal sized maternal Y and one (57.6) was an XYy male with a Y chromosome from each parent. This result strongly suggests that the mutation maps to the Y chromosome for two reasons. First, the phenotype segregates with the Y chromosome and secondly, it is complemented by the small y from the father in the XYy male. (This part of the pedigree from L57 is shown in Fig. 5A.)

One additional XY female (P13), identified in the PGK screen, also proved to be fertile and gave rise to XY female offspring carrying the maternal Y. Subsequent breeding of the offspring of both L57 and P13 have shown that the mutation has segregated with the Y chromosome from the founder XY females without exception in well over 200 informative cases (Lovell-Badge and Burgoyne, unpublished data). The Y chromosome carrying the mutation will be referred to subsequently with the symbol Ɏ.

The XɎ females tend to produce very small litters. They also have a limited reproductive lifespan as might be expected from the reduced number of oocytes. Karyotypic analysis revealed that approximately half of the offspring are sex-chromosome aneuploids (XɎy males; XXɎ and XO females). This implies that there is essentially no pairing between the X and Ɏ chromosomes in female meiosis, with the X and Ɏ segregating at random. XXɎ female offspring show apparently normal fertility and reproductive lifespans. When mated to normal males, for example from the 'small y' strain, they give rise to roughly equal proportions of XX females, XXɎ females, Xy males and XɎy males. This again suggests that the Ɏ chromosome remains unpaired and segregates at random in female meiosis. This is likely to be a property of all Y chromosomes in female meiosis (see Eicher and Washburn, 1986). However, it will be necessary to carefully analyse pairing in XɎy males, some of which have also proved to be fertile, before we can rule out an additional mutation on the Ɏ that affects pairing. A more complete analysis of the breeding data from these animals will be presented elsewhere (Burgoyne *et al.* in preparation).

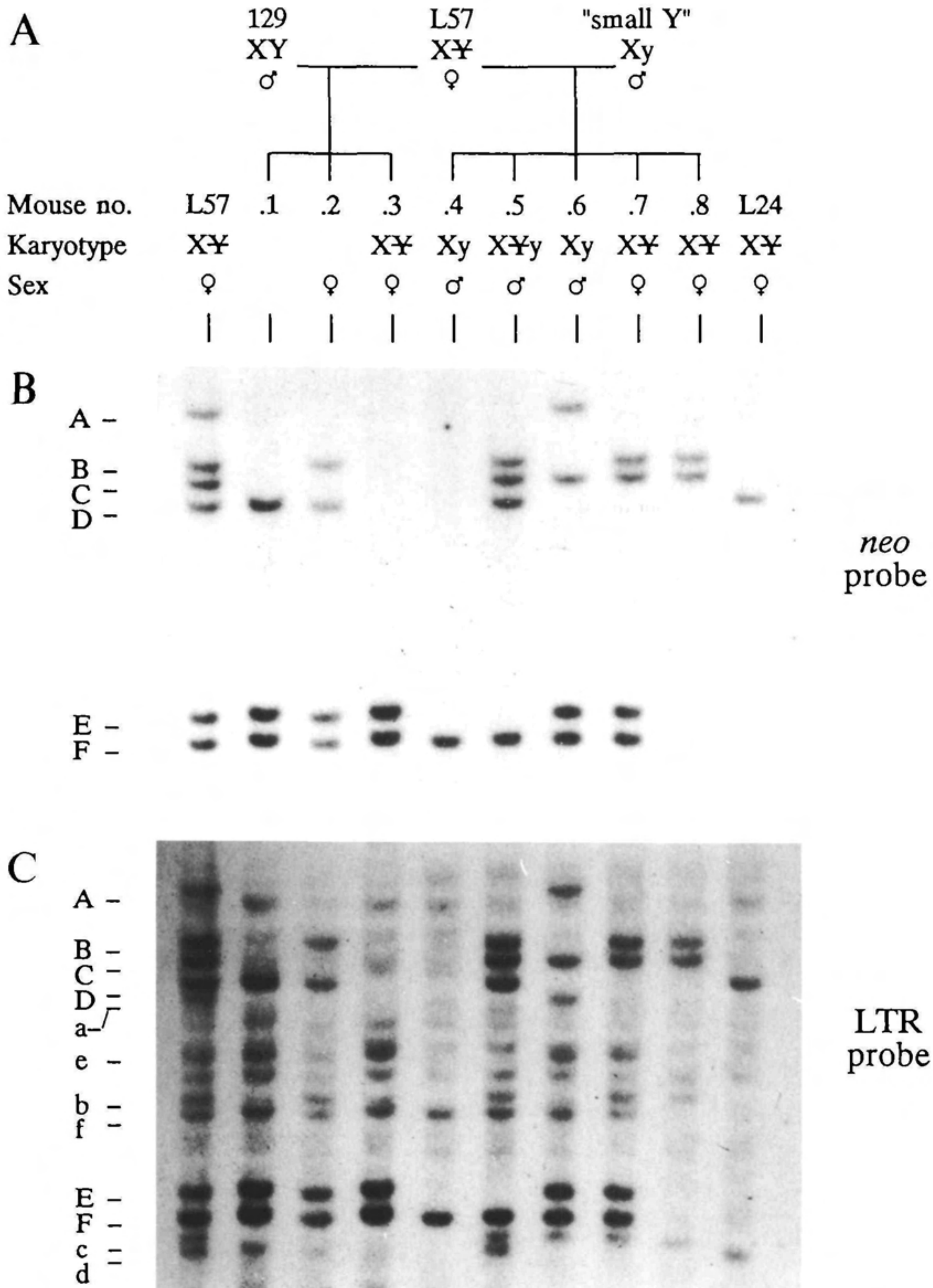
The mutation is not associated with a specific proviral insertion

The availability of F_2 progeny allowed us to readdress the issue as to whether inheritance of the mutation is associated with a specific proviral insertion site. The results of the Southern analysis of genomic DNA samples from the mother (female L57) and her progeny are presented in Fig. 5. Female L57 carried six proviral insertion sites; however, none of the insertions segregated with the mutant phenotype amongst her offspring. Moreover, it is evident that none of the proviruses are linked either to the Ɏ or the X chromosome. In order to check whether a provirus with deleted internal sequences may be responsible for the mutation, the same filter was rehybridised with a probe that detects the LTR region of MPSV. This gave a complex pattern due to the presence of two bands per retroviral insertion and fainter bands corresponding to endogenous proviruses. However, no additional bands could be identified as segregating with the XɎ female phenotype. We have concluded that the mutation is not a direct result of the integration of either an intact or incomplete MPSV vector sequence.

The mutation is complemented by Sxr and Sxr′

The finding that XɎy animals are male shows that the mutation can be complemented in *trans* by the functionally normal y chromosome. We have used a complementation analysis involving Sxr and Sxr′ translocations to map the mutation more precisely. Sxr most likely arose by translocation of the short arm of the Y chromosome onto the pseudoautosomal region of the X or Y (McLaren *et al.* 1988; Roberts *et al.* 1988), and carries genes responsible for at least three male-specific functions namely *Spy*, a gene involved in spermatogenesis (Sutcliffe and Burgoyne, 1989), *Hya*, the gene controlling expression of the minor histocompatibility antigen H-Y (Simpson *et al.* 1981) and *Tdy*. Sxr′ is a variant of Sxr that retains *Tdy* but is deleted for *Hya* and *Spy* as well as for a number of short arm repetitive DNA sequences (McLaren *et al.* 1984; Burgoyne *et al.* 1986; Roberts *et al.* 1988).

XɎ females were mated to either X/Y Sxr or X/Y Sxr′ males. PGK markers were used to follow inheritance of X chromosomes and Southern hybridisation with an Sxr-derived probe pSx1 (Roberts *et al.* 1988) was used to follow inheritance of Sxr or Sxr′. As the results are similar for both, we only present data on Sxr′ as this is the minimal fragment. The essentially random segregation of the sex chromosomes from the mother and of Sxr′ from the father (due to obligatory crossover in the pseudoautosomal region (Evans *et al.* 1982)), means that there are 16 possible combinations of gametes. These should be in equal proportions, although four are non-viable products. The genotypes of the progeny expected from the cross are presented



in Table 2. Given completely random segregation, we would predict that 2 out of 8 males should be PGK-1^b only. Half of these should be X Sxr′/O which are sterile. The other half should be X Sxr′/Y̶ if the mutation has been complemented. Of 24 males screened, 7 were found to be PGK-1^b. Three of these gave a pattern with the pSx1 probe consistent with them being X Sxr′/Y̶. All three of these have proved to be fertile and have given XY̶ females amongst their offspring. We conclude that the mutation has been fully complemented. This places the mutation within the region of the Y chromosome delineated by Sxr′ and which is the minimal portion known to contain *Tdy*. Due to the phenotype and deduced location of the mutation, we can conclude that it has occurred in *Tdy*. In agreement with current terminology, we shall therefore refer to the mutated gene as Tdy^{m1}. The Y chromosome carrying the mutation should be referred

Fig. 5. Analysis of the retroviral inserts transmitted by female L57. (**A**) Progeny from the first two litters from female L57. Analysis of the three pups from her first litter is incomplete as they were found dead shortly after birth. Animal L57.1 contained Y-chromosome DNA sequences (by Southern blot analysis), but no karyotype or phenotypic examination of the gonads was possible. Animal L57.2 was phenotypically female on autopsy and carried Y-chromosome DNA sequences. As it was not possible to obtain a karyotype, this animal is presumed to have been either X¥ or XX¥ (i.e. with the maternal ¥). Her second litter resulted from a mating with a male carrying the R3 Y-del chromosome from the 'small Y' strain (designated 'y'). All were successfully sexed and karyotyped. Female offspring L57.7 and 8 were found to be karyotypically XY and to have inherited the normal sized maternal ¥-chromosome. (**B**) DNA samples from L57 and from all eight progeny were digested with *Bam*HI and probed for *neo* sequences. Also included on this blot is a DNA sample from X¥ female L24. As it had been determined in an earlier analysis that this animal carried a single insertion site, it was of interest to establish whether this insertion segregated with the X¥ female phenotype. There is no correlation apparent between the phenotype and a particular insertion site or combination of insertions, in any of the animals. (**C**) The filter was stripped and rehybridized with a probe that detects the LTR region of the MPSVmos$^-$*neo* vector. Under these conditions of digestion, the LTR probe hybridizes to two unique fragments from each provirus. One of these corresponds to the *neo* fragment detected in panel **B** (indicated by capital letters) while the other represents the additional junction fragment (indicated by lower case letters). This analysis did not reveal any new MPSV-derived insertions. The large and variable number of faintly hybridising bands are due to cross hybridization with endogenous proviruses in the genomes of these mice.

to as $Y^{Tdy.ml}$, however, as this designation is a little cumbersome, we will continue to use the symbol ¥.

Discussion

Sex determination in mammals may involve a number of genes. The most primary of these, *Tdy*, is believed to act within the supporting cell precursors of the genital ridge and to trigger their differentiation along the Sertoli cell pathway (Burgoyne *et al.* 1988). Subsequent differentiation and organization of the testes is thought to depend on one or more key products made by these 'pre-Sertoli' cells. These are perhaps directly regulated by the primary testis-determining gene and may be on chromosomes other than the Y. For example, anti-Mullerian hormone (AMH) may play a key role in testis differentiation apart from its established function in the elimination of Mullerian ducts (Vigier *et al.* 1987).

We report here the generation and establishment of a novel mutation in mice, which causes complete sex reversal in the context of a chromosomally male (XY) genetic background. An extensive pedigree analysis using marked sex chromosomes has shown that the mutation segregates with the Y chromosome, such that mice carrying the mutant Y (given the symbol ¥), and which are either X¥ or XX¥, develop as morphologically normal females. Through the use of appropriate breeding strategies, we have shown that the mutation maps to the testis-determining region of the Y as it is complemented by both the Sxr and Sxr′ translocations. This mutation must, by virtue of its location and phenotype, have occurred at the *Tdy* locus. This is the first case of sex reversal in mammals proven to be due to a mutation in *Tdy*.

Origin of the mutation

Retrovirally infected ES cells have previously been used to introduce mutations into the mouse germline. In these cases, the mutations were found either by selection *in vitro* (Kuehn *et al.* 1987) or by looking for any phenotypic effect associated with the inheritance of particular proviruses (Robertson *et al.* 1986; and unpublished results). We set out here specifically to detect mutations affecting genes involved in the testis-determination pathway by combining this insertional mutagenesis strategy with a simple genetic screen. We were surprised to find a desired mutation after screening relatively few insertions (approximately 180 independent insertion sites derived from six germ line chimeras). However, after an extensive analysis, we were unable to show any association between vector-derived sequences and the X¥ female phenotype.

Moreover, as there were clearly two populations of germ cells that shared the same 8 insertions, but only

Table 2. *Predicted genotypes of offspring from matings of X¥ females with X/Y Sxr′ males (X^a¥♀×X^b/Y Sxr′ ♂)*

Genotype	Sex	PGK	Fertility	Genotype	Sex	PGK	Fertility
X^aX^b	♀	A/B	+	X^aX^b¥	♀	A/B	+
X^a/Y Sxr′	♂	A	+	X^a¥/Y Sxr′	♂	A	(+)
X^a/X^b Sxr′	♂	A/B	–	X^a/X^b Sxr′/¥	♂*	A/B	–
X^aY	♂	A	+	X^a¥Y	♂	A	(+)
X^b¥	♀	B	(+)	X^b0	♀	B	+
¥/Y Sxr′	–	–	–	0/Y Sxr′	–	–	–
X^b Sxr′/¥	♂*	B	+	X^b Sxr′/0	♂	B	–
¥Y	–	–	–	0Y	–	–	–

*These animals would only be ♂ if the mutation was complemented by Sxr′.
a and b refer to the *Pgk*-1 allele.
(+)=Reduced fertility.

one of which carried the mutation, we can conclude that the mutation arose subsequent to the introduction of the ES cells into the host blastocyst (see Fig. 4). It is not feasible to make any inferences from the relative proportions of the three different types of germ cells we see as to the likely point at which the mutation arose because of the possibility of 'founder effects'. The mutation could, therefore, have occurred at any time during the establishment of the germ line, from inner cell mass to spermatogonia. However, as the X¥ females were produced intermittently throughout the life of AL430, the mutation is unlikely to have occurred during later stages of spermatogenesis.

There are two alternative explanations for the origin of the mutation, either (i) a purely spontaneous mutation event, or (ii) an event linked in some way to the retroviral infection strategy. If it is the latter, one possibility is that some other sequence was packaged in the MPSV psi2 cell line, introduced into the ES cell and, through late integration, was carried by only a subset of the descendants of this cell. Alternatively, a high level of reverse transcriptase activity within the ES cells or their derivatives (due in some way to the multiple infection protocol) has allowed retroposition of an endogenous transcript. There has been at least one reported case where, instead of the expected retroviral insertion, an Intracisternal-A particle (IAP) gave rise to a mutation (Stocking *et al.* 1988), and sequences of this sort may be the most likely candidate for the 'mutagen'. IAPs are expressed at high levels in EC cells and early embryos (Morgan *et al.* 1988) and so it is possible that many transcripts would have been around at the time when we think the mutation occurred. However, as there are many thousands of copies of IAPs in the mouse genome, associating a particular one with the mutation would not be trivial. In any case, we have no evidence that the mutation is due to an insertion. The full nature of the mutation will have to wait until *Tdy* has been identified by an alternative route.

Phenotypic effects of the mutation

Other mutations have been described that affect sex determination to give XY female phenotypes. These include mutations in the woodlemming (Fredga, 1988) and horse (Kent *et al.* 1986), which clearly result from mutations at X-chromosome loci. These other loci may represent secondary testis-determining or differentiation genes. The presence of additional secondary genes in the mouse has also been inferred by the study of genetic background effects on certain *domesticus* Y chromosomes (Eicher and Washburn, 1986; Eicher, 1988; Biddle and Nishioka, 1988; Taketo-Hosotani *et al.* 1989). For example, when the Y chromosome from the *poschiavinus* wild mouse strain is crossed into the C57BL/6 background many of the XY progeny develop as females. However, what is striking about this latter set of cases is that they often involve incomplete sex reversal, which results in a range of intermediate hermaphrodite phenotypes. In fact, when fetal stages are examined, the majority of XY gonads show signs of both ovarian and testicular development. The effect is thought to be due either to the combination of a late-acting testis-determining gene on the Y^{pos} chromosome together with early ovarian determination in the C57BL/6 strain (Burgoyne, 1988), or to a failure of interaction between the Y^{pos} *Tdy* and a secondary, autosomal, testis-determining gene in C57BL/6 (Eicher and Washburn, 1986). The mutation described in this report, which segregates with the Y chromosome, results in the differentiation of a normal functional female reproductive system in chromosomally XY individuals. We have looked at the development of the genital ridges from 11.5 days p.c. onwards in X¥ embryos and have no evidence for hermaphroditism or other perturbations at any stage (Gubbay *et al.* 1990; and data not shown).

We have not seen any effects of the Tdy^{m1} mutation apart from that on primary testis determination. Also, at this stage, it is not clear what effect other Y-linked genes may have on female development. However, the X¥ females, and their sex-chromosome aneuploid offspring, will be important in attempts to characterise these genes.

Fertility in the X¥ females

Two of the original X¥ female offspring of chimera AL430 and many X¥ females in subsequent generations have been fertile, although they have small infrequent litters and short reproductive lifespans. Apart from the special case seen with woodlemmings (see below), there have been very few reports of fertile XY female mammals, and it would seem that they are very much the exception. For example, Sharp *et al.* (1980) describe an XY female horse that had one XX female offspring, and an XY^{pos} female mouse that had one litter is mentioned by Eicher and Washburn (1986). It would seem that the infertility is due to a severe reduction in the number of viable oocytes which may be evident from birth (Eicher and Washburn, 1986; Taketo-Hosotani *et al.* 1989). The reasons for oocyte loss are poorly understood, but may be related to the presence of unpaired chromosomes as has been proposed by Miklos (1974) and by Burgoyne and Baker (1984). In support of this idea, we find that there is a very high level of non-disjunction of the X and ¥ apparent amongst the offspring of the X¥ females. This poses the question of why our mice still remain fertile while XY^{pos} females tend not to be? The explanation could simply be the difference in genetic background. XY^{pos} females occur only on a C57BL/6 background, whereas the X¥ females were mostly on mixed backgrounds. Also, out of the five founder X¥ females set up to mate, three were infertile and were blotchy hemizygotes, whereas the two that had offspring were wild type. This would indicate that at least specific genetic background effects can be important. However, an alternative explanation is that the testicular development often apparent in XY^{pos} fetal stages could have an adverse effect on oocyte survival. The XY^{DOM} females of Taketo-Hosotani *et al.* (1989), which had very few

oocytes, often had testis cords remaining in their gonads until well after birth.

All XX¥ females tested have shown normal fertility, with the ¥ apparently segregating at random. It would seem that the unpaired ¥ is relatively unimportant for oocyte survival in this case. These animals may be useful to study the reason why the X and Y fail to pair in female meiosis. The only other case of fertile XY female mammals described is that of X*Y female woodlemmings. However, in these animals their fertility is due to a unique mitotic non-disjunction mechanism that results in all surviving germ cells being X*X* (see Fredga, 1988).

Characterization of the mutant Y chromosome

Karyotypic analysis of the founder X¥ females and their offspring showed that the ¥-chromosome was unaffected at the gross morphological level, with the short arm region remaining cytologically visible. Southern analysis using the 2(8) Bkm probe, which hybridizes to repetitive elements on the short arm (Singh *et al.* 1984), did not reveal any difference in the hybridisation patterns between the X¥ females and normal male siblings. Similarly, the pSx1 probe, which also detects short arm sequences (Roberts *et al.* 1988), and which was used to monitor inheritance of Sxr and Sxr′, failed to reveal differences (data not shown). This would suggest that there was no large deletion or rearrangement affecting *Tdy* expression. We also believe that the ¥ chromosome is completely normal in terms of expression of other Y-linked genes. Thus the spermatogenesis gene *Spy* (Burgoyne *et al.* 1986; Sutcliffe and Burgoyne, 1989) must be active as X Sxr′/¥ males are fertile (data not shown) and the X¥ females are positive for the H-Y transplantation antigen (E. Simpson, unpublished data).

The mutant Y chromosome and candidates for the testis-determining gene

To gain a proper understanding of the process of testis determination in mammals, it is essential to characterise *Tdy* at the molecular level. A number of predictions can be made based on our current knowledge of the gene that any candidate sequence would have to satisfy. The gene is likely to be conserved at least in all mammals shown to have a Y chromosomal sex-determining mechanism, and the candidate sequence must map to the minimal portion of the Y able to confer maleness on an otherwise chromosomally female background, such as the Sxr′ fragment in mice. Furthermore, from the results of Burgoyne *et al.* (1988), we would predict that the gene should be active within the Sertoli cell precursors just prior to their differentiation and aggregation into testis cords (about 11.5 days *post coitum* in the mouse), and to have a structure consistent with a cell autonomous mode of action. Even if a candidate fulfills all of these criteria, they only provide indirect evidence for a role in sex determination. Direct proof of such a role relies on either transgenic or mutation studies. Having described in this paper a mutation known genetically to be within *Tdy*, we can now make a further strong prediction, namely that there should be a molecular basis for this mutation, either at the level of structure or expression of the candidate gene, within the X¥ females.

In the accompanying paper (Gubbay *et al.* 1990), we have taken this latter approach to investigate whether the candidate testis-determining genes *Zfy-1* and *Zfy-2* (Page *et al.* 1987; Mardon *et al.* 1989; Nagamine *et al.* 1989) are altered in mice carrying Tdy^{m1}.

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References

AFFARA, N. A., FERGUSON-SMITH, M. A., MAGENIS, R. E., TOLMIE, J. L., BOYD, E., COOKE, A., JAMIESON, D., KWOK, K., MITCHELL, M. AND SNADDEN, L. (1987). Mapping the testis determinants by an analysis of Y specific sequences in males with apparent XX and XO karyotypes and females with XY karyotypes. *Nucl. Acids Res.* **15**, 7325–7342.

BAKER, B. S. (1989). Sex in flies: the splice of life. *Nature* **340**, 521–524.

BIDDLE, F. G. AND NISHIOKA, Y. (1988). Assays of testis development in the mouse distinguish three classes of *domesticus*-type Y chromosome. *Genome* **30**, 870–878.

BISHOP, C. E., BOURSOT, P., BARON, B., BONHOMME, F. AND HATAT, D. (1985). Most classical *Mus musculus domesticus* laboratory mouse strains carry a *Mus musculus musculus* Y chromosome. *Nature* **315**, 70–72.

BÜCHER, T., BENDER, W., FUNDELE, R., HOFNER, H. AND LINKE, I. (1980). Quantitative evaluation of electrophoretic allo- and isozyme patterns. *FEBS Lett.* **115**, 319–324.

BURGOYNE, P. S. (1988). Role of mammalian Y chromosome in sex determination. *Phil. Trans. R. Soc. Lond.* B **322**, 63–72.

BURGOYNE, P. S. AND BAKER, T. G. (1984). Meiotic pairing and gametogenic failure. In *Controlling events in Meiosis, 38th Symposium of the Society for Experimental Biology* (ed. C. W. Evans and H. G. Dickinson), pp. 349–362. Cambridge: The Company of Biologists Ltd.

BURGOYNE, P. S., BUEHR, M., KOOPMAN, P., ROSSANT, J. AND MCLAREN, A. (1988). Cell autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX↔XY chimaeric mouse testes. *Development* **102**, 443–450.

BURGOYNE, P. S., LEVY, E. R. AND MCLAREN, A. (1986). Spermatogenic failure in male mice lacking H-Y antigen. *Nature* **320**, 170–172.

CATTANACH, B. M., POLLARD, C. E. AND HAWKES, S. G. (1971). Sex reversed mice: XX and XO males. *Cytogenetics* **10**, 318–337.

EICHER, E. M. (1988). Autosomal genes involved in mammalian primary sex determination. *Phil. Trans. R. Soc. Lond.* B **322**, 109–118.

EICHER, E. M. AND WASHBURN, L. L. (1986). Genetic control of primary sex determination in mice. *Ann. Rev. Genet.* **20**, 327–360.

EVANS, E. P., BURTENSHAW, M. D. AND CATTANACH, B. M. (1982). Meiotic crossing over between the X and Y chromosomes of male mice carrying the sex-reversing (Sxr) factor. *Nature* **300**, 443–445.

FEINBERG, A. P. AND VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem.* **132**, 6–13.

Ferguson-Smith, M. A., Affara, N. A. and Magenis, R. E. (1987). Ordering of Y specific sequences by deletion mapping and analysis of X-Y interchange males and females. *Development* **101** (Suppl.), 41–50.
Ford, C. E., Jones, K. W., Polani, P. E., de Almeida, J. C. and Briggs, J. H. (1959). A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). *Lancet i*, 711–713.
Fredga, K. (1988). Aberrant chromosomal sex-determining mechanisms in mammals, with special reference to species with XY females. *Phil. Trans. R. Soc. Lond.* B **322**, 83–95.
Gubbay, J., Koopman, P., Collignon, J., Burgoyne, P. and Lovell-Badge, R. (1990). Normal structure and expression of *Zfy* genes in XY female mice mutant in *Tdy*. *Development* **109**, 647–653.
Hodgkin, J. (1987). Sex determination and dosage compensation in *Caenorhabditis elegans*. *Ann. Rev. Genet.* **21**, 133–154.
Hodgkin, J. (1989). Drosophila sex determination: a cascade of regulated splicing. *Cell* **56**, 905–906.
Hu, N. and Messing, J. (1982). The making of strand-specific M13 probes. *Gene* **17**, 271–277.
Jacobs, P. A. and Strong, J. A. (1959). A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature* **183**, 302–303.
Kent, M. G., Schoffner, R. N., Buoen, L. and Weber, A. F. (1986). XY sex-reversal syndrome in the domestic horse. *Cytogenet. Cell Genet.* **42**, 8–18.
Kuehn, M. R., Bradley, A., Robertson, E. J. and Evans, M. J. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326**, 295–298.
Lovell-Badge, R. H. (1987). Introduction of DNA into embryonic stem cells. In *Teratocarcinomas and Embryonic Stem Cells, a Practical Approach*, (ed. E. J. Robertson), pp. 153–182. Oxford: IRL Press.
Mardon, G., Mosher, R., Disteche, C. M., Nishioka, Y., McLaren, A. and Page, D. C. (1989). Duplication, deletion, and polymorphism in the sex-determining region of the mouse Y chromosome. *Science* **243**, 78–80.
McLaren, A. (1988*a*). Sex determination in mammals. *Trends Genet.* **4**, 153–157.
McLaren, A. (1988*b*). Somatic and germ cell sex in mammals. *Phil. Trans. R. Soc. Lond.* B **322**, 3–9.
McLaren, A., Simpson, E., Epplen, J. T., Studer, R., Koopman, P., Evans, E. P. and Burgoyne, P. S. (1988). Location of the genes controlling H-Y antigen expression and testis determination on the mouse Y chromosome. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6442–6445.
McLaren, A., Simpson, E., Tomonari, K., Chandler, P. and Hogg, H. (1984). Male sexual differentiation in mice lacking H-Y antigen. *Nature* **312**, 552–555.
Meyer, B. J. (1988). Primary events in *C. elegans* sex determination and dosage compensation. *Trends Genet.* **4**, 337–342.
Miklos, G. L. G. (1974). Sex chromosome pairing and male fertility. *Cytogenet. Cell Genet.* **13**, 558–577.
Morgan, R. A., Christy, R. J. and Huang, R. C. C. (1988). Murine A type retroviruses promote high levels of gene expression in embryonal carcinoma cells. *Development* **102**, 23–30.
Nagamine, C. M., Chan, K., Kozak, C. A. and Lau, Y.-F. (1989). Chromosome mapping and expression of a putative testis-determining gene in mouse. *Science* **243**, 80–83.
Page, D. C. (1986). Sex reversal: deletion mapping the male-determining function of the human Y chromosome. In *Molecular Biology of* Homo sapiens. *Cold Spring Harbor Symposium on Quantitative Biology*, vol. **51**, pp. 229–235. New York: Cold Spring Harbor.
Page, D. C., Mosher, R., Simpson, E. M., Fisher, E. M. C., Mardon, G., Pollack, J., McGillivray, B., de la Chapelle, A. and Brown, L. G. (1987). The sex-determining region of the human Y chromosome encodes a finger protein. *Cell* **51**, 1091–1104.
Palmer, M. S., Sinclair, A. H., Berta, P., Ellis, N. A., Goodfellow, P. N., Abbas, N. E. and Fellous, M. (1989). Genetic evidence that *ZFY* is not the testis-determining factor. *Nature* **342**, 937–939.
Reik, W., Weiher, H. and Jaenisch, R. (1985). Replication-competent Moloney murine leukemia virus carrying a bacterial suppressor tRNA gene: Selective cloning of proviral and flanking host sequences. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1141–1145.
Roberts, C., Weith, A., Passage, E., Michet, J. L., Mattei, M. G. and Bishop, C. E. (1988). Molecular and cytogenetic evidence for the location of *Tdy* and *Hya* on the mouse Y chromosome short arm. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6646–6649.
Robertson, E. J. (1987). Embryo-derived stem cell lines. In *Teratocarcinomas and Embryonic Stem Cells, a Practical Approach*, (ed. E. J. Robertson), pp. 71–112. Oxford: IRL Press.
Robertson, E. J. and Bradley, A. (1986). Production of permanent cell lines from early embryos and their use in studying developmental problems. In *Experimental Approaches to Mammalian Embryonic Development*, (eds. J. Rossant and R. A. Pedersen), pp. 475–508. Cambridge: Cambridge University Press.
Robertson, E., Bradley, A., Kuehn, M. and Evans, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* **323**, 445–448.
Russell, L. B. (1976). Numerical sex-chromosome anomalies in mammals: their spontaneous occurrence and use in mutagenesis studies. In *Chemical Mutagens. Principals and Methods for their Detection*, vol. **4** (ed. A. Hollaender), pp. 55–91. New York, London: Plenum Press.
Scherer, G., Schempp, W., Baccichetti, C., Lenzini, E., Bricarelli, F. D., Carbone, L. D. L. and Wolf, U. (1989). Duplication of an Xp segment that includes the ZFX locus causes sex inversion in man. *Hum. Genet.* **81**, 291–294.
Sharp, A. J., Wachtel, S. S. and Benirschke, K. (1980). H-Y antigen in a fertile XY female horse. *J. Reprod. Fert.* **58**, 157–160.
Simpson, E., Edwards, P., Wachtel, S., McLaren, A. and Chandler, P. (1981). H-Y antigen in Sxr mice detected by H-2 restricted cytotoxic T cells. *Immunogenetics* **13**, 355–358.
Singh, L. and Jones, K. W. (1982). Sex reversal in the mouse (*Mus musculus*) is caused by a recurrent nonreciprocal crossover involving the X and an aberrant Y chromosome. *Cell* **28**, 205–216.
Singh, L., Phillips, C. and Jones, K. W. (1984). The conserved nucleotide sequences of Bkm, which define Sxr in the mouse are transcribed. *Cell* **36**, 111–120.
Stocking, C., Löliger, C., Kawai, M., Suciu, S., Gough, N. and Ostertag, W. (1988). Identification of genes involved in growth factor autonomy of hematopoietic cells by analysis of factor-independent mutants. *Cell* **53**, 869–879.
Sutcliffe, M. J. and Burgoyne, P. S. (1989). Analysis of the testes of H-Y negative XOSxr^b mice suggests that the spermatogenesis gene (*Spy*) acts during the differentiation of the A spermatogonia. *Development* **107**, 373–380.
Taketo-Hosotani, T., Nishioka, Y., Nagamine, C. M., Villalpando, I. and Merchant-Larios, H. (1989). Development and fertility of ovaries in the B6.Y^{DOM} sex-reversed female mouse. *Development* **107**, 95–105.
Vergnaud, G., Page, D. C., Simmler, M. C., Brown, L., Rouyer, F., Noel, B., Botstein, D., de la Chapelle, A. and Weissenbach, J. (1986). A deletion map of the human Y chromosome based on DNA hybridisation. *Am. J. hum. Genet.* **38**, 109–124.
Vigier, B., Watrin, F., Magre, S., Tran, D. and Josso, N. (1987). Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro*. *Development* **100**, 43–55.
Weissenbach, J., Levilliers, J., Petit, C., Rouyer, F. and Simmler, M.-C. (1987). Normal and abnormal interchanges between the human X and Y chromosomes. *Development* **101** (Suppl.), 67–74.
Welshons, W. J. and Russell, L. B. (1959). The Y chromosome as the bearer of male determining factors in the mouse. *Proc. natn. Acad. Sci. U.S.A.* **45**, 560–566.

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