Evidence for distinguishable transcripts of the putative testis determining gene (ZFY) and mapping of homologous cDNA sequences to chromosomes X,Y and 9

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Received January 31, 1989; Revised and Accepted March 13, 1989

ABSTRACT

Oligonucleotide sequences based on the amino acid sequence of the putative testis determining gene *ZFY* have been used to isolate a 1.3 Kb Hind III Y genomic DNA fragment CMPXY1 and three human testis cDNA sequences (CMPXY2, CMPXY3 and CMPXY4). These sequences detect at least four potential exons on the Y (Y1, Y3, Y4 and Y5), three on the X (X1, X2 and X3) and three of autosomal origin (A1, A2 and A3) as determined by comparing the fragments detected by different clones. Analysis with subfragments of CMPXY4 shows that Y3 is unique to the Y and that Y4 and X1 are homologous. Y5 and X3 are detected by the same subfragment of CMPXY4. This is also the case for Y1, X2, A1, A2 and A3. Thus these exons may contain further regions of homology between the X, Y and an autosomal locus. The X-linked sequences all lie in Xp21.2−Xp22.1 and studies with XX males have placed the Y-linked sequences in distal Yp adjacent to the Y-autosomal homologous sequence GMGY3. We have confirmed these localizations by in situ hybridization with CMPXY4 and have shown additionally that the autosomal sequences of both the CMPXY4 sequence and GMGY3 map to 9p22−9pter. Restriction analysis demonstrates that CMPXY1/XY2/XY3 differ in sequence from CMPXY4 at three restriction enzyme sites, thus suggesting that they are transcribed from different but closely related genes and that CMPXY4 must be either X-linked or autosomal in origin. This indicates that more than one of the loci containing *ZFY*-related sequences are transcribed and potentially fulfil functionally distinct roles in the human sex determining pathway. Northern blot analysis of human foetal testis RNA has shown that three low abundance transcripts of 5, 6 and 8 Kb can be detected by *ZFY*-related DNA sequences.

INTRODUCTION

The location of the human testis determining factor (*TDF*) has been established in Yp11.3 by studying Yp sequences present in XX males (1−8) and a highly conserved X-Y homologous sequence from this region, which contains an open reading frame capable of encoding a zinc finger nucleic acid binding protein, has been cloned (9). The genetic locus has been designated *ZFY* and evidence has been presented suggesting that it is a strong candidate for *TDF*. However, while *ZFY* and its X homologue *ZFX* show different hybridization patterns in all eutherian mammals tested, no sex differences have been found in birds and reptiles (10) and the homologous sequences in metatherian mammals appear to be autosomal (11). While various hypotheses concerning primary sex determination in mammals have been postulated on these observations (9,12,13), the function of *ZFY* and its homologues remains unknown. Our approach has been to characterise transcripts of *ZFY* from a testis cDNA library to determine which of the various *ZFX* and *ZFY* loci are functional.

MATERIALS AND METHODS

Hybrid Cell Lines

The Horl X X only, 7631 Y only and AMIR2N somatic cell hybrids used in this study were the same as those described previously (14). Somatic cell hybrids EHA9, W2A9 and W5A9 containing deleted X chromosomes have all been described by Florentin (15).

Screening for ZFY-Related Sequences

All library screening, kinase labelling of synthetic oligonucleotide probes and recombinant phage DNA preparation was carried out as described in Maniatis et al. (16). All other recombinant probes were labelled by the method of Feinberg and Vogelstein (17). CMPXY1 was isolated from a Y flow-sorted library constructed in the Hind III site of charon 21A. This was obtained from the Lawrence Livermore Laboratory, California. cDNA clones CMPXY2, CMPXY3 and CMPXY4 were isolated by screening an adult human testis cDNA library, constructed in NM1149 using Eco R1 linkers, with the probe CMPXY1. The library was prepared from a testis removed from a 55 year old male with a testicular tumour who had been undergoing chemotherapy. Examination of sections showed that the testis was much depleted in germ cells. RNA was extracted from the non-tumourous part of the testis using the guanidinium isothyocyanate method (16) and double stranded cDNA prepared in the usual manner.

DNA Sequencing

Dideoxy chain termination DNA sequencing was carried out on double stranded supercoiled plasmid DNA as described in the Pharmacia DNA sequencing handbook using T7 DNA polymerase.

Preparation of Genomic DNA and Southern Blot Analysis

Genomic DNA was prepared from peripheral blood lymphocytes and somatic cell hybrid lines as described by Kunkel et al. (18) and digested and blotted as previously described (7). Hybridization of labelled probes was performed under the conditions of Church and Gilbert (19). All blots were washed in 0.5×SSC, 0.1% SDS at 65°C.

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5´          10          20          30          40          50          60
A AAA AAT TCA TGA GGA GAC CAG AAC TTT GAT TAA GCA CTC ATA CTG CTT TCT TTT CCT TTC

  lys asn ser trm gly asp gln asn phe asp trm ala leu ile leu leu ser phe pro phe

Start of Open Reading Frame of clone pDP1007  ala leu ile leu leu ser phe pro phe


            70          80          90          100         110         120
TTA GCA ATA ATT ATT GGC CCT GAT GGT CAT CCT TTG ACT GTC TAT CCT TGC ATG ATT TGT

leu ala ile ile ile gly pro asp gly his pro leu thr val tyr pro cys met ile cys

leu ala ile ile ile gly pro asp gly his pro leu thr val tyr pro cys met ile cys


            130         140         150         160         170         180 3´
GGG AAG AAG TTT AAG TCG AGG GGT TTT TTG AAA AGA CAC ATG AAA AAC CAT CCT GAA CAC

gly lys lys phe lys ser arg gly phe leu lys arg his met lys asn his pro glu his

gly lys lys phe lys ser arg gly phe leu lys arg his met lys asn his pro glu his
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Figure 1 This figure shows the DNA sequence of the first 180 base pairs of CMPXY1 and the corresponding amino acid sequence. Aligned with this is the sequence published by Page et al (9). It shows a perfect match over this region.

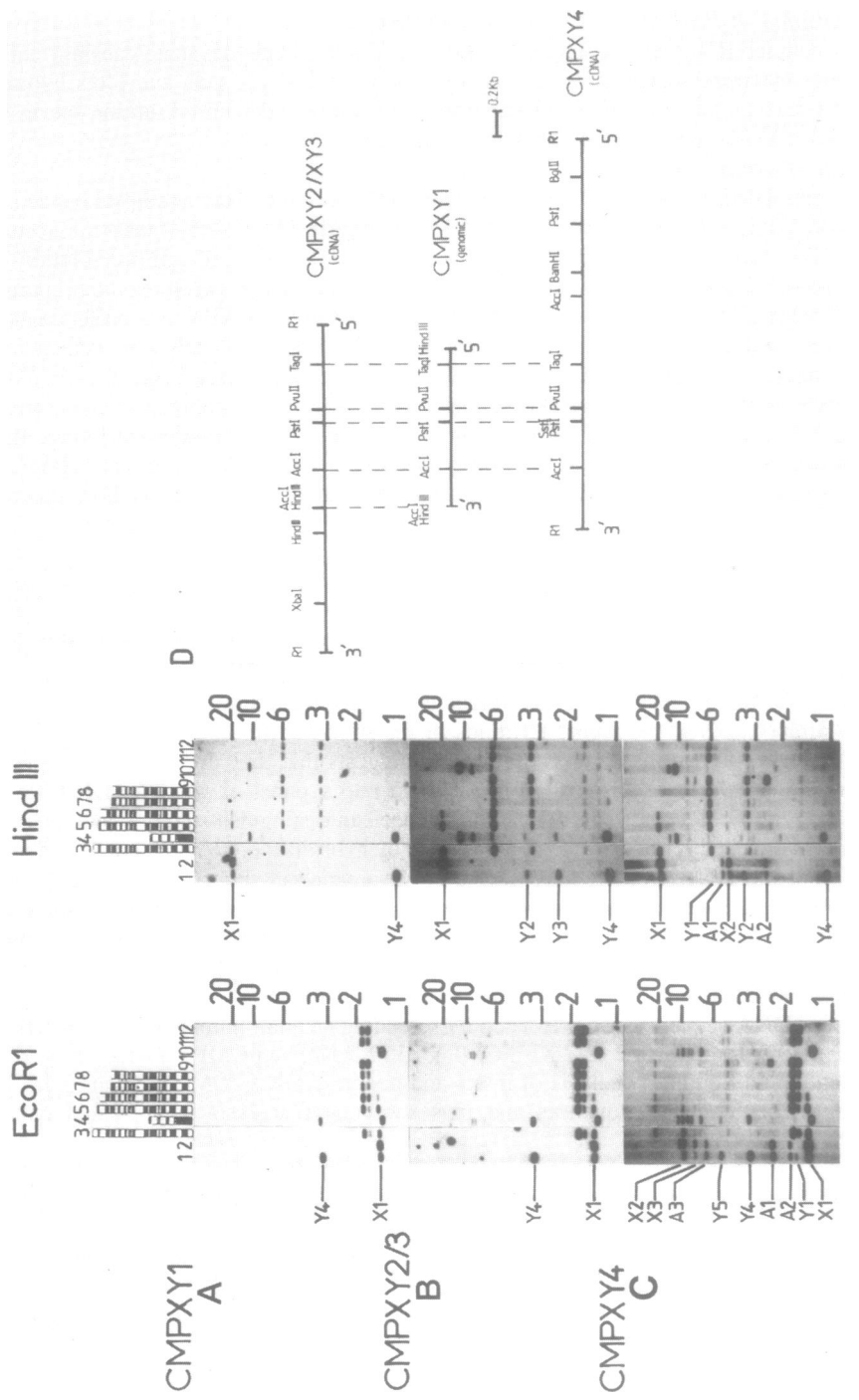
Preparation of RNA and Northern Blot Analysis
Total cellular RNA was prepared by the guanidinium isothyocyanate/caesium chloride method, electrophoresed in denaturing agarose/formaldehyde gels and blots hybridized in 50% formamide, 5×SSC, 1×Denhardts, 100 μgms/ml denatured salmon sperm DNA and 6% PEG as described in Maniatis et al. (16).
In Situ Hybridization
PHA stimulated lymphocytes were fixed with methanol/acetic acid and lipsol banded prior to hybridization. CMPXY4 was labelled with biotin-dUTP by nick-translation according to the BRL protocol. Following RNase treatment (100μl of a 100μgm/ml solution for 1 hour at 37°C) and simultaneous heat denaturation of probe and chromosome preparations (10 minutes at 85°C), the slides were hybridized for 16 hours at 42°C in a buffer containing 50% formamide with a final probe concentration of 6.5 ng/μl. Signals were detected using either the avidin−peroxidase approach (20) with reflection contrast microscopy (21), or the streptavidin−alkaline phosphatase method (22) with phase contrast microscopy. The probe GMGY3 was labelled with tritium by oligonucleotide random priming. In situ hybridization was performed as previously described (23). Slides were autoradiographed for 19 days at 4°C. Relative lengths of chromosomes were based on flow cytometry measurements (24).

RESULTS

Isolation and Characterization of ZFY-Related Sequences
The screening of a Y-specific flow-sorted library with two synthetic oligonucleotide probes of 24 and 30 bases derived from *ZFY* zinc finger domains, yielded the 1.3 Kb genomic sequence CMPXY1. Partial DNA sequencing has provided a perfect match for a stretch of 49 amino acids at the amino terminus of the proposed protein described by Page et al.(9). This is shown in figure 1 and clearly suggests that they are the same sequences. Figure 2A shows the hybridization of CMPXY1 to a panel of genomic DNA samples digested with EcoR1 and Hind III. The sequence can distinguish single X and Y specific fragments (labelled X1 and Y4) as demonstrated by hybridization to human male and female DNA and the DNA from X-only and Y-only somatic cell hybrid lines. However, no autosomal sequences are apparent with these two enzymes. Conservation is evident from the strong hybridization to mouse and hamster DNA. The hybridization pattern to somatic cell hybrids carrying deleted X chromosomes, places the X sequence within Xp21.2−Xp22.1.

Three cDNA sequences were isolated by screening an adult human testis cDNA library with CMPXY1; CMPXY2 (2.7 Kb), CMPXY3 (2.7 Kb) and CMPXY4 (3.2 Kb). (From restriction and Southern analysis of insert and genomic DNA, CMPXY2 and CMPXY3 appear to be identical sequences and thus subsequent analysis is presented only for CMPXY2).

Restriction maps were developed and compared for each clone and are illustrated in figure 2D (EcoR1 sites derive from synthetic linkers used during cDNA cloning). The alignment of these restriction maps (via the Pvu II, Pst I, AccI, Taq I and Hind III sites) reveals the region of overlap and that between them the sequences span 4.3 Kb of potential mRNA. One of the Hind III sites flanking the genomic CMPXY1 sequence cannot be detected within the cDNA overlap of either CMPXY2/3 or CMPXY4, thus suggesting that the 5′ end of CMPXY1 spans an exon/intron junction. Furthermore, the Hind III and AccI sites positioned at the same point in CMPXY1 and CMPXY2/XY3 are not present

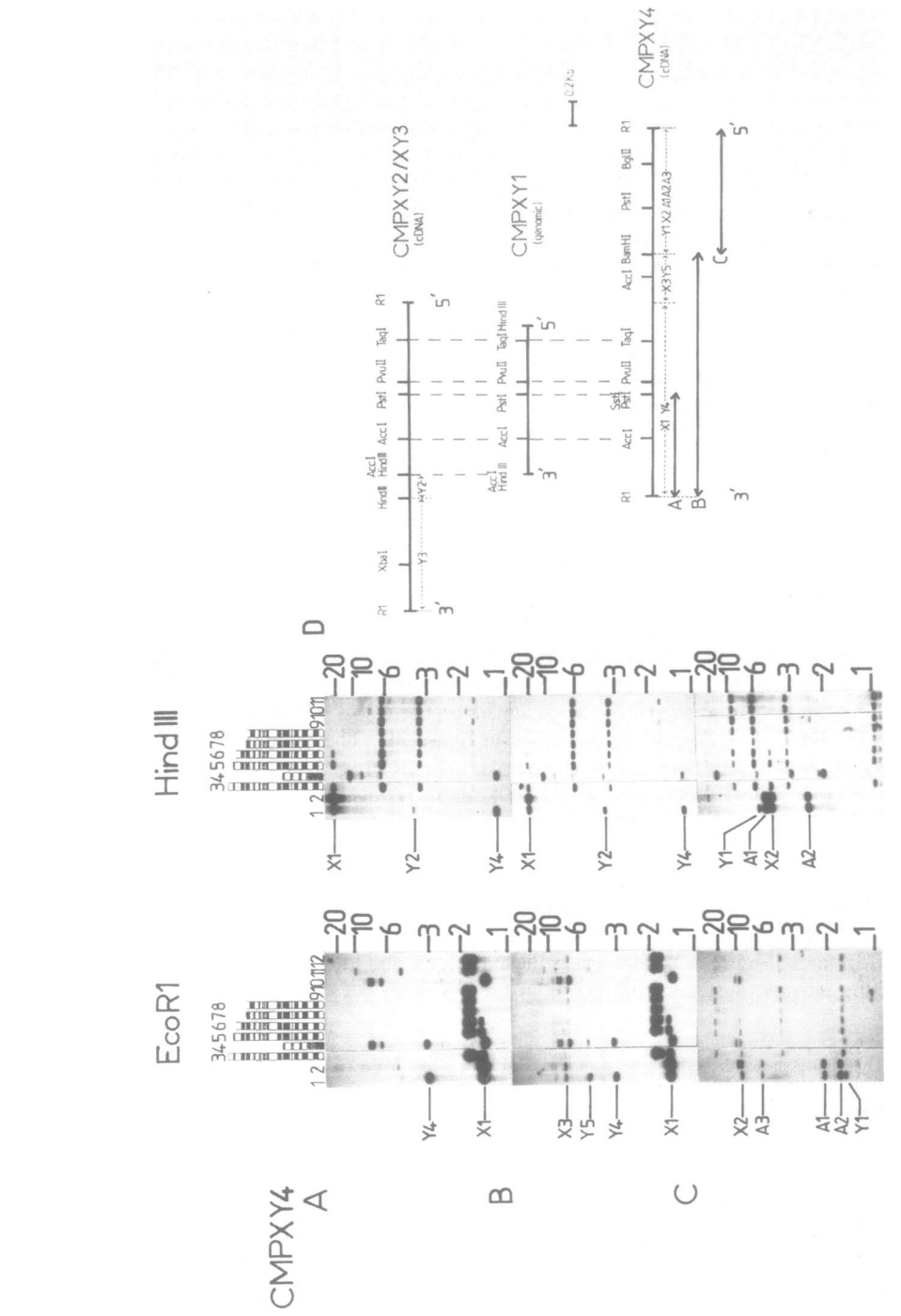

EcoR1
Hind III
CMPXY1
A
CMPXY2/3
B
CMPXY4
C
D
CMPXY2/XY3
(cDNA)
CMPXY1
(genomic)
CMPXY4
(cDNA)
0.2 Kb

in the CMPXY4 cDNA. Similarly, the SSt I site found in CMPXY4 cannot be found in either CMPXY1 or CMPXY2/XY3. Thus these cDNAs appear to differ in sequence and most probably reflect transcription from different homologues. The alternative of differential splicing of exons is possible but less likely in view of the fact that these differences occur within what appears to be the only open reading frame defining a single exon (9).

CMPXY2/CMPXY3 give a pattern of hybridization identical to that of CMPXY1 for EcoR1 (see figure 2B), but detect two additional Y fragments (Y2 and Y3) in Hind III digested DNA. CMPXY4, however, clearly detects a series of additional X and Y fragments (X2, X3, Y1, Y5) which must represent additional X and Y linked exons of the putative TDF gene since this cDNA does not contain any EcoR1 or Hind III sites. Furthermore, this cDNA also detects three autosomal EcoR1 (A1 – A3) and two Hind III fragments since they can be found in both male and female human DNA but not in DNA from X-only and Y-only cell hybrids. The additional fragments seen in hybrid cell lines are rodent specific. All X-linked fragments map in the interval Xp21.2 – Xp22.1. An XX male (MM) shown previously (7) to contain the Y-autosomal homologous probe GMGY3 (found 100 kb distal to *ZFY*) as the only Y-linked sequence is also positive for all Y-linked sequences detected by CMPXY1, XY2/XY3 and XY4. Thus all Y fragments lie in the expected region of the Y chromosome just proximal to the pseudoautosomal segment and most probably represent further domains of the putative *TDF* gene.

Different sub-fragments of CMPXY4 were used in Southern blot analysis to confirm the orientation of the restriction maps and to position the points at which introns must exist. Figure 3 presents the hybridization patterns of the 0.9 Kb EcoR1 – Pst I (3A), 2.1 Kb EcoR1 – BamH1 (3B) and 1.2 Kb BamH1 – EcoR1 (3C) fragments to the panel of DNAs used in figure 1. The 0.9 Kb EcoR1Pst I fragment clearly covers a large part of the region of overlap with CMPXY1 and CMPXY2/CMPXY3 and detects Y2, X1 and Y4. The additional X and Y fragments can be assigned to the Pst 1 – BamH1 (Y5, X3) and BamH1 – EcoR1 (Y1, X2,) regions as shown in figure 3D. The three autosomal EcoR1 fragments are exclusively detected by the BamH1 – EcoR1 region. Given the absence of both EcoR1 and Hind III sites from CMPXY4, these results indicate that at least three potential exons exist on the X and Y and three on a possible autosomal homologue. When one also considers the fragments detected by CMPXY2/CMPXY3 then there must be at least one further Y-linked exon. It should be noted that the failure by CMPXY3 to detect a 0.2 Kb Hind III fragment (generated by digestion at the two Hind III sites in this cDNA) suggests the presence of an intron of approximately 3.4 Kb (fragment Y2) between the two Hind III sites. Figure 3D summarises these findings and indicates the points at which some of the introns must interrupt potential mRNA sequences.

Figure 2 This figure shows the hybridization of (A) CMPXY1, (B) CMPXY2/XY3 and (C) CMPXY4 to the following panel of DNA samples digested with Eco R1 and Hind III. 1—human male genomic DNA; 2—human female genomic DNA; 3—Horl X, X-only somatic cell hybrid; 4—7631 Y-only somatic cell hybrid; 5—AMIR 2N somatic cell hybrid containing Xp22.3 – Xqter; 6—EHA9 somatic cell hybrid containing Xp22.1 – Xqter; 7—W5A9 somatic cell hybrid containing Xp21 – Xqter; 8—W2A9 somatic cell hybrid containing Xp21 – Xqter in addition to an interstitial deletion from Xp21 – Xp11.2 ; 9—Mouse A9 fibroblast cell line; 10—Chinese hamster ovary genomic DNA; 11—Female mouse genomic DNA; 12—Male mouse genomic DNA. The bars on the right indicate the position of molecular weight markers in kilobases and the letters on the left identify different X, Y and autosomal linked sequences. The 7631 hybrid has a hamster background and all other hybrids have a mouse background. The regions of the X and Y chromosomes present in these hybrids is shown schematically above the appropriate track. (D) Summary of restriction maps developed for CMPXY1, CMPXY2/XY3 and CMPXY4.


CMPXY4
A
B
C
D
EcoR1
Hind III
CMPXY2/XY3
(cDNA)
CMPXY1
(genomic)
CMPXY4
(cDNA)
0.2 Kb

In Situ Localisation of the Autosomal Sequences

In situ hybridization to metaphase chromosomes using CMPXY4 and the previously characterized sequence GMGY3 (14) labelled with biotin and tritium respectively, was used to identify the chromosomal site(s) carrying autosomal homology to these sequences. In total, 133 male metaphases were analysed with the biotinylated probe using the alkaline phosphatase and peroxidase detection systems. Forty-five metaphases were analysed after hybridization to GMGY3. Table 1 and figure 4 summarise the findings and clearly illustrate the hybridization of the putative *TDF* sequences to expected positions at Xp21 and Yp11.3. Highly significant hybridization is also found for both probes to the short arm of chromosome 9 in the region 9p22−9pter, suggesting a block of homology between distal Yp and the subtelomeric region of 9p (see table 1). No significant hybridization of GMGY3 is found to Xp21−Xp22.1.

Origin of the CMPXY4 and CMPXY3 Transcripts

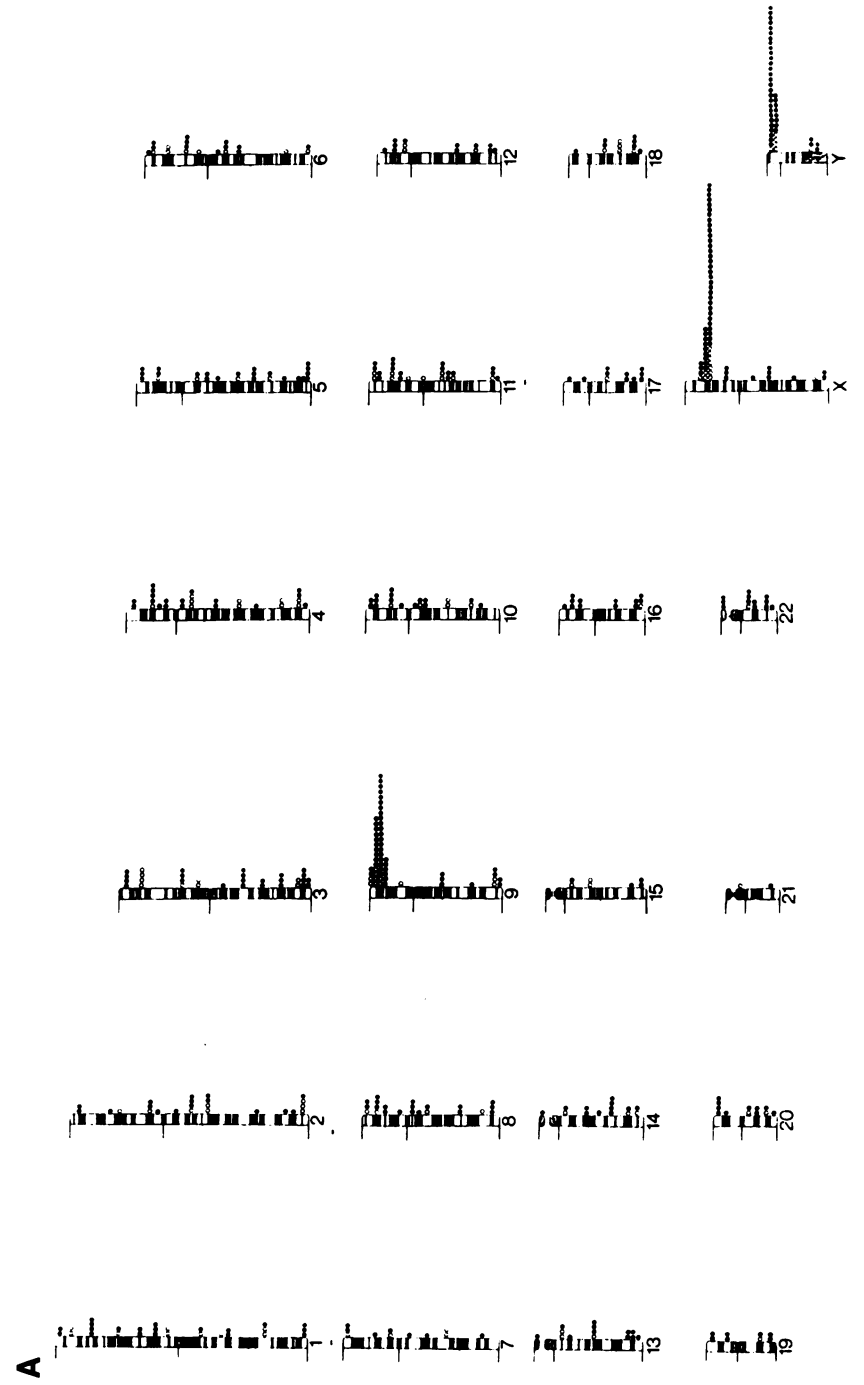
The presence of the Sst I site in CMPXY4 was exploited to investigate the chromosomal origin of this transcript. Figure 5 shows the result of probing double digests (Eco R1 + Sst I and Hind III + Sst I) of human and cell hybrid DNA with CMPXY1. Sst I clearly cuts the fragment derived from the X chromosome but not from the Y. This therefore confirms that CMPXY4 does not represent sequences transcribed from the Y. In addition, it can be seen that in the Eco R1 + SstI digest, CMPXY1 can now detect a fragment in male and female DNA which is not present in the X only hybrid. This suggests the fragment is of autosomal origin. Thus the Sst I site in CMPXY4 must lie on the X and possibly an autosome, therefore indicating that (a) CMPXY4 is either an autosomal or X transcript and (b) that a region homologous to the X and Y zinc finger domain may lie on an autosome. In the Eco R1 and Hind III digests the autosomal fragment must comigrate with the X-linked fragment thus suggesting that the positional relationship of these restriction enzyme sites is highly homologous between the X and autosomal loci. In the double digests the difference in the position of the Sst I site with respect to Eco R1 sites on the X and autosome must be sufficiently different to reveal an autosomal fragment but remains highly conserved with respect to Hind III site where no autosomal fragment is evident.

Since CMPXY1 is a Y genomic DNA fragment, the strict homology between the CMPXY1 and CMPXY2/XY3 restriction maps implies that the latter cDNA sequences represent transcripts from the Y chromosome.

Northern Blot Analysis With CMPXY3

In order to determine the size of *ZFY*-related transcripts, total cellular RNA was prepared from human foetal testis and a male lymphoblastoid tissue culture cell line and subjected to northern blot analysis on agarose/formaldehyde gels. Figure 6 shows the results of probing these blots with CMPXY3. Three RNA species of 5, 6 and 8 kb (as judged by comigration of a marker RNA ladder) are detectable with this probe. CMPXY1 and CMPXY4 detect the same sizes of RNA species. As a control, an actin probe was used to reprobe the same

Figure 3 This figure shows the hybridization of subfragments of CMPXY4 to the same panel of DNA samples used in figure 2 for the Eco R1 digest. For the Hind III digest 1−9 as in figure 2; 10—Male mouse genomic DNA; 11—Female mouse genomic DNA. The fragments in part (D) of the figure marked A (0.9 Kb Eco R1−Pst I), B (2.1 Kb Eco R1−BamH1) and C (1.2 Kb BamH1−Eco R1) were purified from a low melting point agarose gel, labelled by oligonucleotide random priming and used to probe respectively the panels in A, B and C.(D) summarises the regions to which X, Y and autosomal fragments can be localised (and therefore indicating the position of introns) including fragments detected by CMPXY2/XY3 and CMPXY1.


A

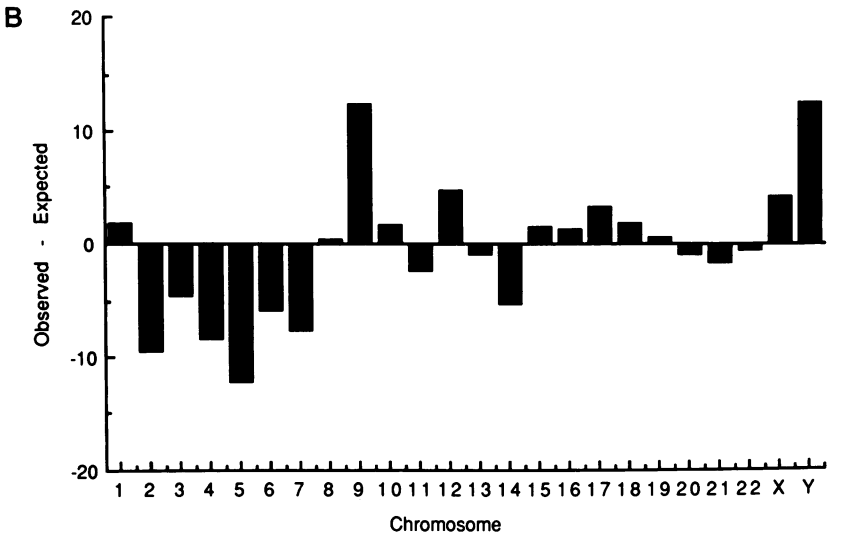


Figure 4 This figure summarises the in situ hybridization data obtained with CMPXY4 (A) and GMGY3 (B). Of the 133 metaphases analysed in (A), 38 (29%) showed signals on Yp, 49 (37%) on Xp21−Xp22.1 35 (26%) on 9p22−9pter and 86 (65%) on one or more of these loci (see table 1); (−) streptavidin−alkaline phosphatase, (−) avidin−peroxidase. In (B), of the 45 cells scored, 10 (22%) had signals on Yp, 12 (27%) on 9p22−9pter and 20 (44%) at one or both of these loci; 4 (9%) had signals on Xp21−Xp22.1, but this latter hybridization was not found to be statistically significant (see table 1). In this experiment the chromosomal distribution of the grains is represented in the form of a histogram which plots the difference between the expected and observed grain counts for each chromosome. The expected grain count for each chromosome is determined by the relative proportion of haploid genome length occupied by the chromosome as measured by flow cytometry.

filter and gave a distinct band at the expected size of 18s. This, together with the fact that the 18 and 28s ribosomal RNA species were intact as judged by staining with ethidium bromide, indicated that the RNA had suffered little degradation. A long exposure of 11 days was necessary to visualize these transcripts, suggesting that they are of low abundance in foetal testis RNA. The 5 and 6 Kb species are weakly visible in RNA from the male lymphoblastoid cell line. The smearing may reflect, partly, some RNA degradation but may also represent cross-hybridization to other RNA species containing conserved zinc finger motifs which begin to come up on prolonged exposure.

DISCUSSION

The CMPXY1, CMPXY2/XY3 and CMPXY4 sequences are strong candidates for the *TDF* gene(s) but this awaits confirmation by demonstration of their mutation in XY females or their ability to determine male sex in a chromosomally female transgenic mouse. The presence of transcripts in foetal and adult (since the cDNA sequences were isolated from an adult testis) human testis would further suggest a role, not only in determining testis development, but also in its maintenance. At least two transcripts can be distinguished on the basis of three restriction enzyme site differences within a highly conserved region and thus are not likely to represent polymorphisms. The divergence of the X, Y and 9 homologues is further indicated by the different restriction fragments detected at these loci, in contrast to reptiles and birds where no sex differences in the arrangement of *ZFY*-like

Table 1: Summary of in situ hybridisation data for CMPXY4 and GMGY3.

Probe	Total		Average background	Locus	Mets. with signals at locus		No. of signals at locus
	Metaphases	Signals			Number	%age of total	
CMPXY4	133	525	2.9	9p22-pter	35	26	46
				Xp21-p221	49	37	54
				Yp	38	29	41
				One or more of the above loci	86	65	141
GMGY3	45	366	7.5	9p22-pter	10	22	13
				Xp21-p221	4	9	6
				Yp	9	20	14
				One or more of the above loci	24	53	33

Table 1 The table summarises the distribution of signals on metaphases and loci to which the probes have been assigned. The average background per metaphase is obtained by subtracting the sum of signals at the mapped loci from the total number of signals and dividing by the number of metaphases analysed. For both probes a standard Chi square analysis was performed to assess the significance of the observed signal distribution. For CMPXY4 the signal distribution is still non-random ($p<0.05$) after excluding chromosome 9, X or Y, or any two of them, but becomes random ($p>0.05$) on excluding all three. For GMGY3 the distribution remains non-random after excluding chromosome 9 or Y, but becomes random on excluding both of them.

sequences is evident suggesting that they may be pseudoautosomal or autosomal in origin (10) and also in marsupials where they have been shown to be autosomal (11). Differential splicing of a transcript from a single functional gene could account for these cDNAs and has been demonstrated to occur during the expression of the *dsx* and *tra* genes of the sex determining pathway in drosophila (25). This, however, is unlikely to be the case here

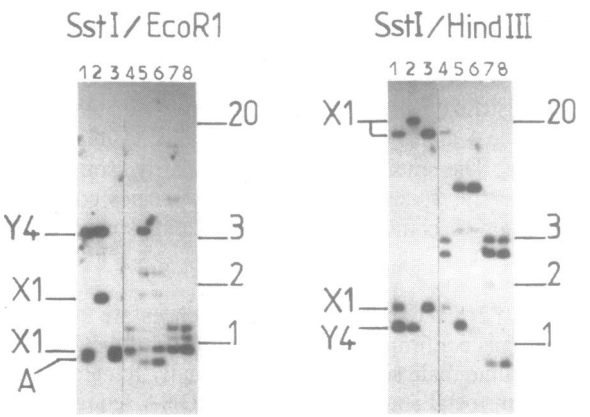


Figure 5 This figure illustrates the fragments detected by CMPXY1 in double digests with Sst I/Eco R1 and Sst I/Hind III. For both panels the order is 1—Human male genomic DNA digested with Sst I/Eco R1 or Hind III; 2—Human male genomic DNA digested with Eco R1 or Hind III; 3—Human female genomic DNA digested with Sst I/Eco R1 or Hind III; 4—Horl X X-only cell hybrid digested with Sst I/Eco R1 or Hind III; 5—7631 Y-only cell hybrid digested with Sst I/Eco R1 or Hind III; 6—Chinese hamster ovary genomic DNA digested with Sst I/Eco R1 or Hind III; 7 and 8 male and female mouse genomic DNA digested with Sst I/Eco R1 or Hind III.

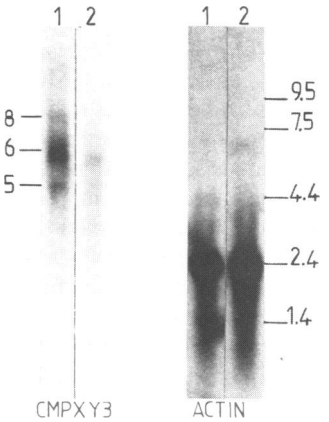


Figure 6 This figure shows the results of northern blot analysis of (1) human foetal testis and (2) male human lymphoblastoid cell line total cellular RNA. 20μg of RNA was denatured in a formamide/formaldehyde mixture and electrophoresed on an agarose / formaldehyde gel. A BRL RNA ladder was run on the same gel to give molecular weight markers. These are indicated on the right by bars. The bars on the left flag the *ZFY*-related transcripts. The CMPXY3 blot was exposed for 11 days and the actin blot for 18 hours.

since these differences would appear to occur within an exon defined by the single open reading frame found by Page et al.(9).

The key Sst I site found uniquely in CMPXY4 has been shown from double digestion experiments to be on the X chromosome and possibly an autosome, thus showing that CMPXY4 is an X or autosomal transcript and conversely that CMPXY2/XY3 are of Y origin. In situ hybridization has shown that autosomal sequences of CMPXY4 are on chromosome 9. However, to unequivocally demonstrate the presence of the zinc finger domain on chromosome 9 it will be necessary to repeat the in situ analysis with CMPXY1 or use it to perform a Southern blot analysis on DNA from a 9 only somatic cell hybrid.

It is clear that the RNA species containing *ZFY*-related sequences detected in northern analysis must represent mRNAs that are at least 5 Kb long, therefore indicating that the putative *TDF* protein(s) is of substantial size. Unless a large proportion of the mRNA is occupied by 3′ and 5′ untranslated regions this therefore implies that the protein coding domain extends beyond the zinc finger region consistent with the presence of additional exons. The chromosomal origin of the transcripts shown in figure 6 is unknown.

From the analysis of cDNA sequences, one is consequently led to propose that more than one of the identified loci (Y, X and 9p22−9pter) are potentially functional, giving rise to distinguishable transcripts. Relative dosage of transcripts from identical functional X and Y genes in mammalian males and females dictated by X inactivation has been suggested as a basis for determining testicular or ovarian differentiation, and can account adequately for sex-reversed phenotypes (12,13). This quantitative model, however, would require to be modified if the transcripts from the X and Y are different, for one could then explain the male on the basis of a heteromeric and the female a homomeric complex.

The sub-telomeric 9p localization of closely related sequences introduces further subtleties. One may not be able to conveniently dismiss this site as a pseudogene in view of the fact that monosomy of 9p22−pter is sometimes associated with anomalous sex differentiation in XY individuals (26−28). In one case where the gonads were examined (27), testicular differentiation had occurred together with the development of uterus and oviducts, but

spermatogonia were absent; the incomplete differentiation of the testis may have failed to suppress the development of the Mullerian ducts. This finding coupled with the demonstration that autosomal sequences detected by GMGY3 (which is about 100 Kb distal to the sequence encoding the open reading frame (9)) are also located in the sub-telomeric region of 9p, implies a functional homology between distal Yp and distal 9p. A caveat with respect to deletions and anomalous sexual differentiation must be added here in view of the fact that several unrelated deletions in the mouse can lead to sex reversal (Bruce Cattenach, personal communication). Thus the deletion of 9p22−9pter in humans may not have a specific effect on sex determination. Nevertheless, the intriguing correlation of anomalous sexual differentiation with the location of CMPXY4 sequences in this region of 9p, demands further study of the locus.

Thus all three sites may well be expressed and a comparison of their gene sequences is necessary to establish this possibility and to determine whether or not each is functionally distinct. The functional *TDF* molecule may be a heterotrimer produced by a contribution from each locus operating in concert. Equally, the three potential gene loci could form part of an initial cascade necessary for the successful initiation and continuation of testis differentiation.

ACKNOWLEDGEMENTS

This work is supported by a Medical Research Council Programme Grant. The flow-sorted Y library was obtained from the Lawrence Livermore National Laboratory, California, USA. We would like to thank Marie Ferguson-Smith for assistance in karyotype analysis and cell culture of lymphoblastoid cell lines.

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