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 (¥) in both adult XY¥ testis and X¥ 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. 1987a). 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. 1987b). 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. (1987b) 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. 1987b). 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, Tdym, 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 Tdym 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♀), and X/X Sxr′ males were from stocks maintained at the Mammalian Development Unit. 129/Sv/Ev males and CA females (an outbred MFI based line homozygous for Pgk-1*) were from stocks maintained at the National Institute for Medical Research.

The origin of the Tdym 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 Tdym mutation has been given the symbol Y and will be referred to as such in this paper. The Y chromosome is of 129 origin and is of the Mus musculus type, and so, where appropriate, it is referred to as Y♀. The Y is maintained most readily by breeding XXY females, usually to outbred MFI males carrying the RH1 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, XXY 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♀X♂ males (Burgoyne et al. unpublished). XY♂Y♀ males were obtained from matings of XX♀×XY♂ females with CD1 males.

XY female embryos from matings of an XY♂ female (No. 57.9.14.8/17) with CA males were identified at 12.5 days post coitum (dpc) by gonad morphology and staining of sex chromatin in amnion 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 XY♀ females, plus a few XY♂ males (S. Mahadeviah 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 XY♀ 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 32P 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 XY♂mY♂♂ males by the method of Affruf and Rougeon (1980). 1 μg was reverse transcribed using 200 units of MuMLV 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′-CTCAT TGCATT GCAGC GATC ACCTT-3′) and 3′ primer (5′-CGTAA ATTT GTGTT TGTT-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 PsI and TaqI, 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 PsI and TaqI and subcloned into the PsI and Accl sites of pBluescript (Stratagene), using standard techniques (Maniatis et al. 1982). Recombinant plasmid inserts were sequenced using Sequence (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′-GTGTT TCGAT TGTG CGTG-3′ and 5′-CTGTG TATAC TTCG ATGC-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 Tdym 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 XY 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 XY 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 Y. Zfy expression has been detected in adult but not fetal testis by Northern (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 Y 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 Y 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<sup>m</sup> with a normal Y chromosome in XXY males. These occur spontaneously amongst the offspring of XY females through non-disjunction of the X and Y, but are more readily obtained by breeding XXX females with normal XY males, where 25% of offspring will be XXY. However, this had to be done in such a way that the products of the Zfy genes on the mutant Y could be distinguished from those on the normal Y chromosome in the XXY 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<sup>d</sup>), 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<sup>m</sup>). As the mutant Y 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. XXX<sup>m</sup> mice were therefore mated with males of the CD1 strain, which are known to carry Y<sup>d</sup>, in order to generate the required XXY<sup>m</sup>Y<sup>d</sup> offspring.

Karyotypes were determined from tail tip cultures to identify the XXY<sup>m</sup>Y<sup>d</sup> 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 Tag1 RFLPs (Mardon et al. 1989). Fig. 2 shows the distinct patterns derived from Y<sup>m</sup> and Y<sup>d</sup> 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<sup>m</sup> and Y<sup>d</sup>. 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 PstI and Tag1 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<sup>d</sup> Zfy-2 sequence, except for one nucleotide difference. We assume this last type corresponds to Y<sup>m</sup> 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 Y at approximately the same rate as they are from the normal domesticus Y chromosome. We conclude from this that the Tdy<sup>m</sup>
Fig. 1. Comparison of Zfy genes in XY female versus XY male mice by Southern analysis. (A) Southern blots were hybridised to the following probes; Probe 1, nucleotides 1 to 788 (EcoRI-BglII restriction fragment) of a full-length Zfy-1 cDNA clone (Ashworth et al. 1989). Probe 2, nucleotides 789 to 1496 (BglII-HindIII restriction fragment) of the Zfy-1 cDNA. Probe 3, a 1.9 kb HindIII 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.
Zfy genes in XY female mice

651

wished to see whether altered Zfy-1 expression in XY 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 XY female, XX female and X¥ male from each of two litters. Zfy-1 expression in XY ovaries was indistinguishable from that in littermate X¥ testes. The three bands seen in the X¥ 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 XY tracks, at equivalent intensities to those seen in X¥ tracks.

Discussion

This paper presents evidence from the study of Tdy<sup>mi</sup> 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<sup>mi</sup> mutation, which causes sex reversal such that affected XY 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 XY 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<sup>mi</sup>. Initially we tested Zfy-1 and Zfy-2 for the presence of major rearrangements (insertions, translocations, inversions, duplications or deletions) by comparing XY

Fig. 2. Identification of XY<sup>m</sup>Y<sup>d</sup> progeny by Southern analysis. Southern blots of Tufl-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<sup>mi</sup> show two bands of approximately 9 kb and 4.5 kb, mice carrying Y<sup>d</sup> 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 XY 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

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Number of clones of each type:

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Fig. 3. Sequences obtained from RT-PCR analysis of Zfy transcripts from XY<sup>m</sup>Y<sup>d</sup> 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.
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 XY 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<sup>mt</sup> 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 Carlson 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<sup>mt</sup> 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 XY<sup>W</sup>/YM testis has shown that both Zfy-1 and Zfy-2 are capable of being transcribed from the Y 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 XY female mice. Similarly, Zfy-1, but not Zfy-2, is expressed in 12.5 dpc XY<sup>W</sup> 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 XY female mice. Thus, the overall regulation of these genes seems to have been unaffected by the Tdy<sup>mt</sup> mutation. A further critical point relating to the demonstration of Zfy-1 expression in XY 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 Y chromosome and from a normal Y chromosome of <i>musculus</i> type (the only available full length sequences are from a <i>domesticus</i> 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<sup>W</sup>W<sup>W</sup> 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 chimaerae, 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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