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Human and mouse *ZFY* genes produce a conserved testis-specific transcript encoding a zinc finger protein with a short acidic domain and modified transactivation potential

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Mammalian *ZFY* genes are located on the Y chromosome, and code putative transcription factors with 12–13 zinc fingers preceded by a large acidic (activating) domain. In mice, there are two genes, *Zfy1* and *Zfy2*, which are expressed mainly in the testis. Their transcription increases in germ cells as they enter meiosis, both are silenced by meiotic sex chromosome inactivation (MSCI) during pachytene, and *Zfy2* is strongly reactivated later in spermatids. Recently, we have shown that mouse *Zfy2*, but not *Zfy1*, is involved in triggering the apoptotic elimination of specific types of sex chromosomally aberrant spermatocytes. In humans, there is a single widely transcribed *ZFY* gene, and there is no evidence for a specific role in the testis. Here, we characterize *ZFY* transcription during spermatogenesis in mice and humans. In mice, we define a variety of *Zfy* transcripts, among which is a *Zfy2* transcript that predominates in spermatids, and a *Zfy1* transcript, lacking an exon encoding approximately half of the acidic domain, which predominates prior to MSCI. In humans, we have identified a major testis-specific *ZFY* transcript that encodes a protein with the same short acidic domain. This represents the first evidence that *ZFY* has a conserved function during human spermatogenesis. We further show that, in contrast to the full acidic domain, the short domain does not activate transcription in yeast, and we hypothesize that this explains the functional difference observed between *Zfy1* and *Zfy2* during mouse meiosis.

INTRODUCTION

In eutherian (placental) mammals, *ZFY* genes appear to play an important role in male development, as they have been found on the Y chromosome in many species including humans and mice (1). *ZFY* genes have a widely expressed X-linked homologue, *ZFX*, that codes a highly similar protein; this has been shown to be a regulator of self-renewal in embryonic and haematopoietic stem cells (2). In metatherian (marsupial) mammals, genes related to *ZFX* and *ZFY* are autosomal (3), and it is thought that *ZFX* and *ZFY* originated more than 100 million years ago, after the separation of eutherian and metatherian lineages, by translocation of an autosomal segment to the pseudo-autosomal region in a common ancestor of extant eutherians (4). *ZFY* and

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ZFX genes code putative transcription activators, characterized by a large N-terminal acidic (activating) domain (approximately 360 amino acids) and a C-terminal DNA-binding domain of 12–13 Cys_2-His_2 zinc fingers, separated by a short basic nuclear localization signal (5,6). The DNA targets and protein partners of ZFY and ZFX remain to be determined.

There are two *ZFY* genes on the mouse Y chromosome, *Zfy1* and *Zfy2* (94% amino acid identity), both of which are transcribed primarily in germ cells in the post-natal testis, but have also been described in some fetal tissues (7–10). Post-natal transcripts from the *Zfy1* and *Zfy2* genes are first readily detected in the testis around the time that the germ cells enter meiosis, between 7 and 10 d.p.p. (days postpartum) (9,11). RNA FISH (fluorescence *in situ* hybridization) detecting ongoing transcription has documented the transcriptional silencing of *Zfy1* and *Zfy2* as they enter pachytene, a manifestation of meiotic sex chromosome inactivation (MSCI), and has identified *Zfy2* transcription in Y-bearing round spermatids (12–14). Reflecting this resumption of *Zfy2* transcription in spermatids, *Zfy2* transcript levels have been observed to increase between 22 and 24 d.p.p. as spermatids appear and develop (11). Strong *Zfy2* transcription in spermatids is directed by an 'acquired' spermatid-specific promoter derived from an X-linked *CYPT* gene (11). The *CYPT* genes form a spermatid-specific gene family (15). The *CYPT* exon of *Zfy2* is most likely derived from *Cypt1*, as it is flanked by 12.1 kb of a sequence (5′: 7.8 kb; 3′: 4.3 kb) with approximately 90% nucleotide identity to the genomic region surrounding *Cypt1* (Genome assembly NCBI37—chrX: 16087265–16107151). *Zfy1* does not have an upstream copy of the *CYPT* promoter, and the presence of *Zfy1* transcripts in spermatids remains to be determined. Furthermore, the nature of the promoter used by *Zfy2* for its early expression and the structure of the *Zfy1* and *Zfy2* transcripts produced at the different stages of spermatogenesis have not been determined.

In mice, the *Zfy* genes are affected by the ~1.3 Mb Sxr^b deletion that occurred in the mouse Y short-arm-derived 'sex-reversal factor' Tp(Y)1Ct^{Sxr-a} (16) via an illegitimate recombination between the *Zfy1* and *Zfy2* genes; this created a transcribed *Zfy2*/1 fusion gene composed of the promoter region, 5′ UTR (untranslated region) and first coding exon of *Zfy2* fused in intron 5 to the remaining six coding exons of *Zfy1* (17). This effectively puts the *Zfy1* coding region under the control of the *Zfy2* promoter (18). For this reason, it has been difficult to assess how ZFY function has been affected by the deletion, if at all. Recently, however, we have shown that ZFY is involved in the apoptotic elimination of spermatocytes in two types of sex chromosomally variant males. First, we have shown that in XYY males the apoptotic elimination of spermatocytes during the mid pachytene stage is due to the mis-expression of *Zfy1* and *Zfy2*; both genes are silenced during pachytene in normal males but escape silencing when the two Y chromosomes are synapsed (13). Second, *Zfy2* (but not *Zfy1* or the *Zfy2/1* fusion gene) has been shown to be required for the efficient apoptotic elimination of spermatocytes at the first meiotic metaphase (MI) that occurs in response to the X univalent in XSxr^aO males that carry the sex reversal factor Tp(Y)1Ct^{Sxr-a} attached to the pseudoautosomal regions (PAR) of the X chromosome (18).

In humans, in contrast to mice, there is a single *ZFY* gene on the Y chromosome; furthermore, the transcription of human *ZFY* is ubiquitous (1). Despite its widespread expression, a rare deletion of *ZFY* and *SRY* in a woman carrying a Y;22 balanced translocation was not associated with Turner syndrome stigmata, indicating that *ZFY* has no critical somatic functions (19). No mutations of *ZFY* have been described in men and there is, therefore, no information concerning its possible contribution to human germ cell development or male fertility.

With the aim of understanding the basis of the functional difference observed between the nearly identical *Zfy1* and *Zfy2* genes in relation to the apoptotic response to X chromosome univalence at MI, we undertook a detailed analysis of mouse *Zfy* transcription during spermatogenesis. We reveal an unexpected complexity of *Zfy* transcription involving multiple splice variants. This led us to reassess *ZFY* transcripts in the human testis where we have identified a testis-specific splice-variant, structurally homologous to the major *Zfy1* variant transcript in mice.

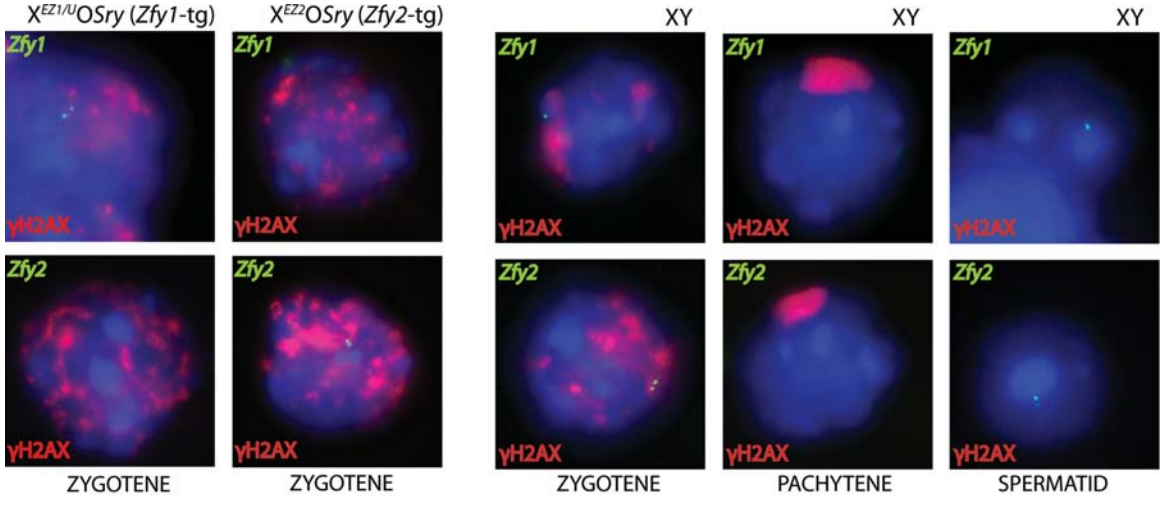
RESULTS

Zfy1 and *Zfy2* are transcribed in early spermatocytes and round spermatids

In order to define the cell types in which the *Zfy1* and *Zfy2* genes are transcribed in the adult testis, we carried out RNA FISH designed to identify nascent transcripts within the nucleus as they are transcribed (20) (Fig. 1). No clear hybridization was seen in nuclei of Sertoli cells or other somatic cells. Weak hybridization signals were seen in nuclei of a few A spermatogonia (*Zfy1*: 1/20, and *Zfy2*: 2/30; data not shown), but appropriate localization to the encoding genes was not confirmed by DNA FISH; no *Zfy* signals were detected in B or intermediate spermatogonia. *Zfy1* and *Zfy2* signals then began to appear during leptotene (data not shown), and robust signals were clearly present in all mid-late zygotene nuclei. As expected, no signals were detected in pachytene nuclei where the *Zfy* genes are silenced by MSCI. In agreement with previous data, *Zfy2* transcription is resumed in (Y-bearing) round spermatids where it localizes to the post meiotic sex chromatin; however, *Zfy1* is also clearly transcribed at this stage, although it was detected in a substantially lower proportion of spermatid nuclei than *Zfy2* (17.5 versus 37%, which equates to 35 versus 74% of Y-bearing spermatids). The higher proportion of spermatids with a *Zfy2* signal probably reflects the higher level of *Zfy2* transcription from the strong *Cypt*-promoter in spermatids. Our results establish that both *Zfy1* and *Zfy2* genes are transcribed in spermatocytes prior to the onset of MSCI, and that both genes are reactivated in spermatids.

5′ UTRs and putative promoters of mouse *Zfy* genes

We analysed the *Zfy1* and *Zfy2* transcripts produced during spermatogenesis, using qualitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) with RNAs extracted from testes at different ages after birth: 3 and 7 d.p.p. (spermatogonia only), 10, 16 and 20 d.p.p.



Probe	Genotype	ZYGOTENE		PACHYTENE		SPERMATID	
		positive	negative	positive	negative	positive	negative
Zfy1	XY	25	0	0	100	35	165
	$X^{EZ1/U}OSry$	25	0	0	50		
	$X^{EZ2}OSry$	0	25				
Zfy2	XY	25	0	0	93	74	126
	$X^{EZ1/U}OSry$	0	50				
	$X^{EZ2}OSry$	35	0	0	82		

Figure 1. The mouse *Zfy* genes are transcribed in early spermatocytes and spermatids. Representative images of germ cells are shown hybridized with probes specific for *Zfy1* or *Zfy2* (green). Zygotene and pachytene spermatocytes were identified by staining with an antibody against γH2AX (red). Nuclei are stained with DAPI (blue). The specificity of the probes for each gene is shown with control zygotene spermatocytes from testis of 30 d.p.p. transgenic males without a Y chromosome but carrying *Zfy1* ($X^{EZ1/U}OSry$) or *Zfy2* ($X^{EZ2}OSry$) as a transgene (germ cells develop to secondary spermatocytes in these mice). The numbers of germ cells (zygotene, pachytene or spermatid) scored as positive or negative for *Zfy1* and *Zfy2* transcription in adult are presented in the table, for the wild-type (XY) adult testis. No significant labelling of *Zfy* transcripts was observed for any other testicular cell types. The double signal observed for *Zfy2* in spermatids probably reflects the high level of *Zfy2* transcription from the *Cypt* promoter in spermatids, since the number of RNA FISH signals per spermatid has been found to increase with transcript levels (44).

(spermatogonia + spermatocytes only) and 27 d.p.p. and adult (spermatogonia + spermatocytes + spermatids). *Zfy* exons are numbered following the published genomic structure of *Zfy1* and *Zfy2* (21), which are shown in Figure 2A.

We first investigated the use of the different putative promoters by amplifying from the first exons of *Zfy1* (exon 1b) or *Zfy2* (exon 1a or 1b) to exon 5, the first coding exon (Fig. 2B). The identification of transcripts at 7 d.p.p. provides some support for the RNA FISH identification of weak *Zfy1* and *Zfy2* signals in some A spermatogonia. The products identified at 7 and 16 d.p.p. show that during early meiotic stages prior to the onset of MSCI, *Zfy1* and *Zfy2* transcripts include exon 1b (transcripts *Zfy1*-b and *Zfy2*-b). We show that *Zfy2* does not, however, use the alternative non-coding exons 3 or 4, as previously described for *Zfy1* (7). There is no evidence that any one 5′ UTR structure of *Zfy1* is specific to a particular stage of spermatogenesis. As shown previously (11), the *Zfy2* transcripts with exon 1a (*Zfy2*-a), produced from the spermatid-specific *Cypt*-promoter, are not detected at earlier stages, appearing only in the testis containing spermatids (27 and 50 d.p.p.).

These results were confirmed and extended by quantitative PCR (qPCR) (Fig. 2C). *Zfy1*-b transcripts were amplified with primers in exons 8 and 10, to avoid the multiple bands generated by PCR from exon 1b for *Zfy1*. As expected, levels of *Zfy2*-a (*Cypt*) were strong only in the testis with spermatids (27 and 50 d.p.p.). However, *Zfy1*-b and *Zfy2*-b are seen to increase between 7 and 20 d.p.p. as spermatocytes appear and develop. *Zfy1*-b levels continue to increase with the arrival of spermatids (27 d.p.p.), consistent with our RNA FISH data showing that *Zfy1* is reactivated in spermatids. For *Zfy2*-b, however, levels remain constant between 16 and 50 d.p.p., suggesting that the *Zfy*-promoter of *Zfy2* is not as strongly reactivated in spermatids as that of *Zfy1*.

The two *Zfy* genes in the mouse are generally held to differ in their 5′ UTRs, and to be transcribed from distinct promoters, with *Zfy2* assumed to be transcribed exclusively from a *Cypt*-promoter, even though *Zfy2* retains sequences nearly identical to the 5′ UTR and putative promoter of *Zfy1* (11,21,22). Here, we show, however, that *Zfy2* does use its *Zfy1*-homologous promoter, and 5′ UTR, in particular for its earlier phase of postnatal transcription, when the *Cypt*-promoter is silent.

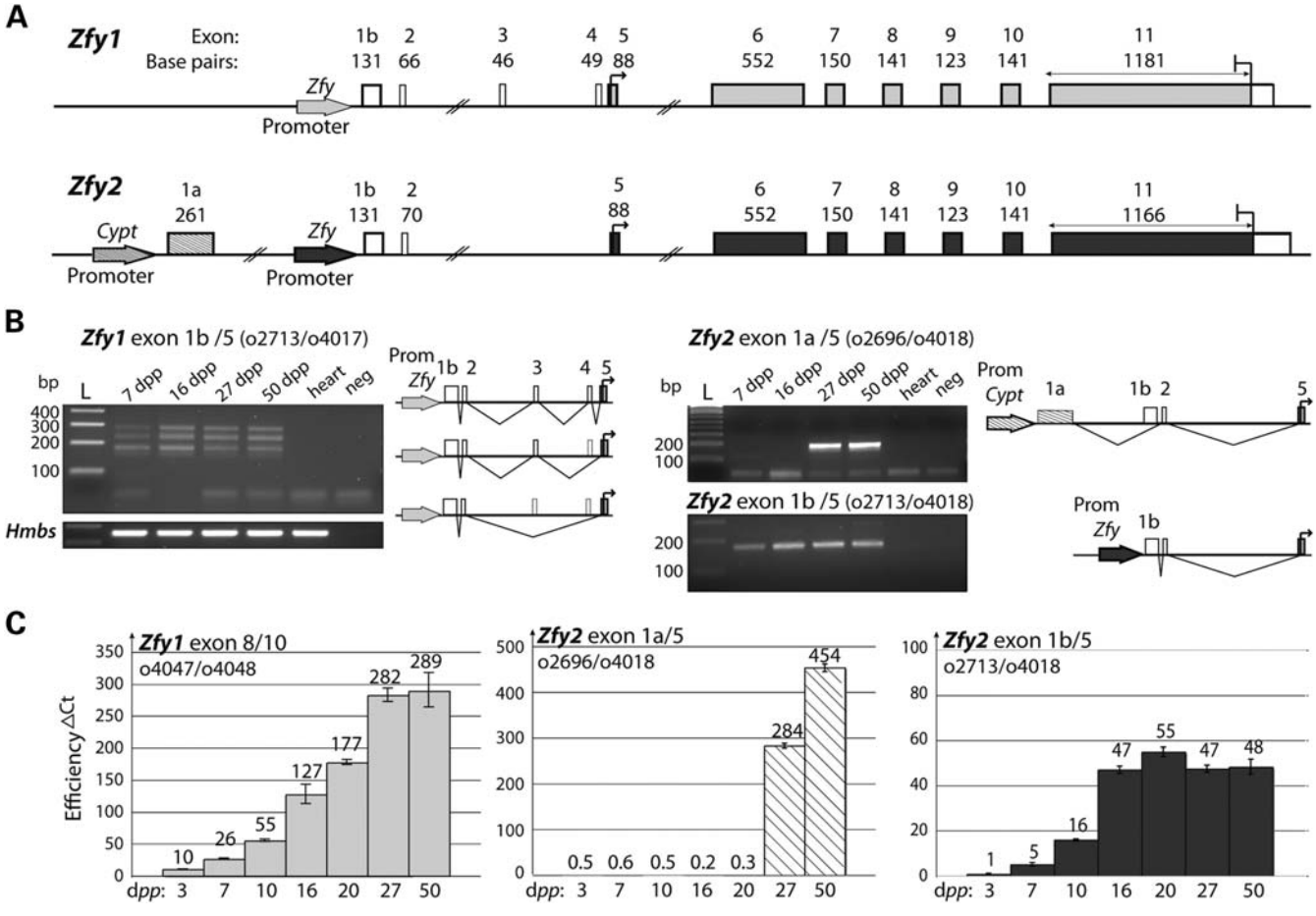


Figure 2. *Zfy2* uses an early (pre-MSCI) and a late (spermatid) promoter during mouse spermatogenesis. (**A**) The genomic structure of the mouse *Zfy1* and *Zfy2* genes. Exons are represented by boxes: coding exons are shaded and non-coding are white. Introns and exons are not to scale. (**B**) RT-PCR analysis of mRNA from wild-type mouse testis of 7, 16, 27 and 50 d.p.p. with primers in exon 1 and exon 5 of the different *Zfy* transcripts: exon 1b for *Zfy1* and exon 1a or exon 1b for *Zfy2*. For the negative controls (neg), Milli-Q water replaced cDNA. The structure of amplified fragments is shown and was determined by sequencing. L = 1 kb plus DNA size ladder (Invitrogen), and sizes of some ladder bands are indicated in base pairs (bp). (**C**) Quantitative RT-PCR analysis of *Zfy1* transcripts and the two *Zfy2* transcripts from the *Cypt*- or the *Zfy*-promoter, in staged testes. The error bars represent the standard deviation of triplicates. Oligonucleotides used for PCRs are indicated as a number prefixed with an 'o', and are listed in Supplementary Material, Table S1.

Coding region variants of mouse *Zfy* transcripts

To investigate coding exon usage for *Zfy1* and *Zfy2*, we performed RT-PCR, on testis RNA from normal XY males, with primers in the first and last coding exons (Fig. 3A). Unexpectedly, the major product for *Zfy1* was ~500 bp smaller than predicted. The expected size was observed for *Zfy2*. Sequencing revealed the major pre-MSCI *Zfy1* product (16 d.p.p.) to have spliced out exon 6, a 552 bp exon that codes for half the acidic domain. Sequencing the major product for *Zfy2* revealed it to include the full exon 6. The lower of the two strongest minor *Zfy2* products at ~900 bp lacks exon 6, whereas the upper product is a doublet that retains 111 or 123 bp of exon 6 (Fig. 3B).

We then zoomed in on this region, using *Zfy1*- or *Zfy2*-specific primers in exon 5 and 7, and confirmed that *Zfy1* transcripts predominantly lack exon 6, whereas the major *Zfy2* transcripts retain exon 6. This is especially evident at 16 d.p.p. (Fig. 3B). We performed qPCR analysis with primer pairs specific for the splice variants with or without exon 6 and specific for *Zfy1* or *Zfy2* (Fig. 3C). This revealed that, for *Zfy1*, the transcript with exon 6 is present at low levels until 20 d.p.p., but increases more than 4-fold between 20 and 27 d.p.p., coincident with the progression of germ cells into spermiogenesis. On the contrary, the concentration of *Zfy1* transcript without exon 6 reaches its peak between 16 and 20 d.p.p. prior to the development of spermatids. For *Zfy2*, the transcript with exon 6 increases between 10 and 20 d.p.p., as spermatocytes accumulate, and further increases between 20 and 27 d.p.p., when the strong *Cypt* promoter becomes active. The *Zfy2* transcript without exon 6 can be seen to increase between 20 and 27 d.p.p., but is expressed at extremely low levels at 7–20 d.p.p., when meiotic stages appear and develop in the testis.

These results show that, in spermatocytes, prior to the onset of MSCI, *Zfy* transcription is strongly characterized by the differential splicing of exon 6 that is predicted to generate two ZFY protein isoforms with very different acidic domains. The quantitative real-time reverse transcription PCR

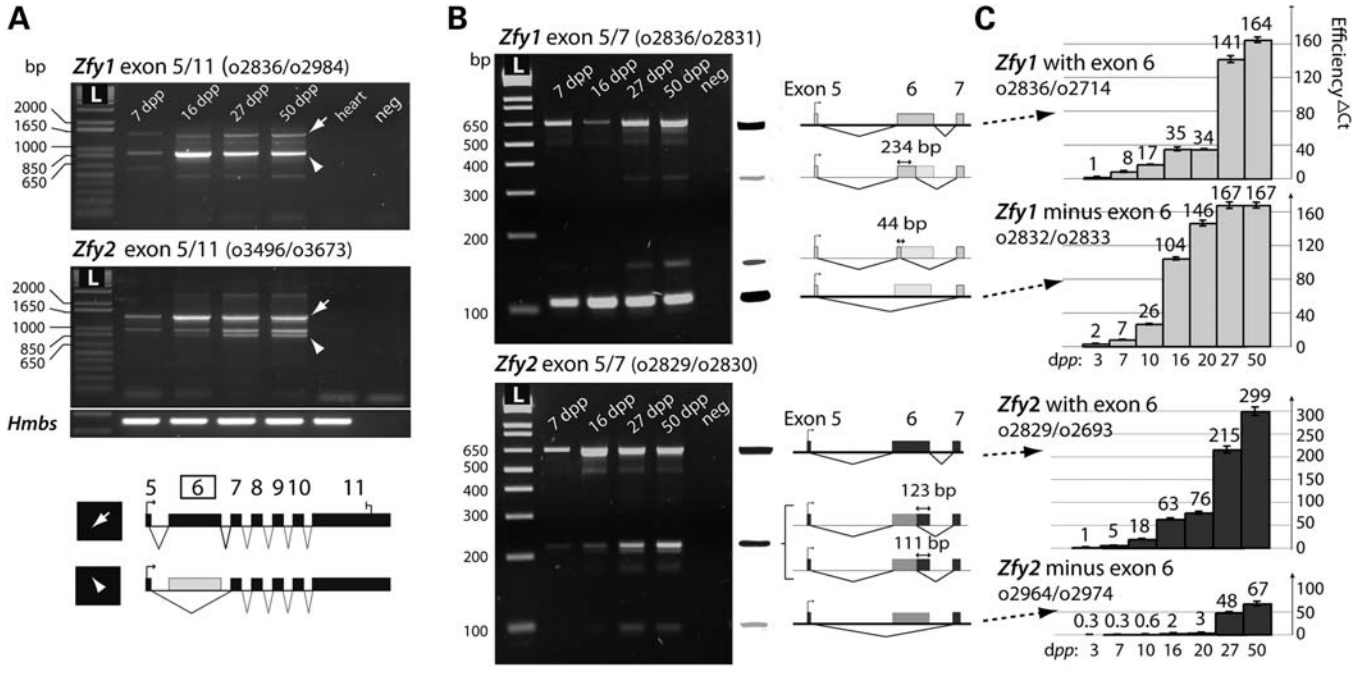


Figure 3. *Zfy1* and *Zfy2* produce major gene-specific transcripts coding distinct putative activating domains during mouse spermatogenesis. (**A**) RT-PCR of mRNA from staged testes of wild-type mice with primer pairs specific for *Zfy1* or *Zfy2* in the first and the last coding exons (exon 5 and exon 11). Amplified fragments were sequenced, and the structure of the amplified fragments are shown. For *Zfy2*, the major product (arrow) is at ~1400 bp, and its sequence shows it to have the same complete structure as the published cDNA sequences of *Zfy1* and *Zfy2*. In contrast, for *Zfy1*, the major product (arrowhead) is ~850 bp and does not include the 552 bp coding exon 6. The minor product at 950 bp for *Zfy2* includes the last 111 or 123 bp of exon 6. The *Zfy2* transcript without exon 6 becomes clearly visible but remains minor at 27 d.p.p. when spermatids are present. White arrow: transcripts with exon 6. White arrowhead: transcripts without exon 6. (**B**) RT-PCR analysis of wild-type mouse testes with primer pairs specific for *Zfy1* or *Zfy2* in exon 5 and exon 7. The exon structures of the products amplified were determined by sequencing and are shown to the right of the graphic representation of the electrophoresed PCR products. (**C**) RT-qPCR analysis was performed with primers specific for the *Zfy1* or *Zfy2* transcripts with or without exon 6 in staged wild-type mouse testes. These results show a complementary pattern of transcription for the *Zfy* genes; up to 20 d.p.p., *Zfy2* produces the transcript with exon 6, whereas *Zfy1* mainly produces the transcript without exon 6. There is an increase of both *Zfy1* and *Zfy2* transcripts including exon 6 between 20 and 27 d.p.p. when spermatids first develop in the testis. For RT-qPCR, Ct values were normalized to the *Hmbs* reference gene. The error bars represent the standard deviation of triplicates. L = 1 kb plus DNA size ladder (Invitrogen), and sizes of some ladder bands are indicated in base pairs (bp).

(RT-qPCR) analysis also shows that the quantity of *Zfy1* transcripts with exon 6 increases in spermatids, suggesting that the predominance of *Zfy1* transcripts without exon 6 may be specific to spermatocytes.

Polyadenylation site usage of mouse *Zfy* transcripts

We also found gene- and stage-specific differences in polyadenylation (polyA) site usage by 3′ RACE (Fig. 4A). Sequencing the major 3′ RACE products showed that *Zfy1* uses two polyA sites, one major (3′ UTR: 196 bp) and the other minor (3′ UTR: 124 bp). The major site is 14 bp downstream of a canonical AATAAA motif. The minor site is 15 bp after a non-canonical AATATAAA site.

Zfy2 also uses two polyA sites. However, a comparison of the 3′ UTRs from *Zfy1* with those of *Zfy2* reveals that the canonical AATAAA polyA signal used by *Zfy1* is absent from *Zfy2*. Instead *Zfy2* uses an upstream site corresponding to the minor *Zfy1* site (3′ UTR: 124 bp) for its late transcripts (mostly *Cypt* transcripts) in spermatids, and a downstream site (3′ UTR: ≥284 bp) for its pre-MSCI transcripts, giving it a 3′ UTR that is longer than *Zfy1* at the early stage. The major downstream site used by *Zfy2* is difficult to define because the major *Zfy2* 3′ RACE product, at 16 d.p.p., has its 3′ end within a genomic polyadenosine tract (dA_{14}) (Fig. 4A). The true polyA site of the *Zfy2* meiotic transcript must, therefore, be situated further downstream.

Conventional PCR from exon 5 to a primer specific for each 3′ UTR in exon 11 of *Zfy1* or *Zfy2* amplified fragments with and without exon 6, showing that the inclusion or exclusion of exon 6 is not associated with a particular 3′ UTR for either *Zfy1* or *Zfy2* (Fig. 4B and C). RT-qPCR shows that the levels of *Zfy1* transcripts with the shorter 3′ UTR increase between 16 and 27 d.p.p., whereas the levels of the medium 3′ UTR increase between 7 and 16 d.p.p. and then remain constant (Fig. 4B). This is also indicated by the 3′ RACE result for *Zfy1* (Fig. 4A), where the short product, at 27 and 50 d.p.p., appears stronger, relative to the medium product, than it does at 7 and 16 d.p.p.. This suggests that the usage of the minor polyA site may increase in spermatids for *Zfy1*, as is observed for *Zfy2*.

Although human *ZFY* also uses alternative polyA sites and the non-canonical AATATAAA site (23), only a short stretch of nucleotide homology remains between the mouse and human 3′ UTR (61 bp with 79% nucleotide identity), beginning, respectively, at 23 and 24 bp after the stop codon. This

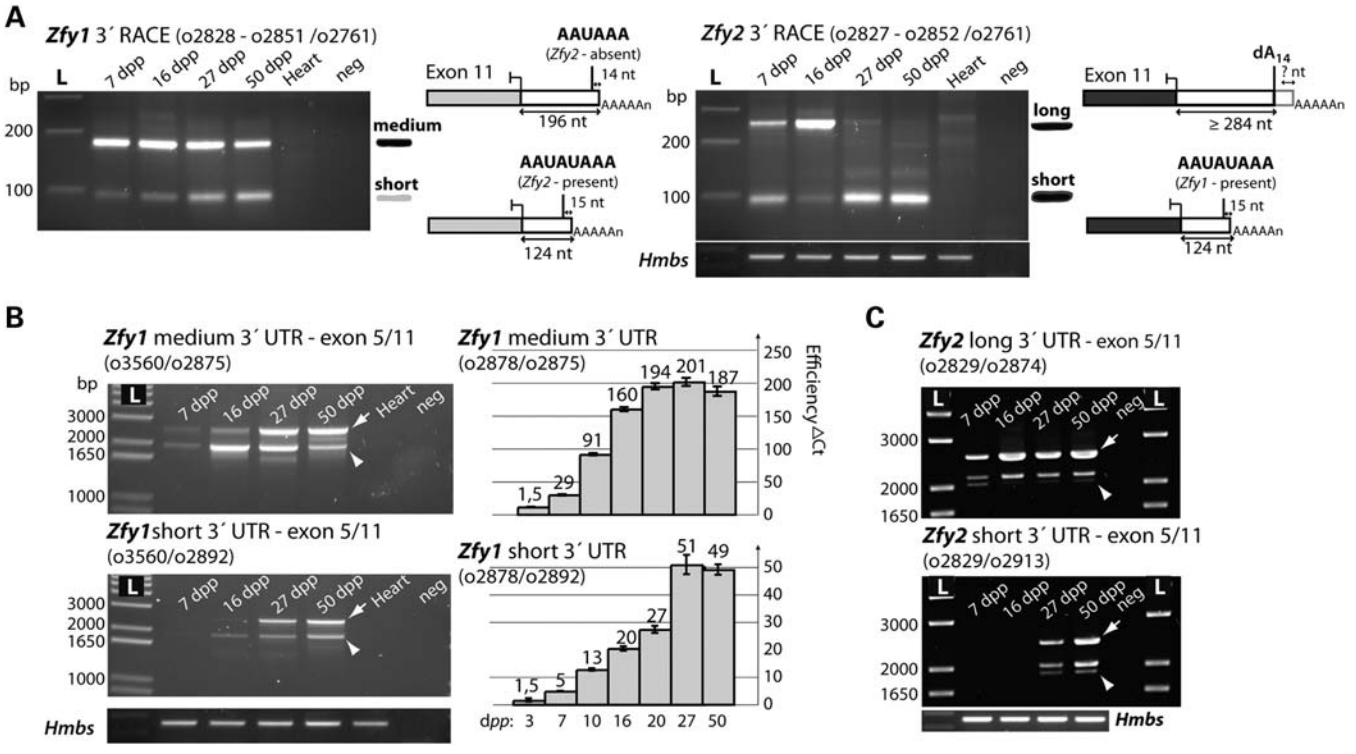


Figure 4. The *Zfy* genes use gene- and stage-specific polyA signals. (**A**) 3′ RACE using nested primers in exon 11 of *Zfy1* or *Zfy2*, with cDNA from staged testes of wild-type mice. The major RACE products were sequenced and their structure is represented to the right of the gel. dA_{14} refers to the poly adenosine stretch present in genomic DNA. nt, nucleotides. (**B**) RT-PCR and RT-qPCR analysis of *Zfy1* transcripts with the short and medium 3′ UTR in staged wild-type mouse testes. RT-PCR from exons 5–11 shows that the expression of the transcript with exon 6 increases between 16 and 27 d.p.p., a further indication that the full *Zfy1* transcript increases in spermatids (Fig. 3B and C). The splicing of exon 6 appears to be independent of the 3′ UTR. The error bars represent the standard deviation of triplicates. (**C**) RT-PCR analysis of *Zfy2* transcripts with the long or short 3′ UTR in staged testes. The long 3′ UTR is used at pre-MSCI stages, since it is amplified from 16 d.p.p. testis, containing only spermatogonia and spermatocytes. The short 3′ UTR is detectable only in testis with spermatids (27 and 50 d.p.p.), suggesting that it is specific to this later stage. The positive control RT-PCR is with *Hmbs*. PCR products with exon 6 (arrow) and without exon 6 (arrowhead) are indicated. L = 1 kb plus DNA size ladder (Invitrogen), and sizes of some bands are indicated in base pairs (bp).

homology does not include the polyA signals. There is, therefore, no strong evidence that *ZFY* polyA choice is achieved by a conserved mechanism.

The *Sxr^b* deletion breakpoints within the *Zfy2/1* fusion gene

The *Sxr^b* deletion is known to have arisen by unequal exchange between intron 5 of *Zfy1* (20 705 bp) and intron 5 of *Zfy2* (11 556 bp) (17). The exact breakpoints, resulting in the generation of the *Zfy2/1* fusion gene, have not been determined. Since the breakpoints map to the intron that precedes the alternatively spliced exon 6, we localized the breakpoints precisely using *Zfy1*- and *Zfy2*-specific STS markers from intron 5 (data not shown), and then amplified and sequenced the *Sxr^b* junction fragment (Fig. 5A and B). This revealed that the *Sxr^b* deletion occurred by an illegitimate cross-over event between *Zfy1* and *Zfy2* in 95 bp of identity. Based on the position of the breakpoints on the mouse Y chromosome contig NT_078925, we estimate the length of the *Sxr^b* deletion interval to be 1 299 537 bp.

The *Sxr^b* junction fragment was confirmed using a PCR RFLP assay (Fig. 5C). This generated a specific *Rsa*I fragment only when the *Sxr^b* interval was present. This will be a useful screening tool for the *Sxr^b* interval in general, and especially for the identification of XY*Sxr^b* carrier males, previously identified through extensive breeding tests.

Zfy2/1 fusion gene transcripts in *Sxr^b* carrier mice

To better define the consequences of the *Sxr^b* deletion for *Zfy* expression, we used RT-PCR to analyse the early and late transcripts expressed from the *Zfy2*/1 fusion gene, in X^E*Sxr^b*O (*Zfy2/1* only) and XY*Sxr^b* (*Zfy1*, *Zfy2* and *Zfy2/1*) mice, in particular with reference to the presence or absence of exon 6. XY*Sxr^b* mice have full spermatogenesis, whereas in X^E*Sxr^b*O mice, interphasic secondary spermatocytes block prior to the second meiotic division (18,24). The block in X^E*Sxr^b*O mice is leaky, however, and between 30 and 60 d.p.p. abnormal sperm appear in testis tubules (24).

Using a *Zfy2*-specific primer for exon 5 and a *Zfy1*-specific primer from exon 10, we specifically amplified the *Zfy2/1* fusion gene from XY*Sxr^b* and X^E*Sxr^b*O testes at early (10–21 d.p.p.) and late (27–73 d.p.p.) stages (Fig. 6A and B). We detected *Zfy2/1* transcripts at both these stages, in *Sxr^b* mice, but not in normal XY males. At early stages, the transcript without exon 6 predominates, showing that the fusion gene is spliced like *Zfy1*, in spermatocytes. The similarity of the *Zfy2/1* and *Zfy1* profiles at later stages suggests that this is also the case in spermatids.

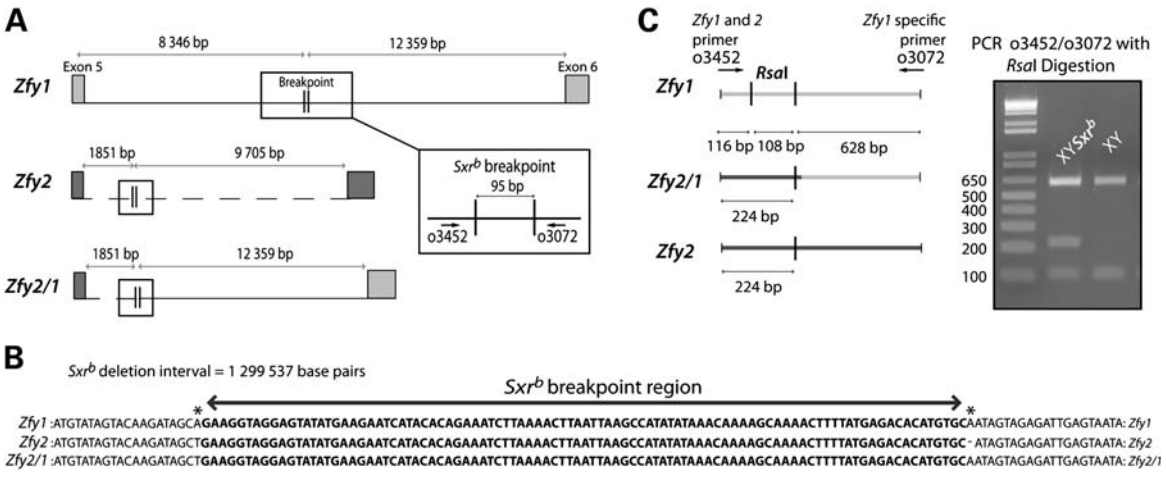


Figure 5. Definition of the *Sxr*^{b} deletion interval breakpoint. (**A**) The deletion breakpoint was fine-mapped within intron 5, using STS markers (data not shown). The junction fragment was PCR-amplified with the primer pair o3452/o3072 and sequenced, localizing the *Sxr*^{b} deletion breakpoints to 95 bp of nucleotide identity between *Zfy1* and *Zfy2*. (**B**) Sequence of the *Sxr*^{b} breakpoint region (bold) within the *Zfy2/1* fusion gene. Twenty bases of the flanking sequences are aligned to *Zfy1* and *Zfy2*, and asterisks indicate the position of mismatches. (**C**) PCR-RFLP test to detect the *Sxr*^{b} junction fragment. *Zfy1* and the *Zfy2/1* fusion genes are amplified from genomic DNA with o3452 (*Zfy1* + *Zfy2*) and o3072 (*Zfy1*), followed by digestion with *Rsa*I, generating a 224 bp *Sxr*^{b}-specific fragment.

Amplification of the *Zfy2/1* gene from staged X^E*Sxr*^{b}O testes with primers in exon 1a or 1b and exon 5 shows the fusion gene to be transcribed from both *Zfy2* promoters (Fig. 6B). Transcripts with exon 1a (*Cypt*) are not detected at early stages (Fig. 6B and C). This is the transcript that was originally detected in adult XY*Sxr*^{b} mice, using primers in exon 1a and exon 6, when the *Sxr*^{b} deletion mechanism was first described (17). It is noteworthy that *Cypt* promoter-driven transcripts are readily detected in 30 d.p.p. testis from X^E*Sxr*^{b}O males (Fig. 6B), because spermatids are not observed by histological examination of X^E*Sxr*^{b}O testis at this stage (18,24). This is, therefore, an indication that, in X^E*Sxr*^{b}O mice, certain spermiogenic processes required for the activation of the spermatid-specific *Cypt* promoter continue independently of the secondary spermatocyte block.

In XY*Sxr*^{b} males, the presence of the *Zfy2/1* fusion gene provides an opportunity to investigate how exon 6 of *Zfy1* is spliced in spermatids. Using primers specific for exon 1a of *Zfy2* and exon 8 of *Zfy1*, *Zfy2/1* fusion gene transcripts were amplified with and without exon 6 (Fig. 6C), showing that the splicing out of exon 6 does occur in spermatids as well as spermatocytes.

The 3′ RACE analysis (Fig. 6D) shows that the *Zfy2/1* fusion gene transcripts predominately use the canonical AATAAA polyA signal, and not the upstream AATATAAA signal used by Zfy2 in spermatids.

In conclusion, we show that, besides the loss of the *Zfy2* coding region, one of the principal consequences of the *Sxr*^{b} deletion for the combined expression of the *Zfy* genes, is a reduced level of transcripts coding the full-length *Zfy* acidic domain, in spermatocytes and spermatids.

A testis-specific human *ZFY* transcript coding a short acidic domain

There is a single widely expressed *ZFY* gene in human suggesting that *ZFY* regulation may vary greatly between humans and mice. We nevertheless investigated the splicing of human *ZFY* in human testis. In human *ZFY*, it is exon 3 that corresponds to exon 6 in mouse *Zfy1*. Using RT-PCR with primers in exon 2 and exon 4, we identified a transcript lacking exon 3, present in the testis but not in the other tissues tested (Fig. 7A). As in the mouse, exon 3 is the second coding exon. It is 573 bp in length and codes for around half of the putative activating domain of *ZFY*.

Testis-specific *ZFY* transcript in men with meiotic maturation arrest

To investigate the possibility that, like its mouse counterpart, the *ZFY* transcript lacking the second coding exon (exon 3) is expressed in germ cells as they enter meiosis, we tested for its expression in a testicular biopsy from two azoospermic men with a histologically determined meiotic maturation arrest. One, Ste-363, with a deletion of the AZFb interval of the Y chromosome, has a pachytene block, whereas the other, Apop12, with no detectable Y chromosome deletion has an earlier block at the leptotene/zygotene transition. Expression of the *ZFY* transcripts with and without exon 3 was tested by RT-PCR with primers in exon 2 and exon 4. Transcripts known to be specific to spermatids (*PRM1* and *TNP1*) were also tested to confirm the absence of post-meiotic germ cells, and the *HPRT* gene was tested as a positive control. RT-PCR analysis showed the presence of *ZFY* transcripts with and, importantly, without exon 3, in the absence of the spermatid markers *PRM1* or *TNP1* (Fig. 7B). These results provide evidence that, like *Zfy1* in mice, *ZFY* in humans produces a transcript in germ cells prior to the onset of MSCI that lacks the 573 bases of exon 3 (mouse exon 6).

ZFY transcripts present in human spermatids

We characterized indirectly the *ZFY* transcripts present in human spermatids by the analysis of RNA extracted from purified spermatozoa. During spermiogenesis, transcription ceases

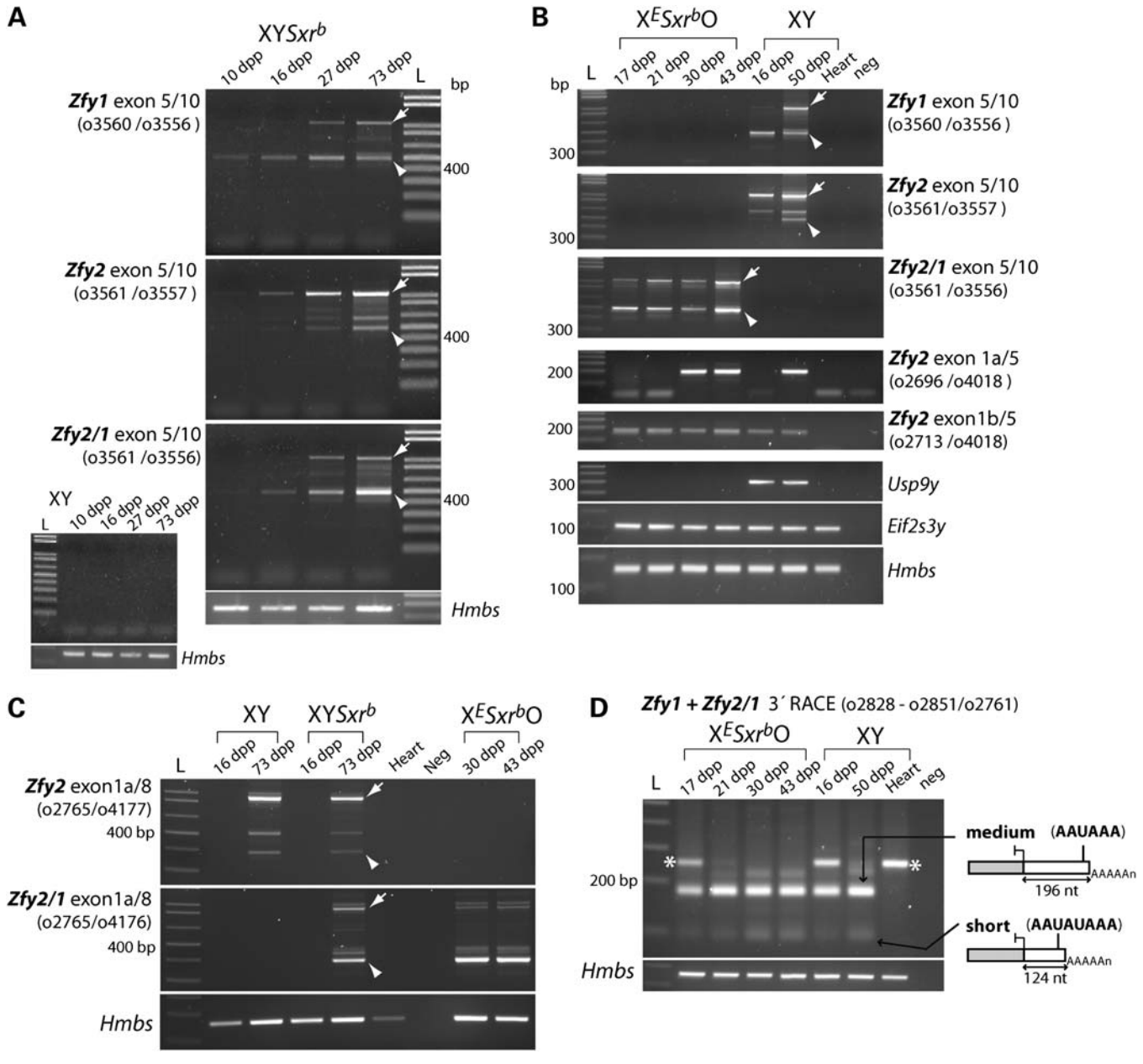


Figure 6. Expression of the *Zfy2/1* fusion gene created by the *Sxr*^{b} deletion in XY*Sxr*^{b} carriers and X^E*Sxr*^{b}O male mice. (**A**) RT-PCR analysis of staged testes from XY*Sxr*^{b} and XY males (same-stage littermates). Exon 6 of the *Zfy2/1* fusion gene is spliced like *Zfy1* and not like *Zfy2*. (**B**) RT-PCR analysis of staged testis from X^E*Sxr*^{b}O and XY males. X^E*Sxr*^{b}O males carry the deleted *Sxr*^{b} region and an *Eif2s3y* transgene on the X chromosome (X^E*Sxr*^{b}), and lack a Y chromosome (O). In X^E*Sxr*^{b}O testis, the *Zfy2/1* fusion gene produces transcripts with exon 1a and exon 1b, showing it to be transcribed from both *Zfy2* promoters, but exon 6 is spliced as for *Zfy1*. The *Usp9y* gene, mapping to the *Sxr*^{b} deletion interval, was included as a negative control. *Eif2s3y* and *Hmbs* are positive controls. (**C**) RT-PCR analysis of *Cypt* promoter-driven transcripts in staged testes from XY*Sxr*^{b} males with same-stage XY littermates, and from X^E*Sxr*^{b}O males, using primers from exons 1a and 8. A substantial proportion of the *Zfy2/1* fusion gene products lack exon 6, an indication that the splicing out of exon 6 is not exclusive to spermatocytes, but also occurs in spermatids. (**D**) 3′ RACE analysis of *Zfy2/1* and *Zfy1* transcripts. As for *Zfy1*, the medium 3′ UTR predominates. The band at 270 bp (asterisk) was sequenced and shown to be the result of priming from contaminating genomic DNA at a poly A tract situated 3′ of *Zfy1*. nt, nucleotides. The positive control RT-PCR is with *Hmbs*. PCR products with exon 6 (arrow) and without exon 6 (arrowhead) are indicated. L = 1 kb plus DNA size ladder (Invitrogen), and sizes of some bands are indicated in base pairs (bp).

when histones are removed from the chromatin as spermatids begin to elongate. A proportion of mRNAs already present in spermatids are nevertheless retained in the mature spermatozoa, representing a sampling of the spermatid transcriptome (25). We tested for *ZFY* transcripts by RT-PCR with primer combinations specific for transcripts with or without exon 3 (Fig. 7C), and detected both transcripts in spermatozoa RNA. However, compared with the whole testis, where the product from the transcript with exon 3 is the stronger, it is the product from the transcript without exon 3 that

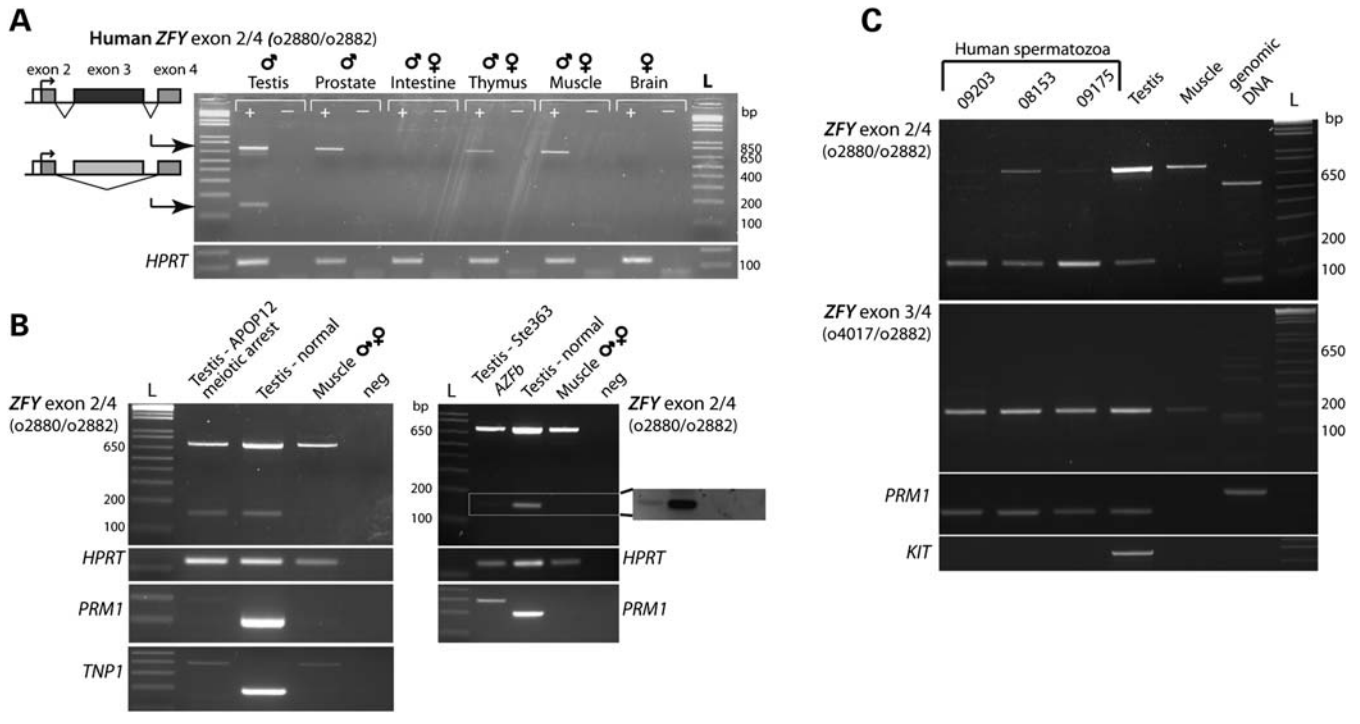


Figure 7. Human *ZFY* produces a major transcript without the second coding exon in the adult testis. (**A**) RT-PCR analysis of *ZFY* transcripts in human tissues. Primers were derived from exons 2 and 4. Exon 3 of human *ZFY* is homologous to exon 6 of the mouse *Zfy1* gene. A product lacking exon 3 was amplified from the testis only, indicating that the *ZFY* transcript without exon 6 is transcribed in the human testis. (**B**) RT-PCR analysis of total RNA extracted from testicular biopsies of patients, Ste-363 and Apop12, with a meiotic maturation arrest. Ste-363 has an AZFb deletion and the arrest is during pachytene. Apop12 does not have an AZF deletion and 97% of the spermatocytes are at leptotene/zygotene transition. The *ZFY* transcript without exon 3 is amplified from the testis with a spermatocyte-stage block. The absence of post-meiotic stages was controlled for by the failure to amplify spliced transcripts from *PRM1* (protamine 1) and *TNP1* (transition protein 1). The negative control sample (neg) was Milli-Q water. (**C**) RT-PCR analysis of RNA extracted from purified human spermatozoa. Transcripts with and without exon 6 were amplified from spermatozoa RNA, showing that they are both present in spermatids. Primers derived from exon 2 and exon 4 were used to detect the transcripts with or without exon 3, and primers derived from exon 3 and exon 4 were used to detect the transcripts with exon 3 only. *HPRT* and *PRM1* served as positive control PCRs, *KIT* as a negative control PCR. L = 1 kb plus DNA size ladder (Invitrogen), and sizes of some bands are indicated in base pairs (bp).

predominates in spermatozoa, indicating that the transcript without exon 3 is a major *ZFY* transcript in spermatids.

The short acidic domain of the ZFY protein does not activate transcription

To investigate the functional differences between the two isoforms of the *ZFY* genes in mice and humans, we exploited the fact that the acidic domains of *Zfy2* and *Zfx* have been shown to activate transcription in the yeast *Saccharomyces cerevisiae* when fused to the DNA-binding domain of the Gal4 transcriptional activator (5). We, therefore, generated six constructs expressing the long or the short acidic domains of mouse ZFY1, mouse ZFY2 and human ZFY fused to the Gal4 DNA-binding domain in the vector pGBKT7 (Clontech). We also generated a construct with the yeast Gal4 acidic domain fused to the Gal4 DNA-binding domain as a positive control, and the Gal4 DNA-binding domain alone (empty vector) as a negative control. These constructs were transformed into the Y187 strain of yeast containing a *LacZ* (β-galactosidase) gene under the control of the Gal4-dependent *GAL1* promoter, and we estimated transcriptional activation by measuring β-galactosidase activity.

Our results with two independent transformants for each construct show a clear difference between the long and the short acidic domains (Fig. 8): whereas the long acidic domain encoded by *ZFY*, *Zfy2* and *Zfy1* activated β-galactosidase production like the acidic domain of Gal4, none of the three short acidic domains activated at all. We were able to confirm the expression of all the ZFY-Gal4 fusion proteins by western blot analysis, although ZFY2-long was extremely faint (Supplementary Material, Fig. S1) and the Gal4-AD fusion protein was not detected at all. We suspect that this may be the result of selection against high expressing clones, since it was the ZFY2-long and Gal4-AD fusion constructs that induced the highest β-galactosidase activity, and these yeast transformants alone gave very small colonies and grew very slowly in liquid culture.

DISCUSSION

ZFY was isolated from the human Y chromosome, over 20 years ago, and was the first gene to be found conserved on the mouse and human Y chromosome (1,8). Most of the early characterization of the two mouse *Zfy* genes focused on their embryonic expression in the developing gonad (7,9,26), with the aim of testing the hypothesis that *ZFY* was the primary testis determinant (1). With the discovery that testis determination was performed by *SRY*, in mice and

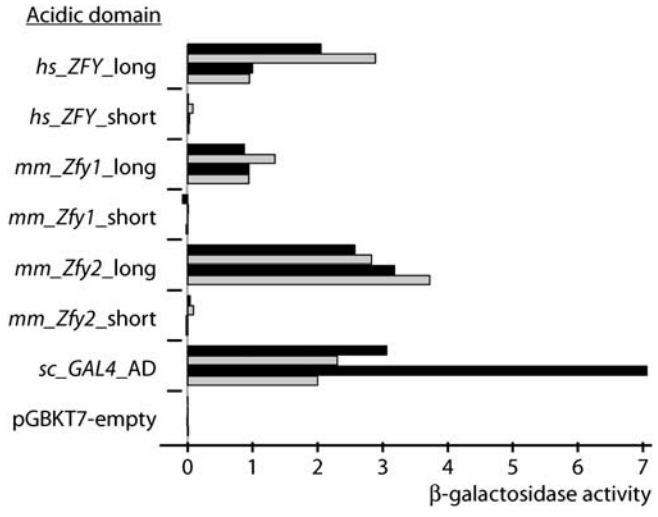


Figure 8. The short acidic domains of ZFY do not transactivate in yeast cells. (**A**) Levels of β-galactosidase induced by the Gal4-DNA-binding domain on its own (pGBKT7—negative control) or fused to an acidic domain from *S. cerevisiae* (*sc*) *GAL4*-AD (positive control) or one of the six different ZFY isoforms (long or short) found in humans (*hs*) or mouse (*mm*). The four bars for each construction on the *y*-axis represent the results of duplicate experiments on two independent transformants (black or grey bars). β-Galactosidase activity (*x*-axis) is expressed as the concentration of *o*-nitrophenol, measured as OD_{420} units, generated from the substrate *o*-nitrophenol-β-D-galactoside per minute per OD_{600} unit of yeast cells $\times 100$.

humans (27,28), interest in *ZFY* waned. As a consequence, the post-natal transcription of *Zfy1* and *Zfy2* has not been fully explored.

Our study builds on this previous work to reveal unsuspected intricacies in *Zfy* transcription during mouse spermatogenesis in XY males and in males carrying the Sxr^b deletion. Our study is the first detailed analysis of *Zfy* transcripts during the early phase of its expression prior to the onset of MSCI (pre-MSCI).

A splice variant specific to *ZFY* genes in humans and mice

The principal finding of our study is that mouse *Zfy1* and human *ZFY* produce a previously unknown major splice variant in the testis that results in the exclusion of the large second coding exon (exon 6 in *Zfy1*) that represents a quarter of the full-length ZFY protein and a half of the acidic domain. This splice variant has not been found among *ZFX* transcripts, including ESTs, in mice or humans (6,29). Only one *ZFX* transcript without the second coding exon has been identified, but it is from a human lymphoblastoid cell line, and its splicing is different from the short *ZFY* transcripts since the first coding exon is also spliced out (6). For the mouse, our own RT-PCR analyses (data not shown) with primers specific for the first and last coding exons of *Zfx* confirmed this, showing that the transcript encoding the full ZFX acidic domain predominates in the testis. A secondary *Zfx* transcript was detected that lacks the 150 nucleotide third coding exon. This latter transcript certainly gave rise to the *Zfa* retrogene which lacks the same exon. The production of the short acidic domain through the splicing out of the large second coding exon would, therefore, appear to be an adaptation that is essentially specific to *ZFY* transcripts in the testis.

Functional consequences of the short acidic domain

Based on their structure, and the fact that the acidic domains of mouse ZFY2 and ZFX have been shown to activate transcription in yeast (5), ZFY proteins are predicted to bind to specific sites in the genome via their zinc finger domain and subsequently recruit regulatory or transcriptional machinery through interactions with their negatively charged acidic domain. We have now shown that, as for ZFY2, the long acidic domain of mouse ZFY1 and human ZFY can activate transcription at Gal4-responsive promoters in yeast. Importantly, however, we show that the short acidic domain from ZFY1, ZFY2 or human ZFY does not activate transcription in yeast.

In yeast, Gal4 is known to activate transcription through the recruitment of a large pre-initiation complex including histone acetyltransferases (HAT) and the TATA-binding protein to the promoter region of target genes, with recruitment dependent on a direct interaction between the Gal4 acidic domain and the Tra1 protein (30). Tra1 is a member of the ATM superfamily, and has been conserved during the evolution of eukaryotes, and its mammalian homologue is TRRAP (transformation/transcription domain-associated protein). TRRAP is common to many HAT complexes and is required for their recruitment to the chromatin during transcription, replication and DNA repair (31). Thus, our data support a basic model in which ZFY-long, but not ZFY-short, recruits TRRAP-containing complexes to specific sites on meiotic chromosomes. ZFY-short still maintains a large acidic domain of 183 amino acids with a net charge of -32 that may recruit distinct complexes to the chromatin during spermatogenesis.

Functional difference between *Zfy1* and *Zfy2* in MI apoptotic response to X-univalence

We have recently established that *Zfy1* and *Zfy2* are functionally distinct in spermatocytes, since we have found that *Zfy2*, but not *Zfy1*, is required for the efficient apoptotic elimination of MI spermatocytes with a univalent X chromosome (18). In $X^E Sxr^b O$ mice, which express *Zfy* exclusively from the *Zfy2/1* fusion gene, there is reduced MI apoptosis in response to the unpaired $X^E Sxr^b$ chromosome. Since the *Zfy2/1* fusion gene has the promoter, 5′ UTR and first coding exon from *Zfy2*, the functional difference between *Zfy1* and *Zfy2* at MI cannot be related to these parts of the genes. The functional differences must, therefore, be related either to the coding region (46 amino acid coding differences) or to the 3′ UTR. Here, we have shown that, in spermatocytes, the *Zfy2/1* fusion gene, the only *Zfy* gene in $X^E Sxr^b O$ mice, mainly produces transcripts lacking exon 6, like *Zfy1*. Furthermore, we show that exon 6 encodes a segment of the acidic domain that is required for at least a part of the transactivating activity of ZFY1 and ZFY2. Thus, we establish that the absence of exon 6 (183 amino acids) could underlie the functional difference between *Zfy1* and *Zfy2*. We, therefore, hypothesize that the potentiation of MI apoptosis in response to a univalent X chromosome requires a ZFY protein with an acidic domain

capable of recruiting TRRAP-containing complexes to the chromatin.

Zfy1 function

Several male mice that have an unpaired X chromosome, and different combinations of *Zfy1* and *Zfy2*, have been created and studied. Comparisons of X*Sxr*^{a}O (*Zfy1* and *Zfy2*) and X^{EZ2}O + *Sry*-tg (*Zfy2* only), or X$^{EZ1/U}$O + *Sry*-tg (*Zfy1* only) and X^{E}O + *Sry*-tg (no *Zfy* gene), have not, however, revealed any effect linked to the *Zfy1* gene, during the first meiotic prophase (18). This raises the question of the role during meiosis of *Zfy1* specifically and of *ZFY* proteins with the short acidic domain in general. In this regard, it is interesting to note that, of the 46 amino acid differences between *Zfy1* and *Zfy2*, 34 are within the 363 amino acid acidic domain, and only 4 map to the 372 amino acid zinc finger domain (excluding the *Mus musculus*-specific six amino acid deletion of the third zinc finger in *Zfy2*). This indicates that the evolution of both the *Zfy1* and *Zfy2* zinc finger domains has been constrained, implying that ZFY1 and ZFY2 may have the same DNA targets, and that *Zfy1* does have a selectable function. Our finding of a *ZFY* transcript coding a short acidic domain in humans is a good indication that the short isoform of ZFY and consequently mouse *Zfy1* do fulfil a conserved role during spermatogenesis.

There is good evidence that the ZFY1 protein is translated in early spermatocytes, as persistent transcription of *Zfy1* in spermatocytes after MSCI, from an autosomally located transgene, causes a mid-pachytene block at stage IV (13). ZFY1 could have a subtle impact on spermatocyte development, improving the efficiency or the fidelity of the spermatogenic process. Like ZFY2, ZFY1 could be involved in the regulation of the apoptotic response at MI, perhaps acting to reduce apoptosis of normal XY spermatocytes by antagonizing ZFY2 binding to its target genes. An anti-apoptotic effect for ZFY1 might be evident as increased MI apoptosis in the absence of unsynapsed chromosomes, in male mice lacking *Zfy1*, but expressing *Zfy2*.

Zfy1 and *Zfy2* in the stage-IV spermatocyte block

The fact that the continued expression of either *Zfy* gene in spermatocytes, during MSCI, results in a mid-pachytene block (13) indicates that *Zfy1* and *Zfy2* could have the same mode of action at this stage, despite their differing capacity to promote apoptosis later at MI (18). This could be a consequence of the highly similar zinc finger domains of ZFY1 and ZFY2 proteins binding to the same promoter regions and reducing access to other transcription factors. However, an alternative explanation is suggested by the fact that a proportion of *Zfy1* transcripts do code for the full-length acidic domain. Our RT-qPCR data for *Zfy1* and *Zfy2* (Fig. 3C) indicate that, during meiosis, transcripts with exon 6 are approximately 2-fold lower from *Zfy1* than from *Zfy2*. However, the *Zfy1*-transgenic animals, in which the stage-IV arrest was seen, carried 14 copies of the *Zfy1* transgene (13), and thus may have produced a level of the full-length protein equivalent to, or greater than, that produced by *Zfy2*, and it was this that triggered the arrest. This explanation is consistent with a model where it is only the ZFY protein with the full-length acidic domain that promotes apoptosis in spermatocytes. The importance of the full acidic domain to the known functions of *Zfy* genes could be tested with transgenes engineered to express *Zfy1* with exon 6, and *Zfy2* without exon 6.

The *Zfy1*/*Zfy2* adaptation from the human perspective

Our data raise the possibility that, as regards *Zfy* function during meiosis in the mouse, the important distinction may not be between *Zfy1* and *Zfy2*, but between full or partial acidic domains. Seen in this way, humans and mice may be understood to be using different means to achieve a similar expression of these two *ZFY* isoforms. Human *ZFY* expression more closely resembles that of *Zfy1* than that of *Zfy2*, in that *ZFY* and *Zfy1* are capable of producing transcripts with and without the second coding exon in germ cells, whereas *Zfy2* almost exclusively produces full-length transcripts. This might be an indication that *Zfy1* assures the core eutherian *ZFY* functions, whereas *Zfy2* has specialized in the elimination of spermatocytes in which the X and the Y have failed to pair through their PAR. Studies in the mouse have recently shown that the reduced size of the PAR has been the selective force for several adaptations, probably established before eutherian radiation, to ensure double-strand break formation and crossing-over in the PAR (32). The mouse PAR is 700 kb (33), which is nearly four times shorter than the human PAR (2.7 Mb) (34). The *Zfy2* gene may, therefore, be an adaptation selected for by the shortening of the PAR in the rodent lineage.

ZFY and human fertility

Our finding that human *ZFY* produces a transcript without the equivalent of exon 6 that predominates in the testis and is present in the testis that lack post-meiotic stages establishes *ZFY* as a candidate gene for human male factor infertility. Interestingly, like *Zfy* in the mouse, *Usp9y* is a testis-specific gene whose transcription increases as germ cells begin to enter meiosis in the testis (35). Furthermore, *Zfy* and *Usp9y* are the only testis-specific mouse Y chromosome genes whose human orthologues are widely expressed (36). Isolated deletions of *USP9Y* (five cases) have only been found in the male partners of couples consulting at infertility clinics, suggesting that they do reduce male fertility (37–40). In three cases, however, the *USP9Y* deletion has been transmitted naturally, and the phenotype in most cases is mild; moderate oligozoopermia in two cases (37), and normospermia in one case (38), indicating that *USP9Y* increases the efficiency of spermatogenesis without being essential for male fertility. *ZFY* mutations could, therefore, have similar slight effects on male fertility.

To begin to evaluate the extent to which mutations in *ZFY* are a cause of human male fertility, we have screened 520 infertile men for the deletion of the *ZFY* gene, using PCR tests for the two ends and the middle of the gene, but have failed to find any deletions (M.J.M. and E.S., unpublished data). The incidence of large deletions affecting *ZFY* is, therefore, low ($< 0.2\%$) in our infertile population. Nevertheless, it is now established that, in the mouse, ZFY2 contributes to the stringency of the pairing checkpoint at MI (18), and ZFY might

Table 1. The semen characteristics of the three samples used for our study, compared with normospermic parameters (last line)

Patients	Volume (ml)	Concentration (10^6/ml) [spermatozoa (round cells)]	Motility [a + b (a/b/c/d)]	Morphology (% normal forms)	Sperm phenotype
08153	2.3	40 (8.3)	20 (0/20/20/60)	19	Astheno-teratospermia
09203	6.2	89 (1.4)	65 (45/20/5/30)	27	Mild teratospermia
09175	3.5	364 (15.6)	40 (5/35/30/30)	51	Asthenospermia
—	>2	>20	>50 or a is >25	>30	Normospermia

play a similar role during human spermatogenesis. If this is the case, it will be important to identify those rare men with a mutation in *ZFY*, as this could have consequences for the stable transmission of the genome to the next generation.

MATERIALS AND METHODS

Mouse samples

Staged testes (day of birth = 0 d.p.p.) were from: (i) XY C57BL/6 males (Charles River, France), (ii) XY*Sxr*^{b} males that carry the variant sex reversal factor Tp(Y)1Ct^{Sxr-b} attached distally to the Y PAR, together with their XY littermates (Random-bred MF1 stock background—NIMR colony) and (iii) X^E*Sxr*^{b}O males that carry an *Eif2s3y* transgene inserted on the X chromosome and *Sxr*^{b} attached distally to the X PAR (also on an MF1 background). The production of the X^E*Sxr*^{b}O males has been described elsewhere (18).

Patient samples: maturation arrest biopsies

Testicular samples were obtained from two azoospermic patients who underwent a testicular biopsy in order to recover testicular spermatozoa within the context of an intracytoplasmic sperm injection (ICSI) attempt. Each patient had a normal karyotype. Patient Ste-363 from St Etienne has a P5/proximal-P1 deletion of the AZFb interval of the Y chromosome and has been described previously (41). Patient APOP12, from Marseille, has no detectable Y chromosome deletion. He gave his informed consent for the storage of the immature testicular cells in our 'Germetheque' biobank, and their use in research. A meiotic study performed on cells from Apop12 revealed an early meiotic block, with 97% of spermatocytes at the leptotene stage (C.M.-G., unpublished data). In both cases, the material was the remains of a testicular biopsy after it had been teased apart to recover spermatozoa for ICSI.

Patient samples: sperm

Human ejaculates were collected by masturbation from normal men, consulting with their partner for couple infertility, through the Sperm Diagnostic Unit, Biology of Reproduction Laboratory, La Conception Hospital, Marseilles, France. Men participating in this study consulted for a sperm analysis as part of the exploration of the infertility of their couple, and gave informed consent that the remaining part of the semen sample could be stored and used in research instead of being discarded. Samples were included in the 'Germetheque' biobank. Sperm analysis was performed according to the WHO criteria and morphology was assessed according to the French DAVID classification (Table 1). After sperm analysis, the remaining sperm was centrifuged at 3000 r.p.m. for 15 min. The pellet was frozen and stored in liquid nitrogen until use.

RNA extraction

For all mouse samples, polyA$^+$ RNA was isolated using the μMACs mRNA Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. For human testis biopsies, total RNA was extracted with TriPure (Roche), as previously described (41). Total human RNAs from normal human testes and other tissues were purchased from Clontech. To extract RNA from spermatozoa, pelleted samples were thawed and washed twice in 2 ml of PBS. They were then resuspended in round cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in RNase-free H_2O) for 10 min on ice and then washed twice in PBS. This treatment preferentially lyses somatic cells and immature germ cells while leaving spermatozoa intact (42). RNAs were extracted from 15×10^6 spermatozoa with 1 ml of TriPure (Roche) following the manufacturer's protocol. RNA was precipitated twice with isopropanol in the presence of 20 μg of glycogen (Roche). The RNA was subsequently treated with DNase I and RNase-free (Roche) and then either purified by passage through a Chroma Spin-200 + DEPC-H_2O column (Clontech) (samples 08153 and 09203) or by isopropanol precipitation (sample 09175).

PCR, RT-PCR and 3′ RACE

For the mouse, in general, 500 ng of mRNA were converted into cDNA with expand RT (Roche) and random nonamers in a 20 μl reaction volume. Less mRNA was used for the X^E*Sxr*^{b}O cDNAs, because RNA was extracted from testis fragments and yields were <500 ng, but in all these cases, the corresponding XY control cDNA was generated from an equivalent amount of RNA. Mouse cDNAs were diluted 10-fold and 1 μl, or a volume corresponding to 2.5 ng of input mRNA, used in a final PCR volume of 12 μl. PCR was performed using standard protocols.

For 3′ RACE, cDNAs were synthesized using an oligo-dT-adaptor primer (GACTCGAGTCGACATCGA-dT$_{17}$). RACE PCR was performed as nested PCR with a single reverse adaptor primer (o2761) and two forward primers specific for *Zfy1* (o2828 then o2851) or Zfy2 (o2827 then o2852). The first PCR was for 30 cycles and then 1 μl was used for a second PCR of 30 cycles.

For humans, except spermatozoa, 1 μg of total RNA was converted to cDNA in a 20 μl reaction, diluted 10-fold and 1 μl used for PCR in a 12 μl reaction. For spermatozoal RNA, all recovered total RNA was converted to cDNA, diluted 2-fold and 1 μl used in a 12 μl PCR reaction. RT reactions were random-primed.

All primers used are listed in Supplementary Material, Table S1.

Sequencing

Individual PCR products were cut from agarose gels and purified using NucleoSpin Extract II (Macherey-Nagel) according to the manufacturer's protocol, and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

Sequences were then analysed with the Sequencher 4.2 software (Genecodes). Comparisons were made with sequences deposited in the NCBI database: mouse *Zfy1* (NM009570), *Zfy2* from its *Cypt* (AK133222) or its *Zfy* promoter (AK030048) and human *ZFY* (NM003411).

Quantitative real-time reverse transcription PCR

Quantitative RT-PCR was performed with the LightCycler 480 SYBR Green I Master Kit (Roche) on the LightCycler 480 (Roche). Reactions were performed in 10 μl. Amplification conditions were 1 cycle at 95°C for 5 min, followed by 43 cycles of 95°C for 10 s, 58 or 60°C for 15 s, 72°C for 15 s. Controls with H_2O were negative for all tests. All reactions were carried out in triplicate per assay and Hmbs was included on every plate as a loading control. The melting curve profiles were analysed for every sample to verify the absence of non-specific products or primer dimers.

For each sample, the mean Ct for Hmbs was subtracted from the mean Ct for Zfy (ΔCt). The value for the Zfy transcript level was calculated from this ΔCt by attributing the arbitrary value of 1000 to the Hmbs transcript level, using the formula $E^{\Delta Ct} \times 1000$, where E is the PCR efficiency. E was calculated for each primer pair, using a series of dilutions of template cDNA. The slope (a) of the standard curve, $Ct = a(\log [conc]) + b$, was obtained by plotting $\log_{10}$ cDNA concentration, [conc], against Ct, and E was calculated as $10^{(-1/a)}$. For each plot, R^2 was >0.99. If a reaction has 100% efficiency, $E = 2$. Values for E ranged from 1.88 to 2 (Supplementary Material, Table S1).

We chose the *Hmbs* gene as control housekeeping gene because it showed low variability in Ct between all testis stages when equivalent quantities of cDNA were used as template.

RNA FISH

RNA FISH was performed as previously described (12,20). Wild-type testis material was from adult MF1 male mice. To provide control material to monitor probe specificity, we used 30 d.p.p. testes from $X^{EZ1/U}OSry$ or $X^{EZ2}OSry$ transgenic males; these males carry BAC transgenes for *Eif2s3y* (^{E}) together with a BAC transgene for *Zfy1* + *Ube1y* ($^{Z1/U}$) or *Zfy2* (^{Z2}), on their single X chromosome, with testis development driven by an autosomally located *Sry* transgene (13,18). The *Zfy2*-specific probe used was the BAC CITB-288D7 (Research Genetics). The *Zfy1*-specific probe used was a modified version of BAC RP24-498K8 (CHORI) from which we had removed the entire *Ube1y* gene by recombineering.

ZFY-Gal4 fusion proteins

ZFY-Gal4 fusion-protein constructs were made by inserting PCR-amplified cDNA segments encoding the different acidic domains into the *Nco*I and *Sal*I restriction sites of pGBKT7 (Clontech), downstream of the *GAL4* DNA-binding domain and the c-myc epitope tag of the vector. Long and short acidic domains were amplified together for each *ZFY* gene from testis cDNA, and each fragment was purified following size fractionation on an agarose gel. The *GAL4* acidic domain (*GAL4*-AD) was integrated into pGBKT7 as a positive control and was amplified from the vector pGADT7 (Clontech). Primers used, with respectively an *Nco*I or *Sal*I adaptor, were *ZFY*: o4108/o4109, *Zfy1*: o4100/o4101, *Zfy2*: o4102/o4103 and *GAL4*-AD: o4106/o4107 (Supplementary Material, Table S1). Recombinants were verified by sequencing the entire PCR product and vector flanks. Two recombinants were selected for each construct and transformed into cells from the *S. cerevisiae* strain Y187, in which the β-galactosidase gene is under the control of the Gal4-responsive *GAL1* promoter. Single-transformed colonies were picked from SD/-trp agar plates and grown in liquid culture to an OD_{600} of 0.6–1.2 in SD/-trp liquid minimal medium. The β-galactosidase assay was performed on ∼1 OD_{600} unit of the culture, using the permeabilized cell assay (43). Another 2.5 OD_{600} units from the same culture were used to prepare protein for standard western blot analysis using an anti-human c-myc tag antibody (Biolegend) (Supplementary Material, Fig. S1).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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