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Normal structure and expression of *Zfy* genes in XY female mice mutant in *Tdy*

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Summary

***Zfy-1* and *Zfy-2* are candidate genes for *Tdy*, the testis-determining gene in mice. We have analysed these genes in a line of XY female mice that have been shown to be mutated in *Tdy*. We have used Southern blot analysis to show that the *Zfy* genes have not undergone any major structural alterations, and have also demonstrated that both genes are transcribed normally from the mutant Y chromosome (Y̶) in both adult XYY̶ testis and XY̶ female embryonic gonads. The fact that these genes show a normal structure and expression pattern in mice with a Y chromosome known to carry a mutation in *Tdy* and that mutant embryos develop into females despite *Zfy-1* expression, strongly supports other recent evidence that *Zfy* genes are not directly involved in primary testis determination.**

Key words: sex determination, *Tdy*, *Zfy*, zinc finger genes, polymerase chain reaction, gene expression.

Introduction

Sex determination in mammals is dependent upon the action of a Y-linked testis-determining gene termed *TDF* in humans and *Tdy* in mice (Goodfellow and Darling, 1988; McLaren, 1988). The study of XX males arising from abnormal X:Y interchange suggested that the human testis-determining gene was located close to the pseudoautosomal boundary on the Y chromosome (Page *et al.* 1987*a*). One such male carried just 280 kb of Y-unique sequences adjacent to the boundary, and this region, termed interval 1A, seemed to be the minimal region of the Y that must contain *TDF* (Page *et al.* 1987*b*). This region was divided into two roughly equal intervals, 1A1 and 1A2, through the study of an XY female (arising from a Y:22 translocation), who carried 1A1 and most Y sequences distal to 1A2. This led Page *et al.* (1987*b*) to propose that at least part of *TDF* must lie within the region deleted in this female, interval 1A2. Cloning of this interval led to the isolation of a gene shown to be conserved on the Y chromosome of a range of placental mammals. Sequence analysis of genomic clones, and subsequently of cDNAs, indicated that the gene encodes a protein with characteristics of a transcriptional regulator, including a putative acidic activating domain and potentially DNA binding zinc finger region with 13 cysteine/histidine zinc fingers. This gene, termed *ZFY*, therefore satisfied a number of predictions that could be made, regarding the location, conservation and cell autonomous action of the testis-determining gene (Burgoyne *et al.* 1988).

However, theories of the mode of action of *ZFY* in testis determination have to take into account the presence of a highly homologous gene (*ZFX*) present on the X chromosome in humans and other mammals (Page *et al.* 1987*b*). In the mouse, the situation is even more complex as there are four homologues to *ZFY*, two that map to the Y chromosome, termed *Zfy-1* and *Zfy-2*, one that maps close to *Xce* on the X chromosome, *Zfx*, and an autosomal copy, *Zfa*, that maps to chromosome 10 (Mardon *et al.* 1989; Nagamine *et al.* 1989). The two Y-linked genes appear to have arisen by a recent duplication event (Mardon and Page, 1989; Ashworth *et al.* 1989). Both genes map to the region of the Y chromosome defined by the Sxr translocation, but *Zfy-2* is not present in the deleted derivative Sxr′ (Mardon *et al.* 1989). As X/X Sxr′ animals are male, *Zfy-2* is clearly not essential for testis determination making *Zfy-1* the best candidate for *Tdy*. However, it is formally possible that each gene could independently cause testis determination, and the similarity of their cDNA sequences led Mardon and Page (1989) to propose that they may be functionally interchangeable.

A classical approach to prove a link between a candidate DNA sequence and a genetic locus is to look for appropriate differences between normal and mutant alleles. Analysis of a number of human XY females shown not to be deleted for *ZFY* failed to reveal any

alterations in this gene (Schneider-Gädicke *et al.* 1989). However, because human XY females are sterile, it is difficult to prove genetically whether these particular cases were due to a mutation in *TDF* itself or in a downstream gene also necessary for testis determination.

In the accompanying paper (Lovell-Badge and Robertson, 1990), we have described a heritable mutation in the mouse, Tdy^{m1}, that gives rise to females with an apparently normal XY karyotype. This mutation maps to the Y chromosome and can be complemented by the Sxr′ fragment. The phenotypic and genetic evidence defines the mutation as being in *Tdy*. In the present report, we have examined both *Zfy-1* and *Zfy-2* for any evidence of mutation in Tdy^{m1} mice. No evidence of altered structure or regulation was found. Taken together with other recent data, these results rule out a direct role for this gene in testis determination.

Materials and methods

Mouse stocks

CD1 males, which carry the *Mus musculus domesticus* Y chromosome (referred to in the text as Y^d), and X/X Sxr′ males were from stocks maintained at the Mammalian Development Unit. 129/Sv//Ev males and CA females (an outbred MF1 based line homozygous for *Pgk*-1^a) were from stocks maintained at the National Institute for Medical Research.

The origin of the Tdy^{m1} mutation and some of the properties of the mice carrying it are described in the accompanying paper (Lovell-Badge and Robertson, 1990). The Y chromosome carrying the Tdy^{m1} mutation has been given the symbol ¥ and will be referred to as such in this paper. The ¥ chromosome is of 129 origin and is of the *Mus musculus musculus* type, and so, where appropriate, it is referred to as $¥^m$. The ¥ is maintained most readily by breeding XX¥ females, usually to outbred MF1 males carrying the RIII del 'small Y' chromosome (referred to here as a lower case 'y'). These crosses normally give rise to roughly equal proportions of XX females, XX¥ females, X¥y males and Xy males. Karyotypic analysis (usually from tail tip cultures of newborn animals) was routinely used to distinguish offspring. X¥ females used in this study were either founder X¥ females or their offspring (for DNA analysis only), or were obtained from breeding selected X¥y (or X¥Y) males (Burgoyne *et al.* unpublished). X$¥^m$$Y^d$ males were obtained from matings of XX¥ females with CD1 males.

X¥ female embryos from matings of an X¥y male (No.57.9.14.8/17) with CA females were identified at 12.5 days *post coitum* (dpc) by gonad morphology and staining of sex chromatin in amniotic cell nuclei (Monk and McLaren, 1981). Embryonic stage was verified by the morphology of the hind limb (Hogan *et al.* 1986). From the previous breeding record of this particular male, we expected roughly equal proportions of XX and X¥ females, plus a few Xy males (S. Mahadevaiah and P. Burgoyne, unpublished).

DNA analysis: Southern blots

Genomic DNA was isolated from adult spleens as described (Lovell-Badge, 1987). 10 μg samples of DNA from X¥ females and 129 XY males were digested with the appropriate restriction enzymes, electrophoresed on 0.7 % agarose gels in TBE and blotted onto Hybond-N (Amersham) according to the manufacturers instructions. Filters were hybridized to probes that had been labelled with ^{32}P using a Multiprime kit (Amersham), and then washed at high stringency (0.1×SSC, 0.1 % SDS at 65°C for 1 h), and exposed to Fuji RX-100 X-ray film for 3–6 days.

RNA analysis: reverse transcription, polymerase chain reaction (PCR) and sequencing

Total RNA was isolated from testes of adult X$¥^m$$Y^d$ mice by the method of Auffray and Rougeon (1980). 1 μg was reverse transcribed using 200 units of MoMuLV reverse transcriptase (BRL) in 30 μl of the supplied buffer containing 500 ng oligo-dT (Pharmacia) at 42°C for 30 min. A 5 μl aliquot was then added to a 50 μl PCR reaction containing 0.5 % NP-40, 500 ng each of 5′ primer (5′-CCTAT TGCAT GGACT GCAGC TTATC-3′) and 3′ primer (5′-CGTAA AGTTT GTCGA TCAGG AGCAAC-3′), and 2.5 units *Taq* polymerase (Anglian) in the supplied buffer. Primer sequences matched both *Zfy-1* and *Zfy-2* cDNA sequences (Ashworth *et al.* 1989; Mardon *et al.* 1989) except for the single mismatches shown in bold type. These were designed to introduce restriction sites for *Pst*I and *Taq*I, respectively (underlined), to facilitate subsequent cloning. DNA was amplified by 30 cycles of 94°C, 5 s, 65°C, 30 s and 72°C, 30 s in a Techne PHC-2 thermocycler. Electrophoresis of 5 μl of each reaction confirmed the amplification of only PCR products of the predicted size. The remainder of each reaction was extracted with phenol/chloroform, digested with *Pst*I and *Taq*I and subcloned into the *Pst*I and *Acc*I sites of pBluescript (Stratagene), using standard techniques (Maniatis *et al.* 1982). Recombinant plasmid inserts were sequenced using Sequenase (US Biochemicals) and T3 primer.

RNA was extracted from single embryonic gonads using a small-scale adaptation of the AGPC method of Chomczynski and Sacchi (1987), with 20 μg glycogen as carrier. The entire yield was reverse transcribed in a 7.5 μl reaction and amplified as described above, but in this case using *Zfy-1* specific primers (5′-GTTAC TCATT TTCAG GTGTT CTGGG-3′ and 5′-GTGTC AGCTG TTATA GGATC AGTGA-3′) (Koopman *et al.* 1989). The hypoxanthine phosphoribosyltransferase (*Hprt*)-specific primers (5′-CCTGC TGGAT TACAT TAAAG CACTG-3′ and 5′-GTCAA GGGCA TATCC AACAA CAAAC-3′) (Melton *et al.* 1984) were included as a positive control for each sample. 1 μl of the completed PCR reaction was transferred to a new reaction containing only 'nested' primers (5′-TGAAG TCTGC AGTAC TTGTC GTCAT-3′ and 5′-TCACT CATCA AGACA TGTTC AGGCA-3′) which further amplify the *Zfy-1* PCR product. 20 μl of the second reaction was visualised by electrophoresis on a 2 % agarose/TBE/ethidium bromide gel.

Results

DNA analysis

The strategy from which the Tdy^{m1} mutation arose was based on using a retroviral vector as an insertional mutagen. However, as described in the accompanying paper (Lovell-Badge and Robertson, 1990), we could not establish linkage between the mutation and any vector-associated sequences. We, therefore, have no *a priori* knowledge of the type of mutation to expect. On the assumption that the mutation may have created an RFLP, we carried out an extensive Southern analysis of both *Zfy-1* and *Zfy-2* genes.

The four ZFY-related genes in mice can be divided into two pairs according to homologies. Thus cDNAs corresponding to the two Y genes are almost identical in sequence along their entire length (Ashworth *et al.* 1989; Mardon *et al.* 1989). Likewise, *Zfx* and *Zfa* cDNA sequences are almost identical (Ashworth *et al.* 1990). However, there are significant differences between the two pairs. Thus, at the amino acid level, *Zfx* and *Zfy-1* are 79 % homologous in the finger region, and just 55 % homologous in the acidic domain. These close homologies have complicated the analysis of the genomic structure of the four genes. A set of three contiguous probes that together constitute a full-length *Zfy-1* cDNA clone was used on Southern blots of XY male and X¥ female DNA cut with a range of restriction enzymes. In some cases, we have determined the origin of each hybridising band by comparing DNA from XX females, XY males and X/X Sxr′ males on additional Southern gels (not shown), allowing us to distinguish Y and non Y-linked bands and further between *Zfy-1* and *Zfy-2* bands (the latter being absent in X/X Sxr′). Fig. 1A shows the results of the comparison between XY male and X¥ female DNA. No differences have been detected with any of the probes covering the entire cDNA. The derivation of the probes used and their relation to Zfy-1 genomic fragments is shown in Fig. 1B. This analysis gives a minimum size for *Zfy-1* of about 26 kb.

RNA analysis

It is conceivable that the mutation is not within the coding region but affects instead the expression of the gene. We therefore wished to look for *Zfy* transcripts from the mutant ¥. *Zfy* expression has been detected in adult but not fetal testis by Northerns (Mardon and Page, 1989; Koopman *et al.* 1989). cDNA cloning and PCR data indicate that both *Zfy-1* and *Zfy-2* genes are expressed in adult testis (Ashworth *et al.* 1989; Mardon and Page, 1989; Nagamine *et al.* 1989). More recent data, obtained using the PCR technique, demonstrated a low level of *Zfy-1* but not *Zfy-2* expression in male genital ridges and fetal testes (Koopman *et al.* 1989). To establish whether the *Zfy* genes on the mutant ¥ were being correctly regulated, it was therefore necessary to look at both adult and fetal gonads.

Expression in adult testis

To test whether transcription of the genes could occur from the ¥ in adult testis, it was clearly necessary to obtain male mice carrying this chromosome. We have described in the accompanying paper that it is possible to obtain complementation of Tdy^{m1} with a normal Y chromosome in X¥Y males. These occur spontaneously amongst the offspring of X¥ females through non-disjunction of the X and ¥, but are more readily obtained by breeding XX¥ females with normal XY males, where 25 % of offspring will be X¥Y. However, this had to be done in such a way that the products of the *Zfy* genes on the mutant ¥ could be distinguished from those on the normal Y chromosome in the X¥Y males.

Comparison of sequences obtained from a partial *Zfy-1* genomic clone, c21 (our unpublished data) with the published sequences of *Zfy-1* and *Zfy-2* cDNAs (Ashworth *et al.* 1989; Mardon and Page, 1989) revealed a number of nucleotide differences. Both published sequences were derived from mice carrying the Y chromosome of the *Mus musculus domesticus* type (referred to here as Y^d), whereas the c21 clone was isolated from a genomic library made with DNA of the 129 mouse strain which carries the *Mus musculus musculus* type Y (Y^m). As the mutant ¥ is of *musculus* origin, this suggested that there may be sufficient differences between it and a *domesticus* type Y to allow all the various *Zfy* transcripts to be distinguished. XX$¥^m$ mice were therefore mated with males of the CD1 strain, which are known to carry Y^d, in order to generate the required X$¥^m$$Y^d$ offspring.

Karyotypes were determined from tail tip cultures to identify the X$¥^m$$Y^d$ sons and Southern blot analysis was used to confirm the presence of both types of Y chromosome by taking advantage of *Zfy-1* and *Zfy-2* *Taq*1 RFLPs (Mardon *et al.* 1989). Fig. 2 shows the distinct patterns derived from Y^m and Y^d and the combined pattern seen in DNA from mice carrying both chromosomes.

A reverse transcriptase-PCR (RT-PCR) strategy was used to amplify a specific region of the *Zfy* gene transcripts known to contain sequence polymorphisms between Y^m and Y^d. Two oligonucleotide primers were designed that fulfilled the following criteria: (i) both recognised identical sequences in *Zfy-1* and *Zfy-2*, according to available sequence data (Mardon and Page, 1989; Ashworth *et al.* 1989 and our unpublished results); (ii) both were located in exons adjacent to the exon to be amplified (J.C. unpublished results). This was to ensure that amplification of contaminating genomic DNA would either not occur, or would yield PCR products of a different size to the predicted band; (iii) both oligonucleotides contained single mismatches to the known sequences, generating convenient restriction sites for subsequent cloning.

RT-PCR gave rise to a single band of the expected size, which was digested with *Pst*I and *Taq*I and subcloned into a bluescript vector (see Material and Methods). 47 subclones were sequenced and all could be assigned to one of four types, shown in Fig. 3. These corresponded to the sequences of both *Zfy-1* and *Zfy-2* of the *domesticus* type, to the *musculus* type *Zfy-1* sequence and to a fourth type that was almost identical to the Y^d *Zfy-2* sequence, except for one nucleotide difference. We assume this last type corresponds to Y^m *Zfy-2* and that the single base difference is a *musculus/domesticus* polymorphism. This is a third-base change within a codon and would not alter the predicted amino acid sequence. The numbers of each type of sequence are also given in Fig. 3, and are likely to reflect the relative proportions of the different types of transcript. This result clearly shows that both *Zfy-1* and *Zfy-2* are transcribed from the mutant ¥ at approximately the same rate as they are from the normal *domesticus* Y chromosome. We conclude from this that the Tdy^{m1}

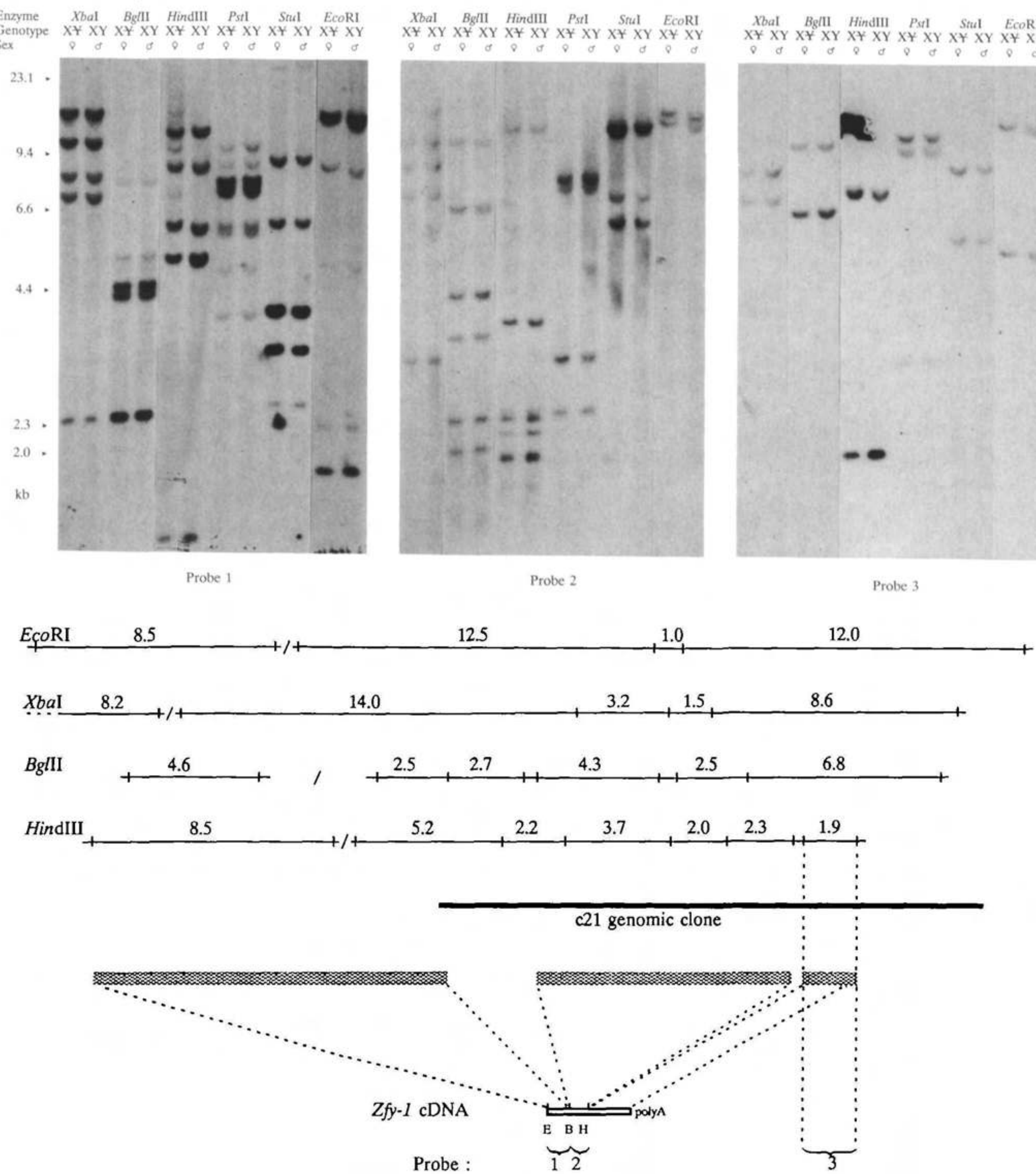

Fig. 1. Comparison of *Zfy* genes in X¥ female *versus* XY male mice by Southern analysis. (A) Southern blots were hybridised to the following probes; Probe 1, nucleotides 1 to 788 (*Eco*RI–*Bgl*II restriction fragment) of a full-length *Zfy-1* cDNA clone (Ashworth *et al.* 1989). Probe 2, nucleotides 789 to 1496 (*Bgl*II–*Hin*dIII restriction fragment) of the *Zfy-1* cDNA. Probe 3, a 1.9 kb *Hin*dIII *Zfy-1* genomic fragment, contiguous with probe 2 and including some 3′ flanking sequences to *Zfy-1*. (B) A map showing the order of *Zfy-1* fragments and the probes to which they hybridise. *c21* is a genomic clone covering part of the *Zfy-1* gene. Bands seen on Southern blots, which are not overlapping with this clone, may not be contiguous with it and are separated by +/+. The stippled bars (▩) represent regions of the genome hybridising to the three probes and which are therefore presumed to contain (all the) exons. The relationship of the three probes used to the *Zfy-1* cDNA is also shown schematically. Bands present on the Southern blots, which are not represented in the map, are from *Zfy-2*. Some faint bands are due to cross hybridization to *Zfx* and *Zfa*.

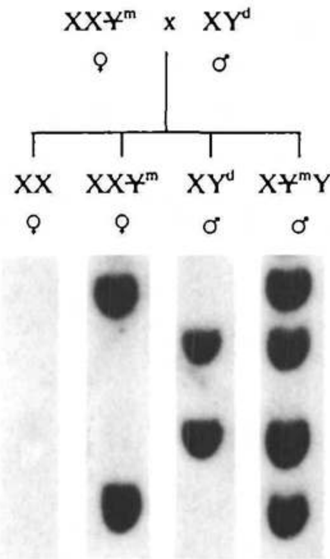

Fig. 2. Identification of X¥^{m}Y^d progeny by Southern analysis. Southern blots of *Taq*I-digested DNA, were hybridized to probe 3 (described in Fig. 1 legend). All the bands seen are absent in females and are therefore Y-linked. Mice carrying Y^m show two bands of approximately 9 kb and 4.5 kb, mice carrying Y^d show bands of 7.5 kb and 5.5 kb. Mice with both Y chromosomes display all four bands.

mutation has not affected regulation of either gene in adult testis.

Expression of Zfy-1 *in X¥ fetal gonads*

We have previously shown that *Zfy-1* is expressed in male mouse fetal gonads from 11.5 days *post coitum* (dpc) until at least 14.5 dpc, with an apparent maximum level at about 13 dpc. In contrast, *Zfy-2* transcripts could not be detected at any of these stages (Koopman *et al.* 1989). This is the period during which the testes differentiate from the genital ridge and it is at this time that *Tdy* is most likely to be expressed. We therefore wished to see whether altered *Zfy-1* expression in X¥ fetal gonads could be associated with their failure to develop as testes.

Embryos at 12.5 dpc were obtained from matings of an X¥y male with CA strain females. RNA was extracted from single gonads and analysed by an RT-PCR method specific for *Zfy-1*. Fig. 4 shows the results of this analysis on gonads from an X¥ female, XX female and Xy male from each of two litters. *Zfy-1* expression in X¥ ovaries was indistinguishable from that in littermate Xy testes. The three bands seen in the Xy tracks are due to alternative splicing in the 5′ non-coding region of *Zfy-1* (Koopman *et al.* 1989). These bands are also apparent in the X¥ tracks, at equivalent intensities to those seen in Xy tracks.

Discussion

This paper presents evidence from the study of Tdy^{m1} mice that neither *Zfy-1* nor *Zfy-2* can be equated with the testis-determining locus *Tdy*. This is distinct from, but complementary to, evidence based on the chromosomal location and expression patterns of *ZFY*-like genes (Koopman *et al.* 1989; Palmer *et al.* 1989). The Tdy^{m1} mutation, which causes sex reversal such that affected X¥ mice are female, is described in detail in the accompanying paper (Lovell-Badge and Robertson, 1990). Although the physical nature of the DNA lesion in these mice is unknown, we would clearly expect any candidate for *Tdy* to display abnormal structure or expression consistent with the mutant phenotype in these mice.

We have analysed the structure and expression of *Zfy-1* and *Zfy-2* in X¥ female mice and find no evidence to indicate that either the genomic organisation of the genes or their regulation is abnormal. Our analysis of these genes was based on a consideration of the type of mutation that may have occurred in Tdy^{m1}. Initially we tested *Zfy-1* and *Zfy-2* for the presence of major rearrangements (insertions, translocations, inversions, duplications or deletions) by comparing X¥

```
Gene    Type    Sequence

        mus     GAAGACCAGAATGTCACTGCCAGTGCTCTCTTAAACCAAGATGAGTCTGG
Zfy-1           ||||| ||||||||||||||||||||||||||||||||||||||||||||
        dom     GAAGATCAGAATGTCACTGCCAGTGCTCTCTTAAACCAAGATGAGTCTGG
                ::::  ::::::: ::::::::::::: : ::: :::: ::::: :::::
        mus     GAAGTGCAGAATGCCACTGCCAGTGCTATGTTACACCATGATGAATCTGG
Zfy-2           |||||||||||||||||||||||||||||||||||||||||||| |||||
        dom     GAAGTGCAGAATGCCACTGCCAGTGCTATGTTACACCATGATGAGTCTGG
```

Number of clones of each type:

	mus	dom	Total
Zfy-1	8	4	12
Zfy-2	15	20	35
Total	23	24	**47**

Fig. 3. Sequences obtained from RT-PCR analysis of *Zfy* transcripts from X¥^{m}Y^d adult testes. Four different sequences were obtained. Critical polymorphisms between these sequences are shown in bold type. Homology between *M. m. musculus* (mus) and *M. m. domesticus* (dom) are indicated by | and between *Zfy-1* and *Zfy-2* by :. The number of clones of each sequence obtained indicates that the total number of *Zfy-1* and *Zfy-2* transcripts originating from each Y chromosome is equal and that the level of *Zfy-2* expression is three times that of *Zfy-1*.

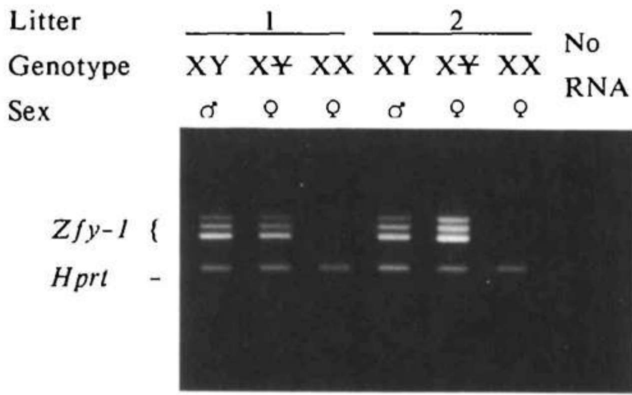

Fig. 4. PCR analysis of *Zfy-1* expression in XY male, X¥ female, and XX female 12.5 dpc embryonic gonads. The three bands corresponding to *Zfy-1* expression were seen in RNA extracted from both normal XY male and mutant X¥ female embryonic gonads but not in normal XX females. The control *Hprt* band was present in all samples. Littermates from two separate litters were analysed.

female DNA against normal XY male DNA on Southern blots, using a variety of restriction enzymes. No differences were seen in *Zfy-1* and *Zfy-2* bands when normal males and X¥ females were compared. The use of probes covering a full-length cDNA means that bands corresponding to all the exons of this transcript should be represented on the blots. This implies that no gross alteration has occurred in the coding regions or adjacent intron sequences of *Zfy-1* and *Zfy-2*. We estimate the limit of resolution of this approach to be approximately 100 bp. Any smaller mutation would not be detected unless it happened to alter the site for one of the restriction endonucleases used. However, previous work using retroviral mutagens has implied that the most likely form of gene disruption to occur in these cases is a gross alteration, which would be visible on a Southern blot, rather than a point mutation. In most cases, the disruption is caused by insertional mutagenesis involving whole virus or just viral LTRs. However, this is not always the case. In a similar mutagenesis protocol to that which gave rise to the Tdy^{m1} mutation, embryonic stem cells were selected for mutations in the *Hprt* gene and a number of lines derived. As expected most of these lines carried inactivating viral insertions, but one line was found not to have viral sequences associated with the mutation and instead to have a 2 kb sequence inserted into the fifth intron of the *Hprt* gene. The origin of this insertion is at present unknown (Mark Carlton and Martin Evans, personal communication). In another study (Stocking *et al.* 1988), growth factor independent variants of a myeloid precursor cell line were isolated after retroviral mutagenesis. In many cases activation of the GM-CSF gene was found to have resulted from retroviral insertion into the locus, however, in one case an intracisternal A particle had integrated into the last exon of GM-CSF, producing a distinguishable band pattern compared to the wild-type allele on Southern blots.

Alternatively, Tdy^{m1} could be a regulatory mutation. While the Southern analysis would have detected gross alterations in 5′ and 3′ control sequences relatively near the exon sequences of the *Zfy* genes, it would not detect alterations in regulatory regions more distant from the genes. However, our analysis of an adult X¥^{m}Y^{d} testis has shown that both *Zfy-1* and *Zfy-2* are capable of being transcribed from the ¥ chromosome. Similar numbers of transcripts of both genes were found from the normal and mutant Y chromosomes. This implies that the elements controlling the regulation of *Zfy* genes in the adult are unaltered in X¥ female mice. Similarly, *Zfy-1*, but not *Zfy-2*, is expressed in 12.5 dpc X¥ female embryonic gonads at the same levels and displaying an identical pattern of alternative splicing as normal male littermates (Koopman *et al.* 1989). We conclude from this finding that the embryonic regulation of *Zfy* genes is also unaltered in X¥ female mice. Thus, the overall regulation of these genes seems to have been unaffected by the Tdy^{m1} mutation. A further critical point relating to the demonstration of *Zfy-1* expression in X¥ female embryonic gonads is that in these embryos ovarian development occurs despite normal expression of *Zfy-1*, clearly indicating that expression of this gene is not sufficient for testis determination.

The possibility remains that there is a point mutation affecting the *Zfy-1* protein product. To exclude this, it would be necessary to determine full length cDNA sequences of *Zfy-1* from both the mutant ¥ chromosome and from a normal Y chromosome of *musculus* type (the only available full length sequences are from a *domesticus* type Y chromosome). However, the conclusions reached in this paper are supported by other recent evidence that *ZFY*-like genes are not the male-determining factor. We have shown that *Zfy-1* is expressed in male genital ridges at the time when gonadal differentiation occurs (Koopman *et al.* 1989), but this expression is associated with germ cells rather than with the somatic portion of the gonad where *Tdy* is thought to act (Burgoyne *et al.* 1988). As testis development can occur quite normally in the absence of germ cells in W^e/W^e mutant embryos, and such embryos lack *Zfy-1* expression, the simplest interpretation of these data is that *Zfy-1* cannot be considered a candidate for *Tdy* (Koopman *et al.* 1989). In a separate study, Palmer *et al.* (1989) analysed several human XX males and hermaphrodites who do not possess *ZFY*. While the majority of these had no detectable Y chromosome sequences, and are therefore presumed to have resulted from mutations elsewhere in the sex-determination pathway, four individuals were found that carried the Y chromosome pseudoautosomal boundary. The authors conclude that, as these four individuals have undergone an abnormal X-Y interchange, sex reversal must have occurred by translocation of *TDF* onto an X chromosome (the chances of having a coincident mutation elsewhere in the pathway as well as an abnormal interchange would be too remote). *ZFY* has not been translocated in these individuals, implying that it cannot be *TDF* in humans. Thus a direct role for *ZFY*-like genes in primary sex determination has been contra-

dicted on the basis of their pattern of expression in mice and chromosomal location in humans. This present study provides a third independent but complementary approach that leads to the same conclusions.

It has been suggested that the correct level of ZFY-like gene expression may be necessary for establishing the correct situation in which *TDF* can act (Burgoyne, 1989). This argument could also hold true for mice if *Zfy* genes are expressed in cell types other than germ cells prior to testis cord formation. This is currently being examined. However, if more than one Y-linked gene is essential for testis determination, mice carrying Tdy^{m1} will provide a useful genetic background in which *Tdy* candidates may be tested.

If ZFY-like genes are not involved in testis determination, we can speculate on possible alternative functions. Mardon and Page (1989) have argued that *Zfy-1* and *Zfy-2* may be interchangeable on the basis of their similar sequences. However, data from Koopman *et al.* (1989) indicate that the genes are regulated differently, *Zfy-2*, being expressed only in adult testes, while *Zfy-1* is expressed both in adult testes and embryonic gonads. In this present study, we have found *Zfy-2* to be expressed at a threefold higher level than *Zfy-1* in adult testis, confirming an earlier suggestion of Nagamine *et al.* (1989), which again implies that the genes are not equivalent. As Koopman *et al.* (1989) have shown that *Zfy* expression is germ cell specific, one or both of these genes may play roles in male germ cell function in the adult, and *Zfy-1* may have a distinct germ cell related function in the embryo. The embryonic role of *Zfy-1* may be quite subtle as XO germ cells in XO↔XY chimaeras, which must lack *Zfy-1*, nevertheless do undergo at least the initial stages of spermatogenesis (Burgoyne, 1987). We have not been able to detect *Zfy* expression in XY female adult ovaries (data not shown), although this may be associated with a general lack of germ cells (Lovell-Badge and Robertson, 1990) rather than the absence of male germ cells in particular.

Clearly any future candidate for *Tdy* will need to satisfy a number of criteria. We feel that showing such a candidate is altered in Tdy^{m1} mutant mice will be convincing evidence for a role in testis determination. Finally, the nature of such a mutation will be a useful tool in the investigation of how *Tdy* itself may function or be regulated.

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References

ASHWORTH, A., SKENE, B., SWIFT, S. AND LOVELL-BADGE, R. (1990). *Zfa* is an expressed retroposon derived from an alternative transcript of the *Zfx* gene. *EMBO J.* **9**, 1529–1534.

ASHWORTH, A., SWIFT, S. AND AFFARA, N. (1989). Sequence of cDNA for murine *Zfy-1*, a candidate for *Tdy*. *Nucl. Acids Research* **17**, 2864.

AUFFRAY, C. AND ROUGEON, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**, 303–324.

BURGOYNE, P. S. (1987). The role of the mammalian Y chromosome in spermatogenesis. *Development* **101 Supplement**, 133–141.

BURGOYNE, P. S. (1989). Thumbs down for zinc finger? *Nature* **342**, 860–862.

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.

CHOMCZYNSKI, P. AND SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156–159.

GOODFELLOW, P. N. AND DARLING, S. M. (1988). Genetics of sex determination in man and mouse. *Development* **102**, 251–258.

HOGAN, B., COSTANTINI, F. AND LACY, E. (1986). *Manipulating the Mouse Embryo*. New York: Cold Spring Harbor Laboratory.

KOOPMAN, P., GUBBAY, J., COLLIGNON, J. AND LOVELL-BADGE, R. (1989). *Zfy* gene expression patterns are not compatible with a primary role in mouse sex determination. *Nature* **342**, 940–942.

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.

LOVELL-BADGE, R. AND ROBERTSON, E. (1990). XY female mice resulting from a heritable mutation in the murine primary testis determining gene, *Tdy*. *Development* **109**, 635–646.

MANIATIS, T., FRITSCH, E. F. AND SAMBROOK, J. (1982). *Molecular Cloning*. New York: Cold Spring Harbor Laboratory.

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.

MARDON, G. AND PAGE, D. C. (1989). The sex-determining region of the mouse Y chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. *Cell* **56**, 765–770.

MCLAREN, A. (1988). Sex determination in mammals. *Trends Genet.* **4**, 153–157.

MELTON, D. W., KONECKI, D. S., BRENNAND, J. AND CASKEY, C. T. (1984). Structure, expression, and mutation of the hypoxanthine phosphoribosyltransferase gene. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2147–2151.

MONK, M. AND MCLAREN, A. (1981). X-chromosome activity in foetal germ cells of the mouse. *J. Embryol. exp. Morph.* **63**, 75–84.

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., BROWN, L. G. AND DE LA CHAPELLE, A. (1987*a*). Exchange of terminal portion of X-Y-chromosomal short arm in human XX males. *Nature* **328**, 437–440.

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*b*). The sex-determining region of the human Y chromosome encodes a finger protein. *Cell* **51**, 1091–1104.

PALMER, M. S., SINCLAIR, A., BERTA, P., GOODFELLOW, P. N., ELLIS, N. A., ABBAS, N. E. AND FELLOUS, M. (1989). Genetic evidence that ZFY is not the testis-determining factor. *Nature* **342**, 937–939.

SCHNEIDER-GÄDICKE, A., BEER-ROMERO, P., BROWN, L. G., NUSSBAUM, R. AND PAGE, D. C. (1989). The *ZFX* gene on the human X chromosome escapes X-inactivation and is closely related to *ZFY*, the putative sex determinant on the Y chromosome. *Cell* **57**, 1247–1258.

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.

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