The two candidate testis-determining Y genes (*Zfy-1* and *Zfy-2*) are differentially expressed in fetal and adult mouse tissues

Claude M. Nagamine,¹ Kaimin Chan,¹ Laura E. Hake,² and Yun-Fai Chris Lau^{1,3}

¹Howard Hughes Medical Institute and Departments of Physiology and Medicine, University of California, San Francisco, California 94143 USA; ²Tufts University, Department of Biology, Medford, Massachusetts 02155 USA

The candidate testis-determining Y genes of the mouse *Zfy-1* and *Zfy-2*, encode proteins containing an acidic amino terminus and a carboxyl terminus composed of 13 zinc fingers. The zinc finger domain is conserved among human and mouse zinc finger X and Y genes. We report a 6-amino-acid deletion in the *Zfy-2* zinc finger domain of laboratory mice possessing *musculus* Y chromosomes. The effect of this deletion on the function of *Zfy-2* is not known. The reverse transcriptase–polymerase chain reaction (RT–PCR) and Northern blot techniques were used to study expression of *Zfy* in adults and fetuses. In adults, the data suggest that *Zfy-1* and *Zfy-2* transcription is linked to spermatogenesis, that transcription increases with the initiation of meiosis, and that high levels of these mRNAs are found in postmeiotic round spermatid cells. The data also suggest that differential expression of these two genes is present with expression of *Zfy-2* being slightly greater than *Zfy-1*. In fetuses, *Zfy* transcripts were detected in several tissues, including the testes. In contrast to the situation in adults, the data suggest that expression of *Zfy-1* is greater than that of *Zfy-2*. The data suggesting that *Zfy-1* expression is present in fetal testes support the hypothesis that this gene plays a role in testis differentiation. However, because the *Zfy* genes are apparently also expressed during spermatogenesis and in fetal organs other than testes, they may serve additional functions besides their postulated role in testis determination.

[Key Words: *Zfy*; *Tdy*; zinc finger protein; testis determination; spermatogenesis]

Received August 22, 1989; revised version accepted November 1, 1989.

The Y chromosome is testis-determining in mammals. The gene(s) on the Y chromosome responsible for initiating testis differentiation has been designated the testis-determining factor (*TDF*) in man and testis-determining Y (*Tdy*) in the mouse (Eicher and Washburn 1986; McLaren 1988). Page and co-workers (1987) have identified a candidate gene for *TDF*. This gene encodes a zinc finger protein, a class of DNA/RNA-binding proteins originally described for the *Xenopus laevis* oocyte transcription factor IIIA gene (Brown et al. 1985; Miller et al. 1985). In the absence of formal proof of its testis-determining capabilities, the candidate *TDF* gene has been designated zinc finger Y (*ZFY*) (Page 1988). Four homologous *ZFY* sequences exist in the mouse: two within the testis-determining region of the Y chromosome (*Zfy-1*, *Zfy-2*), one close to the androgen receptor locus on the X chromosome (*Zfx*); and one on chromosome 10 (*Zfa*) (Mardon et al. 1989; Mitchell et al. 1989; Nagamine et al. 1989a). Although both *Zfy-1* and *Zfy-2* are present in males, testis differentiation is possible with *Zfy-1* alone (Mardon et al. 1989; Nagamine et al. 1989a). The *Zfy-1* and *Zfy-2* genes both encode similar proteins (782 and 783 amino acids, respectively), consisting of an

acidic amino-terminal domain, a short stretch of basic residues, and a carboxy-terminal domain of 13 putative zinc-binding fingers arranged tandemly in two-finger units (Ashworth et al. 1989; Mardon and Page 1989). Although the transcription of both *Zfy* genes in adult testes has been demonstrated by the reverse transcriptase–polymerase chain reaction (RT–PCR) technique (Nagamine et al. 1989a), Northern blot analysis (Mardon and Page 1989), and molecular cloning of the corresponding cDNAs (Ashworth et al. 1989; Mardon and Page 1989; Nagamine et al. 1989a), the relative level of expression of these two genes is unclear. In addition, Northern blot analysis failed to detect any *Zfy* transcripts in fetal mice at the time of gonadal differentiation (Mardon and Page 1989). This finding contradicts the assumption that *Zfy* must be active in male development during testis differentiation.

PCR and RT–PCR are extremely sensitive techniques (Kawasaki et al. 1988; Li et al. 1988; Rappolee et al. 1988; Saiki et al. 1988). PCR allows the exponential amplification of a DNA segment by repeated cycles of in vitro DNA synthesis using gene-specific oligonucleotide primers and *Taq* DNA polymerase (Saiki et al. 1988). RT–PCR is a PCR modification in which mRNAs are first converted to cDNAs using oligo(dT) or random hex-

³Corresponding author.

amer primers and Moloney murine leukemia virus–reverse transcriptase (MMLV–RT). The resulting cDNAs, which are representative of the starting mRNA population, are subsequently subjected to PCR amplification (Kawasaki et al. 1988; Rappolee et al. 1988; Saiki et al. 1988; Doherty et al. 1989). We employed the sensitive RT–PCR technique and conventional Northern blot analysis to study *Zfy-1* and *Zfy-2* expression in adult and fetal mice. We present data suggesting that *Zfy* expression is linked to spermatogenesis in adults. In fetuses, *Zfy* mRNAs were detected in several fetal organs, including the testes, suggesting that these genes play a role in differentiation of several organs. Furthermore, in both adults and fetuses, the data suggest that the *Zfy* genes were differentially expressed. However, although *Zfy-2* transcripts were present at a slightly higher level than that of *Zfy-1* in adults, the reverse pattern was observed in fetuses.

Results

PCR detects a *Zfy*-fragment length polymorphism

To identify useful RT–PCR primers, several oligonucleotide primers were derived from the *Zfy* partial cDNA clone, mYfin. One set of primers, which flank a 600-bp segment, gave four male-specific fragments on polyacrylamide gels (Fig. 1A–D). The male specificity of the fragments (Fig. 1, male versus female) suggests that they are derived from *Zfy-1* and/or *Zfy-2* and not *Zfx* or *Zfa*. To identify the exact origins of these fragments, we correlated their presence or absence with the *Zfy* loci in sex-reversed mice (Cattanach et al. 1971; McLaren et al. 1984). Males carrying the sex-reversed mutation (XYSxr) have a duplication of a segment of their Y chromosome, the Sxr region, which contains the *Zfy-1* and *Zfy-2* loci (Mardon et al. 1989; Nagamine et al. 1989a). Fifty percent of their XX progeny inherit the Sxr region and develop into sex-reversed males (XXSxr). In a variant of the sex-reversed mutation, *Sxr'*, the *Zfy-2* locus in the Sxr region is deleted (Mardon et al. 1989; Nagamine et al. 1989a). Consequently, XXSxr' males have only the *Zfy-1* locus. PCR amplification using genomic DNAs from XXSxr' and mYfin plasmid DNA suggest that fragment C (618 bp) is derived from *Zfy-1*, as only this fragment was amplified using XXSxr' DNA (Fig. 1; XXSxr'). Fragment D (600 bp) must be derived from *Zfy-2*, as it was absent in the XXSxr' sample. Because D was the only fragment amplified using the *Zfy* mYfin cDNA (Fig. 1; mYfin), the mYfin cDNA was derived from the *Zfy-2* locus. We conclude that fragments C and D are derived from the *Zfy-1* and *Zfy-2* loci, respectively, and that our primer set identifies a *Zfy* PCR fragment length polymorphism (PFLP).

Fragments A and B were identified as heteroduplexes composed of C and D complementary strands (Nagamine et al. 1989b). The spurious migration of these two fragments in polyacrylamide gels is attributable to a combination of secondary structure and differences in nucleotide composition, and the capability of polyacrylamide gels to discriminate these differences. Because

Figure 1. PCR-amplified *Zfy* products derived from genomic DNAs of normal male and female BALB/c mice (lanes 1 and 2), XXSxr and XXSxr' sex-reversed males (lanes 3 and 4), YYSxr and YYSxr' males carrying the sex-reversed mutation (lanes 5 and 6), and pUC18 plasmid containing the *Zfy-2* mYfin insert (mYfin, lane 7). Four male-specific fragments are resolved on ethidium bromide-stained polyacrylamide gels (A–D). A and B are PCR artifacts (see text). C and D represent *Zfy-1* and *Zfy-2* loci, respectively.

fragments A and B are PCR artifacts, they will not be considered further [for detailed discussion, see Nagamine et al. (1989b)].

XYSxr' carrier males have two copies of *Zfy-1* but only one copy of *Zfy-2*. An important observation regarding the future use of the *Zfy* primers to study *Zfy* differential expression is their capability to demonstrate this 2 : 1 difference in copy number. This is illustrated by the brighter intensity of the C band relative to D in YYSxr' males (Fig. 1; YYSxr'). This capability is attributable to the *Zfy-1* and *Zfy-2* target sequences being nearly identical (see Materials and methods). Yields of the *Zfy-1*- and *Zfy-2*-amplified products are therefore dependent, in part, on the concentration of their respective templates. We conclude that when used in RT–PCR, the *Zfy* primers should be capable of detecting a differential expression of *Zfy-1* and *Zfy-2* within a sample. However, the differences should only be viewed qualitatively, as the exact ratio of *Zfy-1*/*Zfy-2*-amplified products cannot be determined using ethidium bromide-stained gels. Compared to normal males, YYSxr males have a duplication of their *Zfy-1* and *Zfy-2* genes. PCR could not easily detect this duplication (Fig. 1; male versus YYSxr), except under carefully controlled experimental conditions. Therefore, RT–PCR would not be reliable in detecting differences of *Zfy* expression between samples, except in obvious cases (e.g., Fig. 1; male versus female).

The *Zfy-2* gene of the musculus Y chromosome harbors a deletion in its zinc finger domain

To identify the molecular basis for the *Zfy* PFLP, fragments C and D were isolated, subcloned into pUC plasmid and M13 bacteriophages, and sequenced. As expected, the sequence of fragment D was identical to its corresponding region in mYfin. In the C sequence, three point mutations were identified. Because the C sequence was obtained from a single subclone, it is uncertain whether these point mutations are real or the result of *Taq* DNA polymerase error (Saiki et al. 1988). More significantly, comparing C and D revealed a stretch of 18 bp

that was deleted in D (*Zfy-2*) and was solely responsible for the PFLP. To verify the presence of this deletion in the *Zfy-2* gene, a 5' primer that included the 18 bp (underlined) was synthesized (5'-ATGCTGGGACTTTGTGTACTCAGAAA-3'). PCR amplification using this 5' primer and our original 3' primer should be *Zfy-1*-specific. As expected, PCR amplification of XXSxr', XXSxr, and normal BALB/c male DNAs gave a single fragment representing the *Zfy-1* locus, and no amplification was obtained with either female DNA or the mYfin *Zfy-2* clone (data not shown). We conclude that the *Zfy* PFLP is the result of an 18-bp deletion in the *Zfy-2* locus.

The cDNA sequences of *Zfy-1* and *Zfy-2* were published recently (Ashworth et al. 1989; Mardon and Page 1989). Within the region flanked by the primers (see Materials and methods), except for a single nucleotide mismatch and the 18-bp deletion, the mYfin and published *Zfy-1* and *Zfy-2* sequences are identical. Significantly, the deletion identified in the mYfin *Zfy-2* clone was not present in the published *Zfy-2* sequence. Previous investigators had determined that laboratory mouse strains possess Y chromosomes derived from a minimum of two, and possibly four, subspecies; *Mus mus domesticus*, *M. m. musculus*, *M. m. molossinus*, and *M. m. castaneus* (Lamar and Palmer 1984; Bishop et al. 1985; Nishioka 1987; Nishioka and Lamothe 1987). The *M. m. musculus*, *M. m. molossinus*, and *M. m. castaneus* Y chromosomes are presently indistinguishable (Nishioka 1987) and will be referred to as *musculus* Y chromosomes. The published *Zfy-2* sequence was derived from the FVB/N strain (*domesticus* Y) (Mardon et al. 1989), whereas the mYfin clone was derived from the BALB/c strain (*musculus* Y) (Nagamine et al. 1989a). A logical explanation for the discrepancy is that the deletion is specific to the *Zfy-2* gene of the *musculus* Y chromosome. Indeed, when male genomic DNAs from various mouse strains were subjected to PCR amplification, the PFLP was observed only in strains with *musculus* Y chromosomes (A, BALB/c, CBA, C3H/He, C3H/An, C57BL/6, DBA/1, DBA/2, 129), whereas strains with *domesticus* Y chromosomes [AKR, B6.Y^{Dom}, B6.Y^{AKR}, BFM/2, CD-1, *M. m. domesticus* (Tirano), NCS, SJL, SWR, WLA, WMP] gave a single 618-bp fragment representing both *Zfy-1* and *Zfy-2* loci (Fig. 2). The deletion was also absent in two related *Mus* species, *M. spretus* and *M. caroli* (Fig. 2). We conclude that only laboratory strains with *musculus* Y chromosomes possess the *Zfy-2* deletion and that this deletion probably occurred after the separation of the *domesticus* and *musculus* lineages.

Zfy expression in adult males is restricted to the testes

Using RT-PCR, we had demonstrated previously that *Zfy* transcripts are present in adult testes (Nagamine et al. 1989a). Expression of these candidate testis-determining genes in the fully differentiated adults testis was unexpected and suggested that these genes may play more than one role. Northern blot analyses were performed to confirm and extend our previous findings. When probed with mYfin, Northern blots with poly(A)⁺

Figure 2. PCR-amplified *Zfy* products obtained using genomic DNAs from representative laboratory mice possessing *musculus* Y (A, BALB/c, CBA, DBA/1) or *domesticus* Y chromosomes [AKR, WLA, WMP, *M. domesticus* (Tirano)] and *M. spretus* and *M. caroli* species. A PFLP of *Zfy-1* and *Zfy-2* loci is present only in strains possessing *musculus* Y chromosomes.

RNAs from adult male brains and testes and total RNAs derived from adult male brains, hearts, kidneys, livers, lungs, and testes revealed a strongly hybridizing 3.0-kb band and a weaker 2.7-kb band only in testes samples (data not shown). Similar data were obtained independently by Mardon and Page (1989). The mYfin probe cross-hybridizes with the *Zfx* and *Zfa* homologs, and thus the origin(s) of the 3.0- and 2.7-kb mRNAs cannot be determined using mYfin alone (Nagamine et al. 1989b). However, the mYfin mRNA species are similar with respect to size (3.2 and 2.9 kb) and tissue distribution to mRNAs known to be derived specifically from the *Zfy* gene(s) (Mardon and Page 1989). It is reasonable to assume that the mRNAs observed using the two different probes are the same and that the transcripts obtained with mYfin are from the *Zfy* gene(s). The Northern blots confirmed our RT-PCR data that *Zfy* transcripts are present in testes, suggesting that these transcripts are present only in the testes in adults and indicate that at least two *Zfy* mRNA species (3.0 and 2.7 kb) are present.

To determine whether *Zfy* transcripts were present in other adult organs, but at levels below the sensitivity of Northern blots, we used the more sensitive RT-PCR technique on equivalent amounts of RNAs isolated from adult C57BL/6J (B6) male brains, hearts, kidneys, livers, lungs, and testes. Results from this analysis revealed *Zfy*-amplified products only in the testes sample (Fig. 3, top). In contrast, primers specific for the hypoxanthine phosphoribosyltransferase gene (*Hprt*) gave the *Hprt* fragment with all samples (Fig. 3, bottom), suggesting that the absence of *Zfy*-amplified products was not attributable to RNA or cDNA degradation. Thus, both the Northern and RT-PCR data are in agreement that among the organs tested, only the testes possess high levels of *Zfy* transcripts.

Increase in *Zfy* mRNAs is correlated with the initiation of meiosis

The adult testis possesses both somatic (e.g., Sertoli, Leydig, and myoid cells) and germ cell components. To

Figure 3. Amplified products obtained from the RT-PCR of total RNAs isolated from organs of adult males using *Zfy* (top) and *Hprt* (bottom) primers. *Zfy-1*- and *Zfy-2*-amplified products are visible only in the testes sample. The *Hprt* controls suggest that the absence of *Zfy*-amplified products is not attributable to RNA/cDNA degradation or suboptimal cDNA synthesis. Controls in which the reverse transcriptase was omitted were also amplified as a check for genomic DNA contamination; none was found (data not shown). The testes sample is overloaded.

determine the site(s) of *Zfy* expression in the adult testis, we studied *Zfy* expression in (1) the maturing testis, (2) testes from sterile males of mutant mouse strains, and (3) fractionated germ cells.

As a mouse testis matures, it differs both qualitatively and quantitatively with respect to its somatic and germ cell populations. Prior to 8 days of age, in addition to somatic cells, only spermatogonia are present. Meiosis initiates at 8–10 days of age, and primary spermatocytes reach the early pachytene stage of meiotic prophase at about 14 days of age. Secondary spermatocytes and the earliest postmeiotic germ cell, the round spermatids, are first seen at ~18 days of age. With maturation, the percentage of Sertoli cells in the testis decreases from 84% at day 6 to 29% at day 18 (Nebel et al. 1961; Bellvé et al. 1977). RT-PCR was performed on equal amounts of testicular RNAs derived from newborn, 7-, 14-, 21-, and 35-day-old B6 (Fig. 4a) and newborn, 7-, 10-, 14-, 21- and 28-day-old BALB/c mice (data not shown). *Zfy*-amplified products were only weakly visible in newborn and 7-day-old samples but were conspicuous in samples from mice at 10 days of age and older (Fig. 4a). We conclude that the increase of *Zfy* transcripts in the maturing testis, as suggested by the presence of conspicuous bands on ethidium bromide-stained gels, correlates with the initiation of meiosis.

We studied expression of the *Zfy-1* and *Zfy-2* genes relative to each other by exploiting the capability of the *Zfy* primers to detect differential expression of these genes within a sample. Careful examination of the gels revealed the *Zfy-2* band to be slightly brighter than that of *Zfy-1* (Fig. 4a). This observation suggests that the *Zfy-1* and *Zfy-2* genes are being expressed differentially in the prepubertal and adult testis, with *Zfy-2* being transcribed at a level slightly higher than *Zfy-1*.

Northern blots containing total RNAs isolated from newborn, 7-, 14-, 21-, and 35-day-old B6 mice revealed the 3.0- and 2.7-kb *Zfy* mRNAs to be detectable first at 21 days of age and a stronger hybridization signal to be present at 35 days of age (Fig. 4c). The discrepancy between the RT-PCR and Northern data as to when *Zfy*

transcripts are first detected can be attributed to the greater sensitivity of the RT-PCR technique (Kawasaki et al. 1988; Rappolee et al. 1988). Although incapable of detecting low levels of *Zfy* transcripts, Northern blot analysis can illustrate differences more clearly in high levels of *Zfy* mRNAs, for example, at ages 21 versus 35 days. Thus the RT-PCR and Northern blot techniques complement each other.

Expression of Zfy in the testes of sterile males of mutant mouse strains

The relationship between spermatogenesis and *Zfy* expression was examined in the testes of three mutant mouse strains in which adult males are sterile as a result of blocks at specific stages of spermatogenesis. The testes of adult XXSxr and XXSxr' sex-reversed mice possess Sertoli and Leydig cells but lack germ cells as a result of a degeneration of spermatogonia prior to 10 days of age (Cattanach et al. 1971; Burgoyne et al. 1986). In male mice hemizygous for the X-linked testicular fe-

Figure 4. (a and b) Ethidium bromide-stained gel of amplified products from the RT-PCR of total testicular RNAs derived from C57BL/6 (B6) mice of the following ages: newborn (0), 7, 14, 21, and 35 days postpartum. Amplified products were obtained using *Zfy* (a) and *Hprt* (b) primers. B6 mice possess a *musculus* Y chromosome. Although not discernible here, *Zfy*-amplified products for the newborn and 7-day testes samples were weakly visible on the gel. A slightly higher quantity of *Zfy-2* transcripts relative to *Zfy-1* is suggested by the brighter intensity of the *Zfy-2* band. The *Hprt* controls suggest that the low level of *Zfy*-amplified products in newborn and 7-day testes samples is not attributable to RNA/cDNA degradation or suboptimal cDNA synthesis. No amplified products were obtained with either *Zfy* or *Hprt* primers in samples in which the MMLV-RT was omitted, suggesting that genomic DNA contamination is absent. (+) With MMLV-RT; (–) without MMLV-RT. (c) Autoradiograph of a Northern blot containing total testicular RNAs derived from B6 mice, aged newborn, 7, 14, 21, and 35 days postpartum. Hybridization was with the *Zfy-2* probe mYfin. Ten micrograms of the same RNA preparations used in the RT-PCR analysis in a and b was loaded per lane. Although not shown, rehybridization with a β -actin probe confirmed that equal quantities of RNAs were loaded per lane. The considerable difference in levels of sensitivity between the RT-PCR and Northern blot techniques is clearly demonstrated because *Zfy* transcripts are easily detectable only at 21 days of age in the Northern blot and, then, only weakly.

minizing mutation (*Tfm/Y*), spermatogenesis does not progress past the first meiotic prophase (Lyon and Hawkes 1970). Although spermatogenesis progresses normally up to the spermatid stage, in mice homozygous for the quaking mutation (*qk/qk*), spermiogenesis is abnormal and few spermatozoa form (Bennett et al. 1971). RT-PCR analysis of testicular RNAs from five XXSxr, three XXSxr', two *Tfm/Y*, and two *qk/qk* males revealed conspicuous *Zfy* bands for *Tfm/Y* and *qk/qk* samples but not for sex-reversed male samples (Fig. 5, top). The *Tfm/Y* data support the developmental data, suggesting that *Zfy* expression increases during the first meiotic prophase. In addition, the sex-reversed male data suggest that the *Zfy* genes are not transcribed at high levels in the adult somatic cell population and thus the germ cell population is the probable source of the *Zfy* transcripts.

Northern blot analysis with total testicular RNAs from the mutant mice revealed a hybridization signal in *qk/qk* samples equivalent to normal testes but only a very weak signal from *Tfm/Y* and no signal from sex-reversed male samples (data not shown). The weak Northern signal for *Tfm/Y* testes samples suggests that the concentration of *Zfy* transcripts is lower than in normal testes. Germ cells in *Tfm/Y* testes do not complete meiotic prophase, whereas those in normal testes undergo two meiotic cell divisions. Therefore, a normal testis possesses, at minimum, 4 times more maturing germ cells than a *Tfm/Y* testis. If *Zfy* transcription is linked to maturing germ cells (see below), then considerably less *Zfy* mRNAs will be present in *Tfm/Y* testes. The discrepancy between the RT-PCR and Northern blot data for the *Tfm/Y* sample can be attributed to the higher sensitivity of the RT-PCR technique in detecting these low levels of *Zfy* mRNAs.

Zfy transcription is linked to maturing germ cells

To aid in identifying the source(s) of *Zfy* mRNAs in adult testes, a Northern blot analysis using total RNA

Figure 5. Amplified products from the RT-PCR of total testicular RNAs from normal and mutant mouse strains using *Zfy* (top) and *Hprt* (bottom) primers. *Zfy*-1 and *Zfy*-2 bands are prominent only in B6, *Tfm/Y* (*Tfm*), and *qk/qk* (*Qk*) mice. Occasional sex-reversed male samples gave barely discernible bands in ethidium bromide-stained gels (lane 6). We surmise that this faint band represents rare populations of XOSxr spermatogenic cells that are viable and capable of undergoing spermatogenesis (Cattanach et al. 1971). (+) With MMLV-RT; (–) without MMLV-RT.

from fractionated adult pachytene spermatocytes (90% pure), adult round spermatids (95% pure), spermiogenic by-products (residual bodies/cytoplasmic fragments, 85% pure), and adult testes was performed. The 3.0- and 2.7-kb *Zfy* mRNAs were prevalent in round spermatids (Fig. 6a, RS). Only weak signals were obtained from the pachytene spermatocyte and residual body/cytoplasmic fragments fractions (Fig. 6a; PS, RB, respectively). No evidence of RNA degradation was found either by examining the ethidium bromide-stained agarose gel prior to transfer (data not shown) or stripping and rehybridizing the filter with a human β -actin probe (Fig. 6b). We conclude that the high levels of *Zfy* transcripts in the adult testis can be attributed, in part, if not entirely, to the maturing germ cell population and that these transcripts are most abundant in the round spermatid population.

RT-PCR on total RNAs isolated from fractionated testicular cells resulted in all fractions giving a conspicuous *Zfy* band, with no obvious difference in the quantity of *Zfy*-amplified products obtained from the different fractions (Fig. 6c). These data suggest that the levels of *Zfy* mRNAs in the germ-cell fractions were sufficiently high such that the maximum amount of *Zfy*-amplified products was obtained for all fractions

Figure 6. (a and b) Autoradiograph of a Northern blot containing 10 μ g of total RNAs isolated from cell fractions enriched in adult pachytene spermatocytes (PS), adult round spermatids (RS), and adult residual body/cytoplasmic fragments (RB), and from adult testes (T). The blot was hybridized with the *Zfy*-2 mYfin probe (a), stripped, and rehybridized with a human fibroblast cytoplasmic β -actin probe (b). Exposure was for 4 days and 5 hr, respectively. Arrows indicate the 3.0- and 2.7-kb *Zfy* mRNAs. In addition to giving information on the integrity and loading of the RNAs, the lower β -actin band, which is an mRNA species specific to maturing spermatids (Waters et al. 1985), confirms the staging of the germ cell fractions. (c) *Zfy* amplified products from the RT-PCR of RNAs derived from cell fractions enriched in adult pachytene spermatocytes, adult round spermatids, and adult residual body/cytoplasmic fragments. Because the RNAs were derived from CD-1 males that possess *domesticus* Y chromosomes, the single *Zfy* fragment represents both *Zfy*-1 and *Zfy*-2 transcripts. Although not shown, *Hprt* controls and controls in which the reverse transcriptase was omitted were performed; no evidence of RNA/cDNA degradation or genomic DNA contamination was found.

under our RT-PCR conditions. This interpretation is in keeping with the developmental and mutant mice data, suggesting that sufficient *Zfy* mRNAs are present in pachytene spermatocytes to be easily detected by RT-PCR (Figs. 4 and 5). Furthermore, the Northern blot data on fractionated germ cells suggest that even higher concentrations of *Zfy* transcripts exist in the round spermatid cell fraction (Fig. 6a). Thus, it is not surprising that no discernible difference was noted by RT-PCR among the different cell fractions.

Zfy appears to be silent in the adult XY ovary

Are high levels of *Zfy* transcription restricted to testes or can these Y-encoded genes also be expressed in XY ovaries? To answer this question, we used the B6.Y^{Dom} strain, which exhibits XY sex reversal (Nagamine et al. 1987a,b). XY sex reversal occurs when the Y chromosome of certain populations of *M. m. domesticus* is introduced into the B6 strain (Eicher et al. 1982). Half of the XY progeny develop bilateral ovaries, whereas the remainder develop varying degrees of ovotestes (Biddle and Nishioka 1988; Taketo-Hosotani et al. 1989). It has been proposed that this XY sex reversal is a result of an improper interaction between the *M. m. domesticus* *Tdy* gene and a B6 autosomal gene called *testis-determining autosomal-1* (Eicher et al. 1982; Eicher and Washburn 1986). B6.Y^{Dom} XY gonads can be categorized as testicular, ovotesticular, or ovarian (Nagamine et al. 1987a). Testicular gonads appear as normal testes at the gross anatomical level. Histologically, these gonads often have oocytes among spermatogenic cells, demonstrating that they are, in fact, ovotestes. Ovotesticular gonads are accompanied by both Mullerian- and Wolffian-duct derivatives and possess developing oocytes; spermatogenesis may or may not be present. Ovarian gonads are identical to normal ovaries at the gross anatomical level. Developing oocytes are present, and no evidence of spermatogenesis is found in histological sections. Four adult B6.Y^{Dom} mice of the N15-16 backcross generations were studied. One possessed bilateral testicular gonads; the rest were overt hermaphrodites possessing a left testic-

ular gonad and a right ovotesticular or ovarian gonad. RNA from each gonad was independently isolated and subjected to RT-PCR. In ethidium bromide-stained gels, *Zfy*-amplified products were clearly visible in all B6.Y^{Dom} testicular gonad samples (Fig. 7, lanes 1–3, 5, 7). In contrast, no *Zfy* fragments were visible in the XY ovotesticular (Fig. 7, lane 4) or two XY ovarian gonad samples (Fig. 7, lanes 6, 8). The absence of *Zfy*-amplified products in the ovotesticular gonad sample suggests that spermatogenesis was absent, a finding not unusual for ovotesticular gonads (Nagamine et al. 1987a). The B6.Y^{Dom} data support the previous data suggesting that *Zfy* expression is linked to spermatogenesis. In addition, the data demonstrate that the *Zfy* genes are silent or expressed at very low levels in the adult XY ovarian environment.

Zfy is expressed in fetal tissues

An important question regarding the *Zfy-1* and *Zfy-2* loci is whether these candidate testis-determining genes are indeed involved in testis determination. RT-PCR was performed on RNA isolated from B6 fetal tissues aged 12 days postcoitum (pc), the age when testicular differentiation is first detectable under the dissecting microscope. In contrast to adult samples, two important differences were noted. First, *Zfy*-amplified products were clearly visible in fetal kidney, liver, and testes samples but only weakly or not at all in heart or lung samples (Fig. 8a). Second, in contrast to adult testes, where the level of *Zfy-2* mRNAs was slightly greater than that of *Zfy-1* (Fig. 8c), the reverse was true in fetal tissues (Fig. 8a). Indeed, the *Zfy-2* band was often only weakly visible (Fig. 8a). Identical results were obtained using both random hexamers or oligo(dT) as primers for cDNA preparation. We conclude that *Zfy-1* and *Zfy-2* are expressed in fetal tissues, but their expression is not restricted to the developing testis. Furthermore, the data suggest that although these two genes are again differentially expressed, in contrast to the situation in adults, *Zfy-1* transcription is higher than *Zfy-2* in fetuses.

Discussion

Studies of the Zfy gene illustrate the evolution of the genus Mus

The phylogeny of the genus *Mus* is currently being revised (Potter et al. 1986). In addition, despite the laboratory mouse being one of the most studied animal models, its origin from wild progenitors is not well defined. Indeed, it is still not known whether the *musculus* Y of laboratory mice was derived from one or several subspecies (Nishioka 1987). Because the *ZFY* genes are not normally subject to recombination and are transmitted essentially unchanged from father to son, they serve as a useful molecular tool in evolutionary studies. The *ZFY* genes are conserved among a variety of placental mammalian species, and in most of these species, a single *ZFY* gene exists (Page et al. 1987). Southern blot analyses revealed two *ZFY* loci (*Zfy-1* and *Zfy-2*) in labo-

Figure 7. Amplified products from the RT-PCR of RNAs derived from B6.Y^{Dom} adult XY gonads. The gonads from four B6.Y^{Dom} mice (i–iv) were individually studied. (Lanes 1 and 2) Left and right testicular gonads from i; (lanes 3 and 4) left testicular and right ovotesticular gonads from ii; (lanes 5 and 6) left testicular and right ovarian gonads from iii; (lanes 7 and 8) left testicular and right ovarian gonads from iv. Although not shown, *Hprt* controls and controls in which the reverse transcriptase was omitted were performed; no evidence of RNA/cDNA degradation or genomic DNA contamination was found. Because B6.Y^{Dom} mice possess *domesticus* Y chromosomes, the single *Zfy* fragment represents both *Zfy-1* and *Zfy-2* transcripts (see text).

Figure 8. Amplified products from the RT-PCR of RNAs isolated from organs derived from B6 male fetuses at day 12 postcoitum are shown using *Zfy* (a) and *Hprt* (b) primers. (c) For comparison, *Zfy*-amplified products from the RT-PCR of RNAs from 14-day prepubertal B6 testes. *Zfy-1* transcripts are discernible in kidney, liver, and testes samples. A weak band was visible for heart and lung samples on the gel. Although *Zfy-2*-amplified products can be discerned only in the testes sample, they were also visible in the kidney and liver samples on the original gel, albeit very weakly. All cDNA samples gave the specific *Hprt*-amplified product (b). The quantity of *Zfy-1*-amplified products is greater than that of *Zfy-2* in fetal tissues. This is in contrast to prepubertal and adult testes where *Zfy-2* expression is greater than *Zfy-1* (c). Although not shown, controls in which the reverse transcriptase was omitted were run for all samples; no *Zfy*-amplified products were obtained using these controls.

ratory mice (Mardon et al. 1989; Nagamine et al. 1989a). This duplication is present in the *M. m. domesticus* and *M. m. musculus* subspecies, and related *M. spretus* species, suggesting that the duplication arose in a common male ancestor that gave rise to the *M. spretus* and *M. musculus* lineages (Mardon et al. 1989; C.M. Nagamine et al., unpubl.). It is not known whether other *Mus* species possess the *Zfy* duplication. An RFLP of the two *Zfy* loci further segregates *M. m. musculus*, *M. m. domesticus*, and *M. spretus* species (Mardon et al. 1989; Nagamine et al. 1989b; C.M. Nagamine et al., unpubl.). We have identified a deletion in the *Zfy-2* gene of laboratory mice possessing the *musculus* Y chromosome. The absence of this deletion in *M. m. domesticus* and *M. spretus* suggests that these mice possess the ancestral *Zfy-2* gene. The deletion therefore appeared after the separation of the *M. m. domesticus* and *M. musculus* lineages. Other studies have suggested that four subspecies (*M. m. musculus*, *M. m. molossinus*, *M. m. castaneus*, and *M. m. bactrianus*) are more closely related to each other than to *M. m. domesticus* (Yonekawa et al. 1988). The Y chromosomes of the first three subspecies cannot be differentiated using molecular repeat probes (Nishioka 1987). None of these subspecies has been examined in detail for its *Zfy* gene(s) or the *Zfy-2* deletion. A Southern blot and PCR analysis of the *Zfy* gene(s) in *Mus* species and subspecies is now in progress. These data will complement previous studies on the evolution of the genus *Mus* (Potter et al. 1986) and further our understanding of the role the different *M. musculus* sub-

species has played in the generation of the various laboratory mouse strains.

Disruption of the Zfy-2 zinc finger domain has no obvious biological effect on strains carrying the musculus Y

Studies on the steroid hormone receptors (for review, see Evans 1988), as well as other zinc finger proteins (Hughes et al. 1988; Kadonaga et al. 1988; Redemann et al. 1988), have suggested that the finger domain of zinc finger proteins is responsible for DNA binding. Disruption of the finger domain by mutations can result in loss of biological function of the zinc finger protein (Evans 1988; Hughes et al. 1988; Redemann et al. 1988). Recently, complete cDNA sequences for the mouse *Zfy-1* and *Zfy-2* and human *ZFX* and *ZFY* genes were reported (Affara et al. 1989; Ashworth et al. 1989; Mardon and Page 1989; Schneider-Gädicke et al. 1989; Y.F.C. Lau and K. Chan, in press). Significantly, the finger domains of these zinc finger X and Y proteins were highly conserved. This led to the suggestion that the exact structure of the finger domain is crucial to the function of these proteins (Mardon and Page 1989; Schneider-Gädicke et al. 1989). The deletion of *Zfy-2* effectively disrupts the third zinc finger by removing the first histidine in the Cys-Cys/His-His zinc finger motif. Whether this affects the function of this *Zfy-2* protein remains to be determined. However, because laboratory mice possessing *musculus* Y have not been reported to be aberrant with respect to sex determination or spermatogenesis, the effect of the deletion may be minor or easily compensated by *Zfy-1*.

Expression of Zfy in adult mice is linked to spermatogenesis

Previous Northern blot analyses of fetal, newborn, and adult mouse tissues failed to detect *Zfy* mRNAs (Mardon and Page 1989), except to confirm the presence of these transcripts in adult testes (Nagamine et al. 1989a). The present RT-PCR and Northern blot data confirmed and expanded our initial study (Nagamine et al. 1989a) and studies of Mardon and Page (1989). We demonstrated that levels of *Zfy* transcripts increased during the initiation of meiosis and identified the postmeiotic round spermatid population as a major source of *Zfy* transcripts in the adult testis. Whether the *Zfy* mRNAs in round spermatids represents postmeiotic transcription or the accumulation of *Zfy* transcripts from earlier spermatogenic stages is not known (for a review on postmeiotic gene expression, see Erickson et al. 1981).

The sex-reversed mice data further suggested that the adult somatic cell population is not a major site of *Zfy* transcription. However, because these are mutant mice, one cannot rule out the possibility of *Zfy* transcription in the somatic cell population of normal adult testes. Indeed, studies on the proopiomelanocortin (POMC) gene have suggested that in the mutant mouse strain

atrichosis, POMC expression in their steroidogenically normal Leydig cells requires the presence of germ cells (Gizang-Ginsberg and Wolgemuth 1987). Additional studies using XOSxr and XOSxr' mice (Burgoyne et al. 1986), purified components of the somatic cell population, and/or in situ hybridization may clarify whether *Zfy-1* is transcribed in the somatic cells of the adult testis.

We have presented data suggesting that *Zfy-1* and *Zfy-2* are expressed differentially during spermatogenesis, with *Zfy-2* having a slightly higher level of expression. At present, it is not known whether the *Zfy-1* and *Zfy-2* transcripts are translated or whether the encoded proteins are functional. It is tempting to speculate that the higher transcriptional level of *Zfy-2* may be a compensatory mechanism for a reduced function of this *Zfy-2* protein because of its deletion. Alternatively, the *Zfy-2* may be simply be transcribed at a higher level than *Zfy-1* during spermatogenesis.

When the RT-PCR-amplified products from the various organs of adult males were analyzed using ethidium bromide-stained gels, only testes gave discernible *Zfy* fragments (Fig. 3). This is not to say that *Zfy*-amplified products could not be detected in the other samples. Southern blot analysis is at least 100 times more sensitive in detecting PCR-amplified products than ethidium bromide staining of gels (Saiki et al. 1988). When Southern blots of the RT-PCR-amplified samples were hybridized with a 213-bp probe specific for the *Zfy*-amplified fragment, a hybridization signal was obtained with adult brain, heart, kidney, liver, and lung samples; no signal was obtained in controls in which the reverse transcriptase was omitted (data not shown). Similarly, RT-PCR plus Southern blotting revealed *Zfy*-amplified products in XOSxr and XOSxr' samples. Whether these low levels of *Zfy*-amplified products represent a function for the *Zfy* genes in these tissues or in the case of sex-reversed testes, the presence of rare XOSxr spermatogonia that are viable and capable of undergoing spermatogenesis (Cattanach et al. 1971; Burgoyne et al. 1986; Burgoyne 1987) is not known. Recently, using RT-PCR plus Southern blot hybridization, other investigators have reported a similar low-level expression of genes in tissues in which they were previously thought to be silent (Chelly et al. 1988; Sarkar and Sommer 1989). It has been suggested that this low level of transcription may represent a basal level of expression for all genes (Chelly et al. 1988; Sarkar and Sommer 1989).

Zfy mRNAs can be detected in fetuses

The RT-PCR data suggest that *Zfy* is transcribed in fetal tissues. Because these data were obtained using ethidium bromide-stained gels alone, the expression level noted in fetuses is above the basal level of expression, noted above. Northern blot analyses of fetuses had failed to detect *Zfy* mRNAs previously (Mardon and Page 1989). Their negative results can be partially attributed to their isolation of RNA from whole fetuses. We have demonstrated that all tissues do not express *Zfy*

equally. We attribute our success in demonstrating *Zfy* transcripts in fetal tissues to our analysis of specific organs and, more importantly, in the use of the more sensitive RT-PCR technique. The RT-PCR data further suggest that *Zfy-1* is transcribed at a higher level than *Zfy-2* in fetal tissues. The reverse appears to be true for the expression of these genes during spermatogenesis. Because we examined only one fetal age in detail, the stage at which *Zfy-2* expression decreases during development remains to be determined.

Our data suggest that the *Zfy* genes are expressed both during fetal development and during spermatogenesis and thus may serve two different functions. The *Zfy* genes are not unique in this expression pattern. Other genes known to be expressed during embryonic or fetal development and spermatogenesis include the proto-oncogenes *c-mos* and *int-1* (Propst and Vande Woude 1985; Goldman et al. 1987; Shackleford and Varmus 1987), the homeo box-containing gene *Hox-1.4* (Wolgemuth et al. 1987), the heat shock protein gene *hsp70* (Zakeri and Wolgemuth 1987), and the male-enhanced antigen gene *Mea* (Lau et al. 1989; Lau 1990).

Are the *Zfy-1* and/or *Zfy-2* genes Tdy?

Mapping studies have demonstrated clearly that the human *ZFY* and the mouse *Zfy* genes are located on the testis-determining region of the Y chromosome of both species (Page et al. 1987; Mardon et al. 1989; Nagamine et al. 1989a). However, discrepant results were obtained from studies of other vertebrates. Southern blot and in situ hybridization experiments using the human *ZFY* probe have indicated that the homologous genes are located on the X chromosome or autosomes in reptiles, birds, and marsupials (Page et al. 1987; Bull et al. 1988; Sinclair et al. 1988). Because the marsupial Y chromosome is believed to be testis determining, the autosomal location of the *ZFY*-related sequences suggests that the homologous gene(s) may not be the primary testis-determining gene in marsupials (Sinclair et al. 1988). However, these discrepant data should be interpreted with caution. Southern blot data had suggested that the human *ZFY* gene is more homologous to the mouse *Zfx* and *Zfa* genes than it is to the *Zfy* genes (Mitchell et al. 1989; Nagamine et al. 1989a). Recently, the divergence of the human and mouse *ZFY* genes has been confirmed by sequencing data (Y.-F.C. Lau and K. Chan, in prep.). It is possible that the human *ZFY* probe shares even less homology with the zinc finger Y genes of the even more evolutionarily distant reptiles, birds, and marsupials and that the cross hybridizations reported represent *ZFX* and/or *ZFA* homologs.

Southern blot analyses of XOSxr' male mice have demonstrated that the *Zfy-1* gene, itself, can be correlated with testis determination (Mardon et al. 1989; Nagamine et al. 1989b). One interpretation of this observation is that *Zfy-1* may be the candidate testis-determining gene, whereas *Zfy-2* may be redundant or serve other functions. In support of this view is the present data demonstrating that expression of *Zfy-1* is greater

than *Zfy-2* in fetal tissues. Preliminary RT-PCR data suggest that *Zfy-1* and *Zfy-2* transcripts are also present in the indifferent fetal gonad at day 11 postcoitum with concentrations of *Zfy-1* again being higher than those of *Zfy-2*. In addition, we have also noted that levels of *Zfy-1* and *Zfy-2* are consistently higher in fetal testes as opposed to other fetal organs, albeit only slightly (Fig. 8a). These observations support, but do not prove, the hypothesis that the *Zfy-1* gene plays a special role in testis differentiation. Whether or not *Zfy-1* is the testis-determining gene, our data suggest that both *Zfy-1* and *Zfy-2* play a role in spermatogenesis and in the differentiation of several fetal organs, including the testes. Thus, the function of the *Zfy* genes must be broadened beyond their putative role in testis determination.

Materials and methods

Mice

Laboratory mice or their DNAs were obtained from the Institut Pasteur (Paris, France), Charles River (Kingston, New York), Simonsen (Gilroy, California), and the Jackson Laboratory (Bar Harbor, Maine). Mutant mice and their sources were XXSxr, *Tfm/Y*, and *qk/qk* (Jackson Laboratory); XXSxr and XYSxr (Institut Pasteur); XXSxr, XYSxr, XXSxr', and XYSxr' (Dr. A. McLaren, Medical Research Council, London); *Tfm/Y* (Dr. G. Cunha, University of California, San Francisco). B6.Y^{Dom} and B6.Y^{AKR} mice were described previously (Nagamine et al. 1987a, b).

Fetal dissection

C57BL/6 (B6) females (from Charles River or Jackson Laboratory) were mated with B6/J males and examined on the following morning for vaginal plugs (day of plug = day 0 pc). Females were sacrificed on day 12 postcoitum and the fetuses removed and kept on ice in sterile PBS until dissected. Fetal organs were removed under a dissecting microscope, dissected clear of adjoining tissues, and immediately frozen on dry ice or in liquid N₂. All testes were identifiable by the presence of tunica albuginea and seminiferous tubules. Total RNAs were prepared from pooled tissues according to Chomczynski and Sacchi (1987).

Generation of PCR primers and rationale for our RT-PCR controls

Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer. The *Zfy* primers were derived from the mYfin *Zfy-2* clone, a 1.28-kb partial cDNA derived from a BALB/c adult testis cDNA library (Nagamine et al. 1989a). The mYfin clone is identical to the published *Zfy-2* sequence of Mardon and Page (1989), starting from nucleotide 1297, except for an 18-bp deletion (nucleotides 1438–1455) and 9 nucleotide mismatches. Simultaneous amplification of *Zfy-1* and *Zfy-2* DNAs or cDNAs using the same *Zfy* primers (nucleotides 1336–1361, 1928–1953) is possible because the sequences of the primers are identical in both genes, and except for the deletion in *Zfy-2*, the fragments being amplified differ only by a single nucleotide (nucleotide 1757).

The *Zfy* primers do not span an intron; thus, RT-PCR cannot distinguish amplified products derived from cDNAs from those derived from contaminating genomic DNAs. However, adequate controls were employed to identify genomic

DNA contamination (see below). Comparable RT-PCR data have been obtained with a primer set that spans an intron and is specific for *Zfy-1*. However, the present primer set was preferable, as our goal was to study the transcriptional levels of *Zfy-1* and *Zfy-2* relative to each other.

Two RT-PCR controls were run for each sample. First, duplicate RNA samples similarly treated but omitting the reverse transcriptase were subjected to PCR amplification to check for contaminating genomic DNAs and contamination of our reagents with *Zfy*-amplified products or mYfin plasmid DNA. Because we treated all RNA preparations with DNase prior to reverse transcription, we never found contaminating genomic DNAs to be a problem, and careful experimental technique prevented contaminated reagents (e.g., see controls without MMLV-RT in Figs. 4 and 5). Second, an aliquot of each cDNA preparation was amplified using primers specific for the mouse *Hprt* gene. We chose *Hprt* as a control gene for the following reasons. (1) *Hprt* is X-linked and exists as a single copy in males, making it analogous to the single copies of *Zfy-1* and *Zfy-2*. (2) The *Hprt* primers could be designed to span introns as an additional test for genomic DNA contamination. (3) *Hprt* is ubiquitously expressed, allowing it to be used as a control for all tissues (Stout and Caskey 1985). (4) *Hprt* is expressed at low levels in most tissues (0.005–0.01% of total mRNA) (Stout and Caskey 1985), making it a sensitive indicator for RNA/cDNA degradation and/or suboptimal cDNA synthesis. Two sets of *Hprt* primers were used; both primer sets gave similar results. Two 5' primers were derived from exon 7 (nucleotides 603–626) and exon 8 (nucleotides 638–664), and a common 3' was derived from exon 9 (nucleotides 1260–1285) of the *Hprt* sequence of Melton et al. (1984).

PCR and RT-PCR technique

For PCR and RT-PCR amplification, 0.5 µg of genomic DNA or 1–5 µl of a 30-µl cDNA reaction was added to PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, 1.3 mM dNTP], containing 0.25–0.5 µM of each primer and 1.0 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a final volume of 50 µl. The mixture was overlaid with mineral oil and amplified in a Perkin-Elmer Cetus DNA thermal cycle for 30–40 cycles using a denaturing temperature of 94°C and an extending temperature of 72°C. The annealing temperature for the *Zfy* primers was 60°C and for the *Hprt* primer sets was 65°C. At the end of the cycling, the amplified products were extended at 72°C for an additional 10 min. The amplified products were size-fractionated on 10-cm 4% or 6% polyacrylamide gels and visualized by staining with ethidium bromide and viewing with a UV-transilluminator.

The *Zfy* primers give 600-bp (*Zfy-2*) and 618-bp (*Zfy-1*) products with strains possessing *musculus* Y chromosomes. The *Hprt* primers give cDNA and genomic DNA-amplified products of 683 and 1.48 kb, respectively, for the first primer set, and 648 and 1.25 kb, respectively, for the second set (Melton et al. 1984; Stout and Caskey 1985). Verification that the amplified products were specific was by predicted size analysis of the cDNA and/or genomic DNA-amplified products, restriction enzyme digestion, and, in the case of *Zfy*, Southern blotting using a 213-bp probe specific for the *Zfy*-amplified product.

RNA isolation and cDNA preparation

RNAs were isolated according to published protocols (Chirgwin et al. 1979; Chomczynski and Sacchi 1987). Prior to reverse transcription, the RNAs were treated with RNase-free RQ1

DNase (Promega, Wisconsin) to remove contaminating genomic DNAs. One to two micrograms of DNase-treated RNA was reverse-transcribed with 400–600 units of MMLV-RT (Bethesda Research Laboratories, Maryland) and 0.5–1.0 µg oligo [d(pT)_{12–18}] (Pharmacia, New Jersey) or random hexamers (Boehringer-Mannheim, Indiana) (Haymerle et al. 1986; Noonan and Roninson 1988) as primers in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1.5 units Inhibit-Ace (5 Prime-3 Prime, Inc., California), 3 µg BSA, 0.5 mM each dNTP, and 10 mM dithiothreitol in a final volume of 30 µl for 1 hr at 37°C. The reaction was terminated by heating at 95°C for 5 min.

Sequencing of the Zfy C and D fragments

Fragments C and D were isolated from PCR-amplified XXSxr DNA after resolving on polyacrylamide gels. The isolated fragments were inserted into pUC18 plasmid and subcloned into M13mp18 and M13mp19 bacteriophages (Norlander et al. 1983), and single-stranded DNAs were sequenced by the chain-termination method using ³⁵S-labeled dATP as tracer (Sanger et al. 1977, 1980).

Northern blot analysis

Total RNAs (10–15 µg) or poly(A)⁺ RNAs (3 µg) were glycoxy-lated, fractionated on 0.8% agarose gels, and transferred to Bio-trans nylon membranes (ICN, California) with 20× SSC, as described previously (Lau and Kan 1983). As markers, an RNA ladder (0.24–9.49 kb; Bethesda Research Laboratories) was run simultaneously. The RNAs were cross-linked to the nylon membrane with UV irradiation, baked, and hybridized with nick-translated ³²P-labeled probes. *Zfy-2*-hybridized blots were washed at 50°C in 2× SSC, 1% SDS, for 2× 30 min and exposed to Kodak XAR-5 film with two intensifying screens. Previous experiments demonstrated that washing at higher stringency did not change the results but required a longer period of autoradiography. Northern blots hybridized with human fibroblast cytoplasmic β-actin probe were washed at 65°C in 0.1× SSC, 1% SDS, for 2× 30 min. The *Zfy-2* mYfin clone (Nagamine et al. 1989b) and human fibroblast cytoplasmic β-actin clone (Gunning et al. 1983) have been reported.

Spermatogenic cell fractionation

Adult spermatogenic cells from CD-1 mice were fractionated by sedimentation velocity (Romrell et al. 1976). Samples from the fractions were analyzed under the microscope to identify cell types and to assess degree of purity; like fractions were pooled, and cells subjected to RNA isolation, as described previously (Alcivar et al. 1989).

Acknowledgments

We thank M. Doherty for generation of oligonucleotides, A. McLaren for *Sxr* and *Sxr'* mice, G. Cunha for *Tfm/Y* mice, B. Elder, D. Lin, and J. Gampell for helpful discussions, and G. Martin and Z. Werb for carefully reviewing this manuscript. This research was supported, in part, by the Howard Hughes Medical Institute and a National Institutes of Health grant (HD-24384) to Y.-F.C.L.

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The two candidate testis-determining Y genes (Zfy-1 and Zfy-2) are differentially expressed in fetal and adult mouse tissues.

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Genes Dev. 1990, **4**:

Access the most recent version at doi:[10.1101/gad.4.1.63](https://doi.org/10.1101/gad.4.1.63)

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