Am. J. Hum. Genet. 45:942–952, 1989

The Putative Testis-determining Factor and Related Genes Are Expressed as Discrete-sized Transcripts in Adult Gonadal and Somatic Tissues

Yun-Fai Chris Lau and Kaimin Chan

Howard Hughes Medical Institute and Departments of Physiology and Medicine, University of California, San Francisco

Summary

The zinc-finger-Y (*ZFY*) gene is a candidate for the testis-determining-factor gene (*TDF*) on the human Y chromosome and is postulated to initiate testis differentiation during embryogenesis. However, the present study indicates that the *ZFY* gene and its X homologue (*ZFX*) are differentially expressed in adult tissues. A human testis-specific *ZFY* cDNA was isolated and completely sequenced. The corresponding *ZFY* transcript encodes a protein that has 801 amino acids and a calculated molecular weight of 90.6 kD. Expression analysis demonstrated that *ZFY* is transcribed primarily as 3- and 5.7-kb mRNA in testis and somatic cells, respectively. In contrast, the *ZFX* gene is expressed as a 5-kb transcript in the testis and as 6.7- and 8-kb transcripts in both ovarian and somatic tissues. With sets of gene-specific oligonucleotides, the origin and relative amount of the respective transcripts can be demonstrated in both Northern hybridization and reverse transcriptase–polymerase chain reaction analysis. Significantly, the 3-kb *ZFY* transcript was also detected in other mammalian adult testes. The testis-specific transcription of the *ZFY* gene hence suggests that it serves a conserved function in this organ.

Introduction

The mammalian Y chromosome plays an important role in the differentiation of the indifferent gonads to testes during embryogenesis. The gene(s) responsible for testis determination, the testis-determining factor (*TDF*), has been mapped to the short arm of the human Y chromosome and is postulated to be responsible for this developmental event (Eicher and Washburn 1986; Goodfellow and Darling 1988; McLaren 1988). Recent chromosome walking experiments reported by Page et al. (1987) have identified a candidate gene for *TDF,* a gene termed zinc-finger-Y (*ZFY*), on this chromosome (Page 1988). Furthermore a highly homologous gene, zinc-finger-X (*ZFX*), has also been identified and mapped to the short arm of the X chromosome.

Received July 26, 1989; revision received August 21, 1989.

Address for correspondence and reprints: Dr. Yun-Fai Chris Lau, Howard Hughes Medical Institute, University of California, U-426, San Francisco, CA 94143.

0002-9297/89/4506-0014$02.00

ZFY sequences are present in most sex-reversed XX males and absent in some XY females. Hence, the *ZFY* gene is directly related to testis formation and male phenotype development in humans. The X and Y chromosome locations of the *ZFY*-related sequences are evolutionary conserved in most eutherian mammals (Page et al. 1987). In the mouse, two copies of the *ZFY*-related sequences, the *Zfy-1* and *Zfy-2* genes, are present in the sex-determining region of the Y chromosome and are considered as candidates for the mouse testis-determining-Y (*Tdy*) locus, the equivalent of *TDF* in man. Two other related sequences, one on the X chromosome (*Zfx*) and one on chromosome 10 (*Zfa*), are also present in the mouse genome (Mardon et al. 1989; Nagamine et al., submitted). Both *Zfy* genes are transcribed primarily as a 3-kb mRNA in adult testis (Mardon and Page 1989; Nagamine et al. 1989), and their expression is directly related to the spermatogenic activities (Nagamine et al., submitted). We have now isolated and sequenced a human testis-specific cDNA of the *ZFY* gene and have demonstrated that the *ZFY* and *ZFX* genes are differentially expressed as discrete-size

transcripts in adult gonadal and somatic tissues. The adult testis-specific transcription of the *ZFY* gene was also detected in other mammalian species, suggesting an evolutionarily conserved and important function for the *ZFY* gene in this organ.

Material and Methods

Material

All human tissues were obtained from the Cooperative Human Tissue Network, Southwestern Division, University of Alabama, Birmingham. Testis specimen A was from a 66-year-old male with mild atropy, and specimen B was from a 55-year-old male with atropy and focal fibrosis. According to the pathology reports, the spermatogenesis was judged to be good for A and moderate for B. All ovarian specimens were from normal females. The chimpanzee testes were obtained from deceased animals through the Yerkes Regional Primate Center, Atlanta. Other mammalian testes were purchased from Pel-Freeze, Inc. (Rogers, AK). Mouse testes were obtained from C57BL/6J mice (Simensen Laboratory, Gilroy, CA).

The TM4 cells were obtained from Dr. J. Mather, Genentech, Inc. (South San Francisco, CA). Although the TM4 cells were derived from mouse Sertoli cells, Southern and polymerase chain reaction (PCR) analyses indicated that the *Zfy* sequences were absent from these cells. Normal hybridization pattern was detected for *Zfx* and *Zfa* sequences. The somatic cell hybrid 3E7 harboring the human Y chromosome in mouse background was a gift from Dr. P. N. Goodfellow, Imperial Cancer Research Fund, London. The GM6317 and GM6318 hybrids, harboring the human Y and X chromosome, respectively, in Chinese hamster background, were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. The human cell lines—HT144 from a male melanoma, MG63 from a male osteosarcoma, Colo320DM from a female colon carcinoma, and other human tumor cell lines—were obtained from the American Type Culture Collection. All cell lines were cultured according to conditions recommended by the suppliers.

RNA Isolation and Northern Hybridization

Total RNA was isolated from frozen tissues and cell pellets according to a modified protocol of Chirgwin et al. (1979; Chomczynski and Sacchi 1987). Poly (A)+ RNA was selected with oligo-dT cellulose chromatography (Maniatis et al. 1982). Northern hybridization was performed according to a method described elsewhere (Lau and Kan 1983). In brief, 3 μg from each sample was glycoxylated and size-fractionated in a 0.8% agarose gel, blotted onto Biodyne membrane (ICN), and hybridized with ^{32}P-labeled zinc finger, hYfin, probe in 50% formamide at 41°C for 20 h. The filters were washed in 0.1 × SSC and 0.1% SDS at 50°C–55°C for 30 min. For relaxed hybridization conditions, the formamide concentration was reduced to 42% and the filters were washed in 2 × SSC and 0.1% SDS at 50°C. For gene-specific oligonucleotide hybridization, the filters were hybridized with end-labeled probes in 6 × SSC solution at 37°C for 16 h and were washed in Me_4NCl solution according to the calculated melting temperature of the respective oligonucleotide probe (Wood et al. 1985).

cDNA Isolation and Sequence Determination

An adult human testicular cDNA library constructed previously (Lau et al. 1988, and in press) in bacteriophage vector, lambda gt11 (Young and Davis 1983), was screened with the Benton and Davis (1977) protocol, using the human *ZFY* genomic probe, hYfin. Additional cDNA was isolated from the same cDNA library by oligonucleotide probe (Wood et al. 1985). Inserts from all cDNA were subcloned into either pUC or pEMBL plasmids and were mapped with restriction enzymes. They were inserted into the single-stranded bacteriophage M13 and sequenced with the chain termination procedure using ^{35}S-dATP as tracer (Sanger et al. 1977; Biggin et al. 1983). The sequences were analyzed with the sequence analysis programs from the Department of Biochemistry, University of California, San Francisco, and with the Microgenie Sequence Analyzer (Beckman, Inc.).

PCR and Reverse Transcriptase–PCR (RT-PCR) Analyses

PCR was performed according to the procedure of Saiki et al. (1988). All primers were derived from the pYF-3 and pYF-2 sequences (see Results). For the genomic DNA analysis, the 5′ primer was a common sequence: 5′-ATTTGTTCTAAGTCGCCATATTCTCT-3′; The 3′ Y-specific primer sequence was 5′-CATCAG-CTGAAGCTTGTAGACACACT-3′. The 3′ X-specific primer sequence was 5′-AGACACACTACTGAGCAA-AATGTATA-3′. The expected sizes of the *ZFY*- and *ZFX*-specific PCR fragments were 340 and 488 bp, respectively. PCR was performed in 50 μl of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 1% gelatin, 20 μM dNTP, 5 pM primers, and 1.25 U *Taq* DNA

polymerase. The mixture was denatured at 94°C for 45 s, reannealed at 63°C for 60 s, and polymerized at 72°C for 10 s for 40–50 cycles. Ten percent of the amplified products were analyzed in a 1.3% agarose gel.

RT-PCR was performed according to a method described elsewhere (Nagamine et al. 1989). For the transcript-specific primers, the common 5′ primer was 5′-TCTTGCACATAGATGAGTCTGCTGGC-3′. The *ZFY*-specific 3′ primer was 5′-TGCTTGTTTTTATATGTGTTTTCAA-3′. The *ZFX*-specific 3′ primer was 5′-GGATTCTCATGTGCTTTTTGAGCTCT-3′. These primers flanked a small intron in both *ZFY* and *ZFX* genes and produced PCR fragments of 654 and 567 bp for the cDNA and of 1,350 and 1,800 bp for the genomic DNA, respectively. Therefore, both 654- and 567-bp fragments were specific for the *ZFY* and *ZFX* transcripts, respectively. Complementary DNA was synthesized from 1 μg poly (A)+ RNA by using a random-priming cDNA synthesis kit (Boehringer/Mannheim). Five percent of the resulting cDNA were subjected to PCR amplification as described above. Ten percent of the amplified products were analyzed on a 1% agarose gel.

Results and Discussion

Isolation of ZFY- and ZFX-specific cDNAs

Using a genomic probe (hYfin) that harbors the zinc-finger domain of the *ZFY* gene, we had isolated three cDNA clones from an adult human testicular cDNA library constructed in the bacteriophage vector, lambda gt11 (Lau et al. 1988, and in press). They contained inserts of about 1, 2, and 3 kb and were designated as pYF-1, pYF-2, and pYF-3, respectively. A poly (A) tail is present at the 3′ end of pYF-1. By sequence analysis, pYF-1 was completely homologous to the 3′ end of the pYF-3 clone, suggesting that both cDNAs were derived from transcripts of the same gene. Furthermore, both pYF-3 and pYF-1 sequences were identical to the coding sequence of the genomic probe (hYfin) and its deduced protein. Since hYfin was isolated from a sorted human Y chromosome library (Nagamine et al. 1989), we postulated that both pYF-3 and pYF-1 were derived from the *ZFY* gene. In contrast, the pYF-2 sequence was homologous, but not identical, to those of pYF-1, pYF-3, and the genomic clone, hYfin. Since only one copy each of the *ZFY* and *ZFX* genes is present in the human genome (Page et al. 1987; Page 1988), we surmised that pYF-2 was derived from transcripts of the *ZFX* gene. In order to confirm this assumption, we selected two sets of specific oligonucleotide primers from the pYF-3 and pYF-2 sequences and used them in PCR amplification (Saiki et al. 1988) of DNA isolated from normal human placenta and from somatic cell hybrids harboring either the human X or Y chromosome. Results from such analyses demonstrated that pYF-3 was Y specific and that pYF-2 was X specific, and they also confirmed that pYF-3 and pYF-2 were indeed derived from transcripts of the *ZFY* and *ZFX* genes, respectively (fig. 1).

The Human ZFY Gene Encodes a Protein with Two Domains in the Adult Testis

Sequence analysis revealed that pYF-3 consisted of 3,214 bp with an open reading frame of 2,406 nucleotides capable of encoding a protein that has 801 amino acids and a molecular weight of 90.6 kD (fig. 2). The 3′ end of the ZFY transcripts contains 250 nucleotides of nontranslated sequence and a consensus polyadenylation signal (AATAAA) 21 nucleotides before the poly(A) track. Southern mapping studies detected about 400 bp of autosomal sequences at the 5′ end of the pYF-3 cDNA. Although Affara et al. (1989) had suggested that sequence homologous to chromosome 9 is present in the *ZFY* transcripts, the exact origin of the autosomal sequences in pYF-3 cDNA is still unknown. However, when specific primers were synthesized from the 5′ autosomal sequence and from the Y-coding sequence and were used in RT-PCR analysis of human testis mRNA, no transcript-specific fragments were amplified. This observation suggests the possibility of a cloning artifact in which an irrelevant stretch of autosomal sequence was ligated to the *ZFY* cDNA. In order to establish the authenticity of the open reading frame, the testicular cDNA library was rescreened with an oligonucleotide corresponding to amino acid positions 200–208 (5′-AGCATCCAACGAAATCATTAGGT-3′). An additional cDNA clone, pYF-4, with an insert of 1,213 bp, was isolated. Sequence analysis established that the pYF-4 sequence was identical to part of the pYF-3 sequence from −33 nucleotides at the 5′ nontranslated region to amino acid position 394, 78 nucleotides upstream from the first zinc-finger repeat domain. Multiple stop codons in all three reading frames were present immediately preceding the putative ATG start codon in both pYF-3 and pYF-4 sequences. The present observations hence confirmed the open reading frame and the corresponding protein sequence in pYF-3.

The deduced protein encoded by pYF-3 is composed of two domains. The carboxyl-terminal end of the mol-

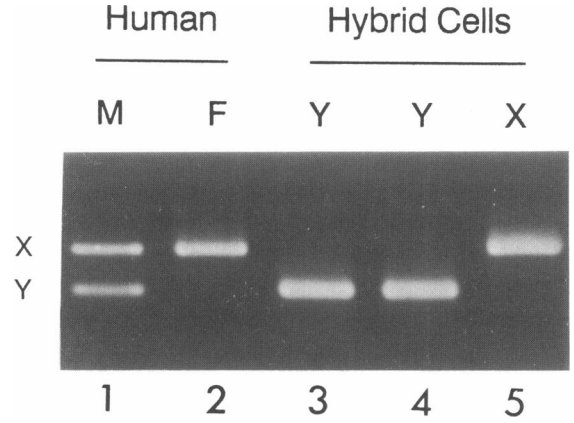


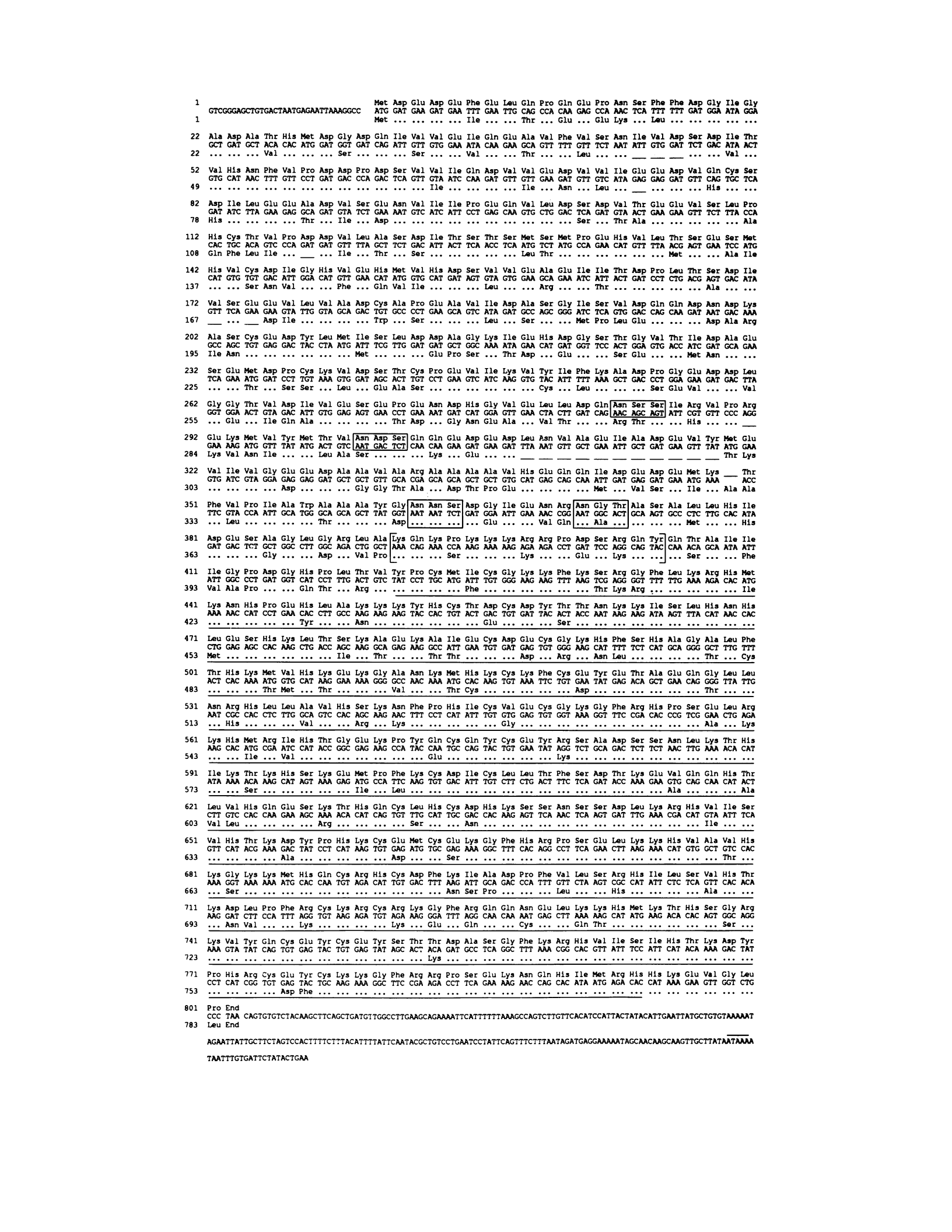
Figure 1 PCR amplification of *ZFY*- and *ZFX*-specific sequences. Specific oligonucleotide primers deduced from the respective sequences of pYF-3 and pYF-2 were synthesized and used in PCR analysis of genomic DNA isolated from male and female placenta (lanes 1 and 2) and from somatic hybrid cell lines which harbor either the human Y chromosome (3E7 in mouse background, lane 3; GM6317 in Chinese hamster background, lane 4) or the human X chromosome (GM6318 in Chinese hamster background, lane 5). As expected, the *ZFY*-specific fragment (340 bp, labeled as Y) is present in DNA of the male placenta (lane 1) and in 3E7 and GM6317 (lanes 3 and 4). Similarly, the *ZFX*-specific fragment (488 bp, labeled as X) is present in both the male and female samples (lanes 1 and 2) and in the somatic hybrid, GM6318 (lane 5). Such correlation suggests that pYF-3 and pYF-2 cDNAs were derived from the *ZFY* and *ZFX* genes, respectively.

ecule (at amino acids 420–795) consists of 13 zinc-finger repeats (fig. 2, underlined sections), as predicted from previous analysis of the genomic sequence (Page et al. 1987). The amino-terminal end of the protein consists of 98 residues (26%) of either aspartic acid or glutamic acid and hence is highly acidic and negatively charged. A short stretch of basic amino acids (at positions 391–405) seems to define the boundary of these two domains. Four putative N-glycosylation sites (fig. 2, boxed sections) are present at positions 284–286, 300– 302, 362–364, and 371–373, preceding the first zinc-finger unit. Recently, sequences of the *Zfy-1* and *Zfy-2* cDNAs from the mouse Y chromosome have been reported (Ashworth et al. 1989; Mardon and Page 1989). These investigators also observed a similar organization of the acidic domain, the basic amino acid region, and the zinc-finger domains in the mouse proteins. On the basis of sequence homology to other nuclear proteins, Mardon and Page (1989) postulated the basic region to be the nuclear localization signal for the Zfy-2 molecule. Despite such structural similarities, differences are discernible between the two sequences. The human *ZFY* and mouse *Zfy-2* cDNA share 82% and 73% sequence homology at their zinc-finger and acidic domains, respectively. The homology is further reduced at the protein level, to 80% and 59%, respectively. Only two of the four putative N-glycosylation sites (positions 362–364 and 371–373) are conserved at the corresponding positions in the mouse Zfy-2 protein. Although both human and mouse cDNAs contain the same putative translation start site, the mouse genes encode only 783 amino acids as compared with the 801 amino acids encoded by the human gene. Such divergence is mainly due to in-frame deletions of the corresponding amino acids in the mouse protein, noticeably at positions 45–47 and 309–319 of the human protein (fig. 2). It is uncertain whether such deletions represent any significance in its function.

The pYF-2 sequence contained 1,874 bp and a partial open reading frame of 1,695 nucleotides which starts at the first nucleotide at its 5′ end and terminates at the same stop codon (TAA) of the ZFY protein. In contrast to the mouse *Zfy-2* gene, the human *ZFX* gene, as indicated by the sequence of pYF-2, exhibits (*a*) 95% and 97.4% homology to the *ZFY* gene at the DNA and protein levels, respectively, at their zinc-finger domains and (*b*) a 94% homology at both DNA and protein levels at their acidic domains. Besides the stretch of basic amino acids immediately preceding the zinc-finger domain, all four putative N-glycosylation sites are also conserved in the deduced ZFX protein. Although the exact molecular structure of the ZFX protein has yet to be determined, its close homology to the ZFY protein at the zinc-finger domain, the putative DNA-binding site, suggests that both proteins may interact with the same or similar DNA sequences. Since only partial sequence of the amino-terminal end is identified in pYF-2, it is still unknown whether the missing portion of the molecule (*a*) represents a structure different from that of the ZFY protein and hence (*b*) may serve functions different from those of the ZFY protein.

The Human ZFY and ZFX Genes Are Differentially Expressed in Adult Gonadal and Somatic Tissues

To study the expression of the *ZFY* and *ZFX* genes, we used the Northern blot analysis of poly (A)+ RNA samples isolated from human testes, ovaries, somatic tissues, and established cell cultures. With the zinc-finger probe, a 3- and a 5-kb band were consistently detected in most testicular samples (figure 3*a*, lanes 2 and 3).



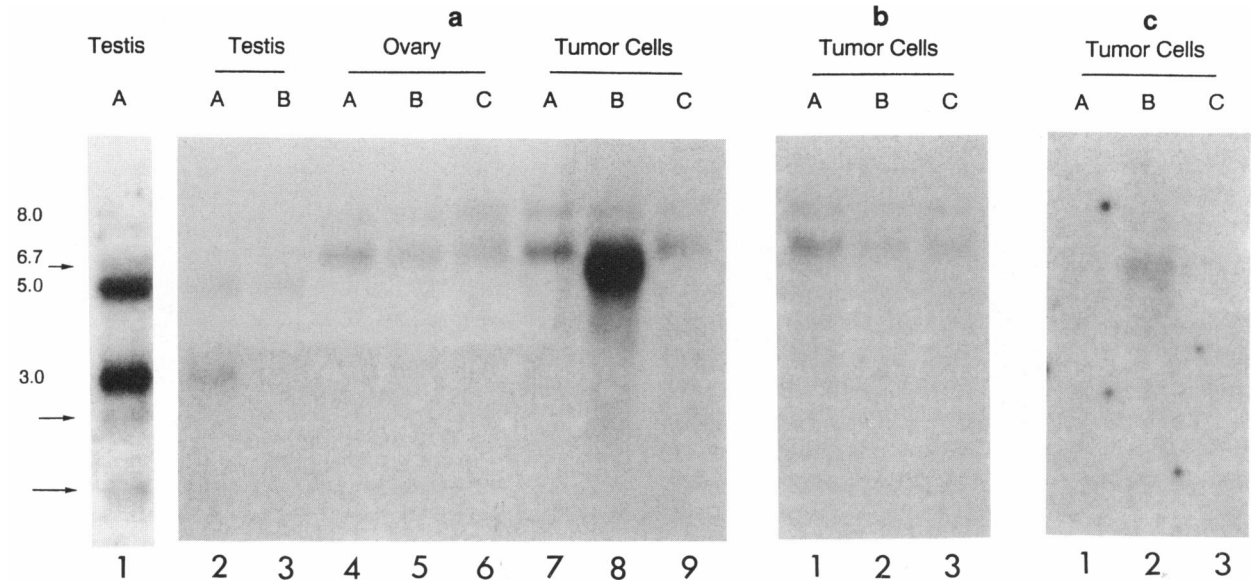


Figure 3 Northern analysis of *ZFY* and *ZFX* transcripts in testes, ovaries, and tumor cells. *a,* 3 μg poly (A)+ RNA from each sample was analyzed by Northern blotting and hybridized with ^{32}P-labeled zinc-finger (hYfin) probe. A prominent 3-kb band and a minor 5-kb band were consistently detected in most testis samples (lanes 2 and 3). Under relaxed conditions, minor 1.5-kb (long arrow), 2.7-kb (medium arrow), and 5.7-kb (short arrow) bands were also detected in testis A (lane 1). All ovarian samples (lanes 4–6) and all somatic cells of both sexes (lanes 7–9) showed hybridization of RNA at 8.0 and 6.7 kb and presumably were derived from the *ZFX* gene. Furthermore, a 5.7-kb transcript was also detected in a male cell line (tumor B, lane 8). *b,* Autoradiogram showing hybridization of a *ZFX*-specific oligonucleotide to the 6.7- and 8-kb bands in the somatic cells. *c,* Autoradiogram showing hybridization of a *ZFY*-specific oligonucleotide to the 5.7-kb transcripts (lane 2). Identical filters were used in all Northern hybridizations in panels *a–c.* An RNA ladder (0.24–9.49 kb; BRL) was used as a size marker. Tumor A (HT144) was derived from a male melanoma; tumor B (MG63) was derived from a male osteosarcoma; tumor C (Colo320DM) was derived from a female colon carcinoma. Similar results were obtained from several other human tumor cell lines derived from neuroblastoma and leukemia. HT144 and MG63 were chosen as representative samples, respectively, for low and high expression of the *ZFY* gene.

The 3-kb band was previously detected in adult mouse testis (Mardon and Page 1989; Nagamine et al., submitted) and was linked to spermatogenesis (Nagamine et al., submitted). Hence, the reduced intensity of the 3-kb band in human testis B (lane 3) probably reflects the suppressed spermatogenic activities in this sample, as indicated by the pathologist's report (CHTN, University of Alabama). Under relaxed hybridization conditions, other minor transcripts of 1.5 (long arrow), 2.7 (medium arrow), and 5.7 (short arrow) kb were also observed (fig. 3*a,* lane 1). At present, the exact origins of these minor transcripts are unknown. However, the 5.7-kb band was probably derived from transcription of the *ZYF* gene in the somatic cells of this organ, since similar-size *ZFY*-specific transcript was detected in other somatic cells (see below). Two transcripts, of 6.7 and 8 kb, were consistently detected in the ovarian samples (fig. 3*a,* lanes 4–6) and in male and female somatic cell cultures (fig. 3*a,* lanes 7–9). We presumed that the 6.7- and 8-kb mRNA were derived from the *ZFX* gene. This inference was confirmed by Northern hybridization of the somatic cell RNA by a *ZFX*-specific oligo-

Figure 2 Sequence of the testis-specific pYF-3 cDNA derived from the *ZFY* gene. The 5′ end of the DNA sequence (middle line) starts at the nucleotide position corresponding to the first nucleotide of pYF-4 sequence. An open reading frame starts at nucleotide 34 and ends at nucleotide 2440, encoding a protein that has 801 amino acids and a calculated molecular weight of 90.6 kD (top line). The amino-terminal domain (position 1–390) is highly acidic and negatively charged. The carboxyl-terminal domain (position 420–795) contains a DNA-binding motif of 13 zinc-finger repeats (underlined). A segment rich in basic amino acids (position 391–405, open brackets) separates the two domains. Four putative N-glycosylation sites (boxed) are present immediately preceding the zinc-finger domain. The bottom line indicates the deduced 783-amino-acid sequence of a mouse *Zfy-2* gene as reported by Mardon and Page (1989). Several in-frame deletions (———), notably at position 309–319, render mouse Zfy-2 protein smaller than the human ZFY protein. Ellipses (. . .) denote identical amino acids between the two proteins.

nucleotide probe (fig. 3*b*, lanes 1–3). Frequently, an additional 5.7-kb transcript was also detected in some male cell cultures, by both the zinc-finger and acidic-domain probes (fig. 3*a*, lane 8). We believe this 5.7-kb transcript was derived from the *ZFY* gene, since it hybridized strongly with a *ZFY*-specific oligonucleotide (fig. 3*c*, lane 2). The lack of hybridization of this Y-specific probe to tumor A and tumor C (fig. 3*c*, lanes 1 and 3) was due to the low level in the tumor A sample (lane 1) and to the absence, in female sample (lane 3), of the *ZFY* transcripts. Similar analysis of RNA samples derived other somatic tissues, such as liver and placenta, revealed the 6-8 kb transcripts at reduced levels (data not shown). On the basis of their sizes, these transcripts in the tissue samples seem to correspond to those of the *ZFX* gene detected in tumor cells.

In order to confirm the relative amount of transcripts from both *ZFY* and *ZFX* genes in the testes, we used the highly sensitive RT-PCR analysis of the same poly (A)+ RNA preparations. We adopted a strategy which can detect relative amount of transcripts from the respective genes. In this approach, cDNA molecules derived from the poly (A)+ RNA samples are generated with a random-priming and reverse-transcription kit (Boehringer/Mannheim). The resulting cDNA samples are subjected to a PCR procedure using sets of gene-specific primers which harbor at least one intron in the corresponding genes, such that PCR fragments from the cDNA will be shorter than those from genomic DNA. This step will eliminate any false signals from the contaminant genomic DNA in the RNA preparation. To establish the relative amount of transcripts from two closely related genes (such as *ZFY* and *ZFX*), a common primer at one end (i.e., the 5′ end) and two gene-specific primers at the other end (i.e., the 3′ end) are used in the PCR analysis. The gene-specific amplification of the cDNA will be specified by both 5′ and 3′ primers. However, since the 5′ primer is common to both genes, its binding to the substrates is dependent on the relative concentration of the respective cDNA species in the sample. Therefore, the intensities of the *ZFY*- and *ZFX*-specific PCR fragments can be interpreted to represent indirectly the relative amount of corresponding transcripts within the same RNA preparation.

Using this RT-PCR approach, we confirmed the transcription of both the *ZFY* and *ZFX* genes in the testes (fig. 4, lanes 3 and 4). More significantly, the relative intensities of the X- and Y-specific PCR fragments in the two testis samples seem to correlate directly to those of the 5- and 3-kb bands, respectively, in the Northern

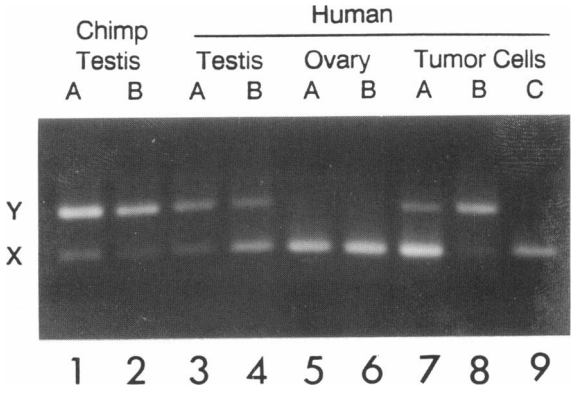


Figure 4 Detection of *ZFY* and *ZFX* transcripts by the RT-PCR method. Complementary-DNA samples were synthesized from 1 μg poly (A)+ RNA of various tissues and cells by using a random-priming cDNA synthesis kit (Boehringer/Mannheim). Five percent of the resulting cDNA was subjected to PCR amplification as described in Material and Methods. *ZFY*- and *ZFX*-specific oligonucleotide primers were selected from pYF-3 and pYF-2 sequences and produced transcript-specific fragments of 654 and 567 bp for the *ZFY* (labeled as Y) and *ZFX* (labeled as X) genes, respectively. The relative intensity of the Y and X fragments in the human testis samples (lanes 3 and 4) were proportional to the Northern signals of 3- and 5-kb transcripts, respectively (fig. 3*a*, lanes 2 and 3). As expected, the ovarian (lanes 5 and 6) and the female tumor C (Colo-320DM; lane 9) samples showed only the *ZFX*-specific fragments. The male tumor A (HT144) transcribed mostly the *ZFX*-specific 6.7- and 8-kb mRNA (fig. 3*a*, lane 7 and fig. 3*b*, lane 1) and showed higher amount of X-specific fragment (lane 7). Conversely, the male tumor B (MG63) showed a high level of *ZFY*-specific 5.7-kb transcript (fig. 3*a*, lane 8, and fig. 3*c*, lane 2) and a brighter Y-specific PCR fragment (lane 8). Lanes 1 and 2 show RT-PCR amplification of chimpanzee testicular cDNA by the human primers.

autoradiogram (fig. 3*a*, lanes 2 and 3), suggesting that the 5- and 3-kb transcripts might be derived from the *ZFX* and *ZFY* genes, respectively, in the testes. Similarly, the *ZFX*- and *ZFY*-specific transcripts in the ovarian samples and in somatic cells could also be confirmed by the RT-PCR method (fig. 4, lanes 5–9). Again, the relative intensities of the *ZFX*-specific PCR fragment (labeled as X in fig. 4, lanes 5, 6, and 9) were directly proportional to the Northern signals of the 6.7- and 8-kb transcripts (fig. 3*a*, lanes 4, 5, and 9), and those of the *ZFY*-specific fragment (fig. 4, lanes 7 and 8) were directly proportional to the signals of the 5.7-kb transcript (fig. 3*a*, lanes 7 and 8). These observations suggest that both *ZFY* and *ZFX* genes are differentially expressed as discrete-size transcripts in somatic and gonadal tissues. The *ZFX* gene is expressed primarily as 6.7- and 8-kb transcripts in most somatic and ovarian tissues and is probably expressed as a 5-kb transcript in the testis. In contrast, the *ZFY* gene produces

a major 3-kb transcript in the testis and a 5.7-kb transcript in somatic cells. Such transcript size variation between testis and somatic tissues has been well documented in several mammalian genes, notably in some proto-oncogenes (Ponzetto and Wolgemuth 1985; Duggal et al. 1987; Goldman et al. 1987; Mutter and Wolgemuth 1987; Propst et al. 1987; Serrentino et al. 1988). It is still uncertain whether such differential expression of both *ZFY* and *ZFX* genes was the result of alternate RNA processing or of transcriptional initiations. Furthermore, whether the gonadal and somatic mRNA encode proteins of different molecular weights and/or different functions has yet to be elucidated.

The Testis-specific Transcripts of the ZFY Gene Are Present in Other Mammals

To determine whether the 3-kb transcripts were present in the testes of other mammalian species, we examined the poly (A)+ RNA from the testes of bull, dog, guinea pig, rabbit, rat, chimpanzee, and mouse by Northern analysis (fig. *5a*). With either the human zinc-finger (hYfin) or the acidic-domain (pYF-4) probe, the 3- and 5-kb bands were only detected consistently in the chimpanzee testicular RNA (fig. *5a,* lanes 6 and 7). With the *ZFY*- and *ZFX*-specific primers, both Y- and X-specific fragments could be amplified in the RT-PCR analysis of the chimpanzee RNA samples (fig. 4, lanes 1 and 2). Significantly, the sizes of these fragments were similar to those of the human samples, suggesting that both *ZFY* and *ZFX* genes are conserved between human and chimpanzee. Again, the intensities of the X- and Y-specific fragments were proportional, respectively, to the relative signals of the 5- and 3-kb bands on the Northern autoradiogram (fig. *5a,* lanes 6 and 7). Testicular RNA from other species showed little or no hybridization at the 3-kb region. Instead, high-molecular-weight transcripts at about 5–6 kb were observed in guinea pig and rabbit testes (fig. *5a,* lanes 3 and 4). The higher-molecular-weight transcripts were probably derived from the *ZFX* gene, as inferred by the Northern and RT-PCR analyses of the human and chimpanzee testes. In addition, the absence of the 3-kb transcripts in these mammalian testes is unlikely to be due to suppression of their spermatogenic activities, since rehybridization of the same filter by a human cDNA probe of another phylogenetically conserved and spermatogenic gene, the male-enhanced antigen (*MEA*) gene (Lau et al. 1988, and in press), showed that comparable amounts of *MEA* transcripts were present in all testicular samples (fig. *5a,* lower panel). Therefore, the lack of appreciable hybridization of the human probe to the 3-kb transcripts in the other testes was probably due to the sequence divergence between the human *ZFY* gene and the *ZFY* genes of other species. We believe the testis-specific 3-kb transcripts would be discernible on hybridization with *ZFY* gene probes of the respective species. The present assumption can be further supported by analyzing RNA preparations from the mouse testis and from the somatic cell line TM4. Transcripts of 7.5 and 6.0 kb were readily detected with the human probe in the TM4 sample (fig. *5a,* lane 9). These large transcripts probably originated from the mouse *Zfx* gene, since Southern blot analysis indicated that the *Zfy* sequences were absent from the TM4 DNA (see Material and Methods). Under identical experimental conditions, only weak hybridization at the 3-kb region was detected in the mouse testis samples (fig. *5a,* lane 8). However, this 3-kb transcript and another minor mRNA band at 2.7 kb (arrow) were readily detected in the mouse testis on rehybridization of the filter with the mouse *Zfy-2* cDNA probe, mYfin (fig. *5b,* lane 1). Although additional investigations will be needed to confirm our presumption that the 3-kb testis-specific *ZFY* transcripts would be discernible in testes of other mammalian species with the respective *ZFY* gene probes, the detection of the *ZFY* transcripts in human, chimpanzee, and mouse testes seems to support such a differential expression of the *ZFY* gene in adult testes of other mammals.

Despite the sex-chromosome locations of the *ZFY* and *ZFX* genes in most eutherian mammals (Page et al. 1987), our studies indicate that the human *ZFY* and *ZFX* genes have diverged from each other more recently than either gene has from the corresponding mouse genes (Nagamine et al. 1989; present report). Since the sequences for the full-length cDNAs from the *ZFX* gene are not available at the present time, it is uncertain whether the close homology between the human *ZFY* and *ZFX* genes represents an example of concerted evolution. However, preliminary sequence analysis of a putative cDNA from the mouse *Zfx* gene indicates that the mouse *Zfx* sequence is more homologous to the human *ZFX* and *ZFY* sequences (about 90%) than to those of *Zfy-1* and *Zfy-2* (about 78%), suggesting that such concerted evolution may not be valid for the mouse. In addition, both Southern blotting and sequence analysis of other mammalian species, e.g., the wood lemming (*Myopus schisticolor*), showed that the *ZFX* gene is evolutionary more conserved than the *ZFY* gene (Lau et al., submitted). If this interpretation is indeed true, it will explain some of the discrepant findings by other investigators who demonstrated that *ZFY*-homologous

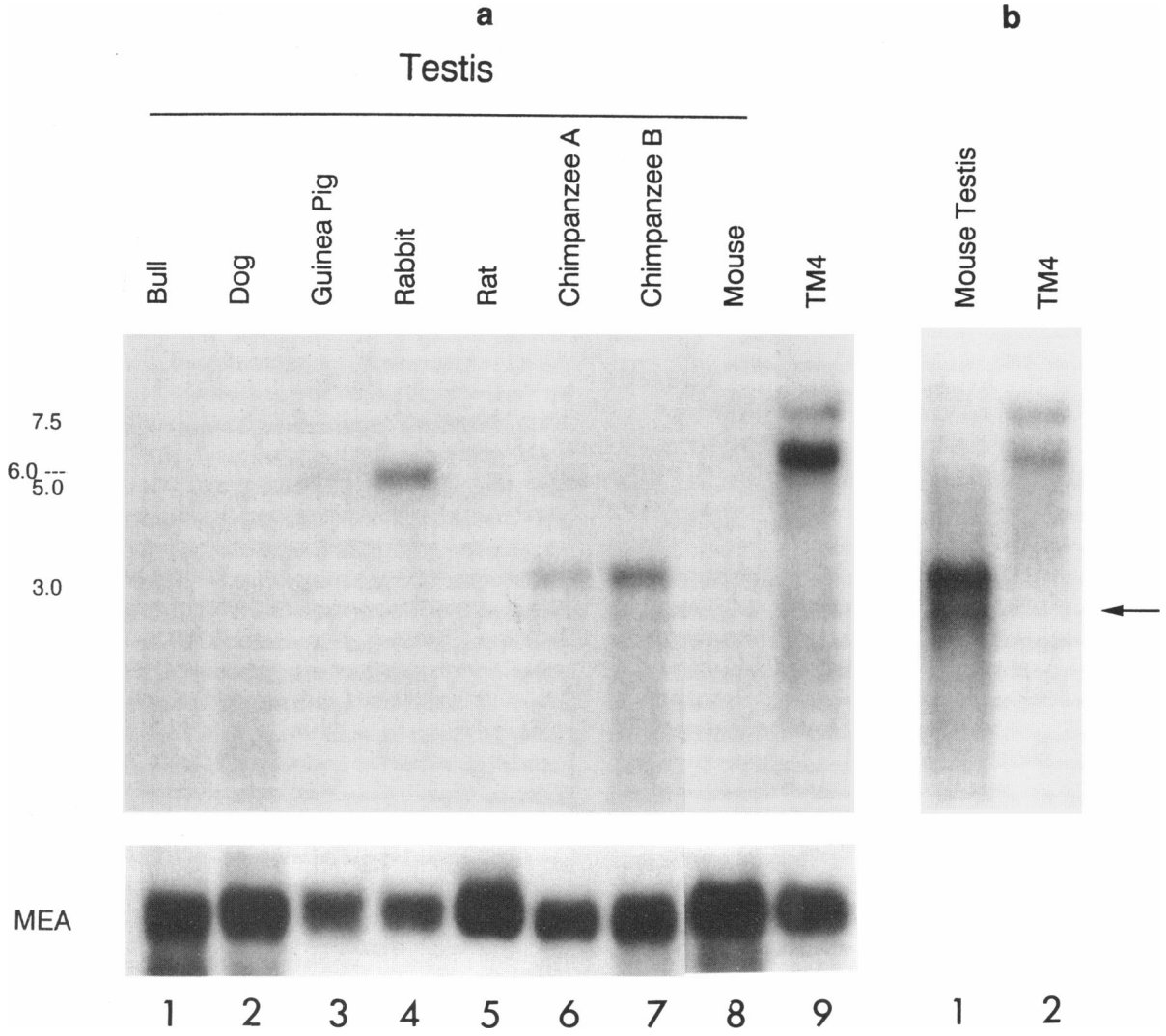


Figure 5 Detection of *ZFY*-specific transcripts in mammalian testes. *a,* 3 μg poly (A)+ RNA isolated from various mammalian testes processed for Northern blotting and hybridized with ^{32}P-labeled human acidic-domain (pYF-4) probe. Only the chimpanzee testes (lanes 6 and 7) showed readily detectable hybridization at the 3- and 5-kb regions. Only a 5–6-kb band was detected in guinea pig and rabbit testes (lanes 3 and 4). A weak 3-kb band was detected in the mouse testis sample (lane 8), but prominent *Zfx* bands of 6 and 7.5 kb were readily discerned in the mouse somatic cell, TM4 (lane 9). The *ZFY* probe was dissociated from the filter which was rehybridized with a human *MEA* cDNA probe. The 1-kb *MEA*-specific RNA band was detected in all samples, indicating that comparable spermatogenic activities were present in each sample. *b,* Autoradiogram from a rehybridization of the filter with a mouse *Zfy-2* cDNA probe, mYfin, showing the major 3-kb and a minor 2.7-kb (arrow) *Zfy*-specific transcript in the mouse testis sample (lane 1) and relatively less intense *Zfx* bands in TM4 (lane 2). These results were interpreted to indicate that the mouse *Zfx* transcripts were more homologous to the human *ZFY* probe than were the mouse *Zfy* transcripts.

sequences are located on the autosomes of reptiles, birds (Bull et al. 1988), and marsupials (Sinclair et al. 1988). Although the sex determination mechanisms in reptiles and birds may be different from those in mammals, the autosomal location of *ZFY*-homologous sequences in marsupials contradicts the postulated sex-determining role of this gene, since the Y chromosome is essential for testis differentiation in metatherian mammals (O et al. 1988). Two explanations have been advanced: (1) the *ZFY* gene is not the primary testis-determining gene, and (2) the *ZFY* gene is sex determining in eutherian mammals but not in marsupials (Hodgkin 1988). In view of the present observations that the *ZFY* gene has indeed diverged among human, mouse, and wood lem-

ming, in contrast to the situation for the *ZFX* and *MEA* genes, a third possibility—that the corresponding *ZFY* gene is undetectable on the marsupial Y chromosome because of sequence divergence from the human *ZFY* probe (pDP1007)—has to be included. Whether the autosomal sequences detected in the marsupials correspond to the human *ZFX*-homologous sequence has yet to be established.

Although Mardon and Page (1989) did not detect any *Zfy* transcripts in fetal mice RNA samples at the time of sexual differentiation, using the highly sensitive RT-PCR analysis we had demonstrated the expression of the *Zfy* genes in testes and somatic tissues from fetal mice at similar embryonic stages (Nagamine et al., submitted). Therefore, the sex-determining role of the *ZFY* and related genes cannot be ruled out completely. Irrespective of whether *ZFY* is the *TDF* gene or not, our studies demonstrate that both human *ZFY* and *ZFX* genes are differentially expressed in adults and that they hence may serve separate functions in gonadal and somatic tissues. The presence of abundant *ZFY* transcripts in adult testis suggests that the *ZFY* genes may play an important role in the normal physiology of this organ. Detailed analyses of RNA derived from testes of normal and mutant mice have demonstrated that the expression of the *Zfy* genes is linked to the spermatogenesis of the animals. More significantly, the 3-kb transcripts are present most prevalently in round spermatids (Nagamine et al., submitted). Whether the similar-sized transcripts detected in other mammalian testes are distributed in the germ cells as those in the mouse testis has yet to be elucidated. The molecular isolation of the cDNA from both human *ZFY* and mouse *Zfy* genes has provided the means for further investigations—such as those using gene-targeting inactivation and transgenic mouse construction—of their roles in mammalian testis determination and/or spermatogenesis.

Acknowledgments

We thank M. Doherty for the synthesis of oligonucleotides, and we thank C. Nagamine, B. Elder, and J. Gampell for critical reading of the manuscript. This work was partially supported by the Howard Hughes Medical Institute and by National Institutes of Health grant HD24384 to Y.-F.C.L.

References

Affara NA, Chambers D, O'Brien J, Habeebu SSM, Kalaitsidaki M, Bishop CE, Ferguson-Smith MA (1989) Evidence for distinguishable transcripts of the putative testis determining gene (*ZFY*) and mapping of homologous cDNA sequences to chromosomes X, Y and 9. Nucleic Acids Res 17:2987–2999

Ashworth A, Swift S, Affara N (1989) Sequence of cDNA for murine *Zfy-1*, a candidate for *Tdy*. Nucleic Acids Res 17:2864

Benton WD, Davis RW (1977) Screening lambda gt recombinant clones by hybridization to single plaques in situ. Science 196:180–182

Biggin MD, Gilson TJ, Hong GF (1983) Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80:3963–3965

Bull JJ, Hillis DM, O'Steen S (1988) Mammalian *ZFY* sequences exist in reptiles regardless of sex-determining mechanism. Science 242:567–569

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

Chromczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Duggal RN, Zakeri ZF, Ponzetto C, Wolgemuth DJ (1987) Differential expression of the *c-abl* proto-oncogene and the homeo box-containing gene Hox 1.4 during mouse spermatogenesis. Ann NY Acad Sci 513:112–127

Eicher EM, Washburn LL (1986) Genetic control of primary sex determination in mice. Annu Rev Genet 20:327–360

Goldman DS, Kiessling AA, Millette CF, Cooper GM (1987) Expression of c-mos RNA in germ cells of male and female mice. Proc Natl Acad Sci USA 84:4509–4513

Goodfellow PN, Darling SM (1988) Genetics of sex determination in man and mouse. Development 102:251–258

Hodgkin J (1988) Right gene, wrong chromosome. Nature 336:712

Lau Y-F, Chan K, Kan YW, Goldberg E (1988) Male-enhanced expression and genetic conservation of a gene isolated with an anti-H-Y antibody. Trans Assoc Am Physicians 100: 45–53

Lau Y-FC, Chan K, Sparkes R. The male-enhanced antigen gene is phylogenetically conserved and expressed at late stages of spermatogenesis. Proc Natl Acad Sci USA (in press)

Lau Y-F, Kan YW (1983) Versatile cosmid vectors for the isolation, expression and rescue of gene sequences: studies of the human alpha-globin gene cluster. Proc Natl Acad Sci USA 80:5225–5229

Lau Y-FC, Yang-Feng T, Elder B, Chan K, Fredga K, Wiberg U. Possible involvement of the *Zfx* gene in sex determination: a case study of the wood lemming (submitted)

McLaren A (1988) Sex determination in mammals. Trends Genet 4:153–157

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Mardon G, Mosher R, Disteche CM, Nishioka Y, McLaren

A, Page DC (1989) Duplication, deletion, and polymorphism in the sex-determining region of the mouse Y chromosome. Science 243:78–80

Mardon G, Page DC (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

Mutter GL, Wolgemuth DJ (1987) Distinct development patterns of *c-mos* protooncogene expression in female and male mouse germ cells. Proc Natl Acad Sci USA 84:5301–5305

Nagamine CM, Chan K, Hake LE, Lau Y-FC. The two candidate testis-determining Y genes (*Zfy-1, Zfy-2*) are differentially expressed in fetal and adult mouse tissues (submitted)

Nagamine CM, Chan K, Kozak CA, Lau Y-F (1989) Chromosome mapping and expression of a putative testis-determining gene in mouse. Science 243:80–83

O W-S, Short RV, Renfree MB, Shaw G (1988) Primary genetic control of somatic sexual differentiation in a mammal. Nature 331:716–717

Page DC (1988) Is *ZFY* the sex-determining gene on the human Y chromosome? Phil Trans R Soc Lond [B] 322: 155–157

Page DC, Mosher R, Simpson EM, Fisher EMC, Mardon G, Pollack J, McGillivray B, et al (1987) The sex-determining region of the human Y chromosome encodes a finger protein. Cell 51:1091–1104

Ponzetto C, Wolgemuth DJ (1985) Haploid expression of a unique *c-abl* transcript in the mouse male germ line. Mol Cell Biol 5:1791–1794

Propst F, Rosenberg MP, Iyer A, Kaul K, Vande Woude GF (1987) *c-mos* proto-oncogene RNA transcripts in mouse tissues: structural features, developmental regulation, and localization in specific cell types. Mol Cell Biol 7:1629–1637

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn KB, Mullis KB, et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sanger F, Nicklin S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 75:5463–5467

Serrentino V, McKinney MD, Giorgi M, Geremia R, Fleissner E (1988) Expression of cellular protooncogenes in the mouse male germ line: a distinct 2.4-kilobase *pim-1* transcript is expressed in haploid postmeiotic cells. Proc Natl Acad Sci USA 85:2191–2195

Sinclair AH, Foster JW, Spencer JA, Page DC, Palmer M, Goodfellow PN, Marshall-Graves JA (1988) Sequences homologous to *ZFY*, a candidate human sex-determining gene, are autosomal in marsupials. Nature 336:780–783

Wood WI, Gitschier J, Lasky LA, Lawn RM (1985) Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci USA 82:1585–1588

Young RA, Davis RW (1983) Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA 80:1194–1198