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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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]

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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 carboxyl-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-
amer primers and Moloney murine leukemia virus–reverse transcriptase [MLV–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, XYSxr' males have only the Zfy-1 locus. PCR amplification using genomic DNAs from XYSxr' and mYfin plasmid DNA suggest that fragment C [618 bp] is derived from Zfy-1, as only this fragment was amplified using XYSxr' DNA [Fig. 1, XYSxr']. Fragment D [600 bp] must be derived from Zfy-2, as it was absent in the XYSxr' 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).

Figure 1. PCR-amplified Zfy products derived from genomic DNAs of normal male and female BALB/c mice (lanes 1 and 2), XYSxr and XYSxr' sex-reversed males (lanes 3 and 4), XYSxr' 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 XYSxr' males [Fig. 1, XYSxr']. 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, XYSxr males have a duplication of their Zfy-1 and Zfy-2 genes. PCR could not easily detect this duplication [Fig. 1, male versus XYSxr'], 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′-ATGCTGAGACCTTT-

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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.

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Zfy expression in mice

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. 1989a]. 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/6 [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

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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 adult 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]*

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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 3.3- 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 XKSxr and XKSxr’ 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; Burgoane et al. 1986]. In male mice hemizygous for the X-linked testicular fe-
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), spermatogenesis is abnormal and few spermatzoa 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 testis. Therefore, a normal testis possesses, at minimum, 4 times more maturing germ cells than a testis. Therefore, a normal testis possesses, at minimum, 4 times more maturing germ cells than a testis. Therefore, a normal testis possesses, at minimum, 4 times more maturing germ cells than a testis. 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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.YDom 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.YDom 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.YDom mice of the N15-16 backcross generations were studied. One possessed bilateral testicular gonads; the rest were overt hermaphrodites possessing a left testicular 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.YDom 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.YDom 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-
Mus expression is greater than that of adult testes where controls in which the reverse transcriptase was omitted were run. 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. 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. 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.

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. 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. 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.

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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 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 [see 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-Gädde 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-Gädde 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].
proteins are functional. It is tempting to speculate that all tissues do not express Zfy-1 and Zfy-2, which may be simply be transcribed at a higher level than Zfy-1 during spermatogenesis.

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. These data were obtained using ethidium bromide-stained gels, only testes gave discernible Zfy fragments (Fig. 3). This is not to say that Zfy-2 is transcribed 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 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 XXSxr and XXSxr' samples. Whether low levels of Zfy-amplified products represent a function of the Zfy genes in these tissues or in the case of sex-reversed testes, the presence of rare XXSxr 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 Varma 1987), the homeobox-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 ZFY homologs.

Southern blot analyses of XXSxr' 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.
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), Simonson (Gilroy, California), and the Jackson Laboratory (Bar Harbor, Maine). Mutant mice and their sources were XXSxk, T7m/Y, and qk/qk [Jackson Laboratory]; XXSxr and XYsr [Institut Pasteur]; XXSxr, XYSr, XXSxk', and XYSr' [Dr. A. McLaren, Medical Research Council, London]; T7m/Y [Dr. G. Cunha, University of California, San Francisco]. B6.YDoom and B6.YARK mice were described previously [Nagamine et al. 1987a, b].

Fetal dissection

CS7BL/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 males were sacrificed on day 12 postcoitum and the fetuses prepared from pooled tissues according to Chomczynski and in liquid N2. All testes were identifiable by the presence of tunica albuginea and seminiferous tubules. Total RNAs were organs were removed under a dissecting microscope, dissected clear of adjoining tissues, and immediately frozen on dry ice or for an 18-bp deletion (nucleotides 1438-1455) and 9 nucleotide mismatches. Simultaneous amplification of DNAs or cDNAs using the same primers are identical in both genes, and except for the deletion in Zfy-I determining gene, our data suggest that both Zfy-1 and Zfy-2 are consistent in their putative role in testis determination.

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 mYin Zfy-2 clone, a 1.28-kb partial cDNA derived from a BALB/c adult testis cDNA library [Nagamine et al. 1989a]. The mYin 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 mYin 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 603–636] 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 Tag 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
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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 (Be- thedesa Research Laboratories, Maryland) and 0.5-1.0 µg oligo [d(T)]12-18 [Pharmacia, New Jersey] or random hexamers [Boehringer–Mannheim, Indiana] [Haymerie 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

Fragment 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 [Norrandet al. 1983], and single-stranded DNAs were sequenced by the chain-termination method using 32P-labeled dATP as tracer [Sanger et al. 1977, 1980].

Northern blot analysis

Total RNAs [10–15 µg] or poly(A)+ RNAs [3µg] 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 (0.24–4.9 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 RNAs were washed at 50°C in 2 × SSC, 1% SDS, for 2 × 30 min and ex- posed to Kodak XAR-5 film with two intensifying screens. Pre- vious experiments demonstrated that washing at higher string-ency did not change the results but required a longer period of autoradiography. Northern blots hybridized with human fibro- blast cytoplasmic β-actin probe were washed at 65°C in 0.1 × SSC, 1% SDS, for 2 × 30 min. The Zfy-2 mYin clone [Naga- mine et al. 1989b] and human fibroblast cytoplasmic β-actin clone [Gunning et al. 1983] have been reported.

Spermatogonic 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 pre- viously [Alcivar et al. 1989].

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