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Differential expression of *Rfx1–4* during mouse spermatogenesis

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Abstract

The regulatory factor X (RFX) family of transcription factors has been recently implicated in gene regulation during spermatogenesis. However, the relative expression of individual members during this developmental process is not completely characterized, particularly in the case of *Rfx4*, which has multiple transcript variants in the testis. We used reverse transcriptase-dependent real-time PCR, 5'-RACE cloning, and western blotting to compare transcripts and protein levels for this family in cell populations from the three major phases of spermatogenesis (mitotic, meiotic, and haploid). Transcripts for *Rfx1–4* were present at trace to low levels in spermatogonia prepared from 8-day-old mice. Transcripts for both *Rfx2* and *Rfx4* were elevated in mid-late pachytene spermatocytes; however the dominant *Rfx4* transcript present begins at a downstream exon and lacks the DNA binding domain. Transcripts for all four genes were elevated in early haploid cells (round spermatids). In these cells *Rfx4* transcripts originate primarily from a newly described promoter with intron 1 but are expected to be translationally compromised due to a poorly situated start codon. Western blotting confirmed that RFX2 is greatly elevated beginning in meiosis and also confirmed that full length RFX4 protein is not prevalent in mouse testis at any stage. These results imply that RFX2 is the most likely X box binding factor to influence novel gene expression during meiosis, that RFX1–3 may all play roles in haploid cells but that RFX4 is much less prevalent than implied by its high transcript levels.

Keywords

Alternate promoter; Gene regulation; Meiosis; Mouse; Real-time PCR; Spermatid; Spermatogenesis; Spermatogonia; Transcription factor; 5'-RACE

The regulatory factor X (RFX) family of transcription factors includes 5 proteins that share a conserved amino-terminal DNA binding motif as well as a common DNA binding site consensus (Gajiwala et al., 2000; reviewed in Emery et al., 1996; Reith and Mach 2001; Zhang et al., 2007). RFX1–4 generally form homo- or heterodimers and have diverse gene targets. In contrast, RFX5 lacks the conserved carboxyl dimerization domain and is primarily associated with regulation of major histocompatibility class II (MHCII) and some examples of MHCI gene expression (reviewed in Reith and Mach, 2001; Nekrep et al., 2003; Rousseau et al. 2004). Although it has been recognized that *Rfx1–4* are up-regulated in the mammalian testis for some time (Reith et al., 1994; Morotomi-Yano et al., 2002; Zhang et al., 2007), investigation of the roles of RFX factors in expression of particular genes during spermatogenesis is relatively recent. RFX binding sites (X boxes) have been identified in the immediate promoter

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of the spermatocyte-specific histone *H1t* gene, the germ cell specific variant of general transcription factor IIA (*Alf* or *Gtf2a1l*), and several other genes with expression beginning primarily or uniquely in spermatocytes (Wolfe et al., 2004; Horvath et al., 2004; Kim et al., 2006).

Northern blots identified testis as the organ with maximum expression of *Rfx1–3* (Reith et al., 1994), but expression patterns in other organs varied for each gene. *Rfx1* was widely expressed. In contrast, Rfx2 had no more than trace expression outside the testis. *Rfx3* transcripts were plentiful in brain, intestine, lung and ovary. Cloned later, the cellular *Rfx4* gene is expressed at high levels in testis (Morotomi-Yano et al., 2002; Araki et al., 2004; Matshushita et al., 2005). However its only clear functional role is associated with dynamic and wide-spread expression in the embryonic central nervous system (Blackshear et al., 2003) and with the suprachiasmatic nucleus in adult brain (Araki et al., 2004). *Rfx4* was reported to lack a transcriptional activation domain (Morotomi-Yano et al., 2002), but recent work indicates that RFX4-v3 can stimulate the *Cx3Cl1* promoter (Zhang et al., 2008). Knockout mice for both *Rfx3* (Bonnafe et al., 2004) and *Rfx4* (Blackshear et al., 2003) have severe central nervous system defects, and affect genes required for cilia formation in the case of *Rfx3* (Bonnafe et al., 2004; Ait-Louinis et al., 2007).

Because of the diversity of the RFX family, and the likelihood that each protein requires unique patterns of interaction with co-regulators and other transcription factors for effective modulation of promoter function, it is important to understand the expression of individual members throughout spermatogenesis. Prior immunohistochemical study showed that RFX1 is prominent both in the somatic Sertoli cells of seminiferous tubules and also detected in early haploid germ cells. In contrast, RFX2 was apparent in germ cell nuclei only beginning in the early pachytene phase of meiosis and extending through the early haploid period of development (Horvath et al., 2004). In the present study we used reverse transcriptase-dependent real-time PCR and western blotting to examine *Rfx* patterns in the three major phases of spermatogenesis. We have also used 5'-RACE cloning to ask if novel promoter use accounts for the up-regulation in testicular expression.

1. Results and discussion

1.1 Relative expression of RFX1–4 during spermatogenesis determined by RT-dependent real-time PCR

Spermatogenesis in rodents occurs according to a precisely controlled developmental schedule (Oakberg, 1956; Russell et al., 1990). Germline stem cells located just inside the wall of the seminiferous tubules generate mitotically dividing, differentiating spermatogonia, which then enter meiosis as spermatocytes. Meiotic cells spend much of this period as large cells in the mid-late pachytene (thick-strand) phase. Following the two meiotic divisions, haploid cells (spermatids) form, which retain a round morphology for about half the time required to transform into a sperm, which is finally released from the seminiferous epithelium. To determine the general pattern of *Rfx* factor expression during spermatogenesis we isolated enriched populations of spermatogonia, large pachytene spermatocytes, and round spermatids. We then used RT-dependent real-time PCR to estimate relative *Rfx* transcript levels. We used primer pairs located in or near the coding region in the most downstream exons. This approach measured transcripts without regard to possible upstream promoter or splice variants, although it would miss transcripts resulting from alternative premature polyadenylation sites. Transcripts were in the low to barely detectable range for all four genes in spermatogonia (Fig. 1). In contrast, all four genes were greatly up-regulated in post-meiotic, early haploid cells. Only *Rfx2* and *Rfx4* were up-regulated during meiotic prophase in pachytene spermatocytes.

1.2 Use of 5'-RACE cloning to identify possible alternate promoters for Rfx1–3

Use of alternate promoters is relatively common in testis-expressed genes (reviewed in Eddy and O'Brien, 1998; Kleene, 2001; DeJong, 2005). In order to screen for alternative cap sites that might account for the significant increase in *Rfx* mRNAs during spermatogenesis, 5'-RACE clones were constructed using a commercial preparation of RACE-ready cDNA. A reverse primer was used in exon 2 of each gene to focus on cap sites that would give rise to full-length proteins.

The reference sequence for *Rfx1* (NM_0090055) begins with a non-coding 144 bp exon. Six RACE clones had shortened versions of this exon, of about 48 or 105 bp (data not shown). These cap sites would have no effect on the normal translational start codon and are considered to originate from the usual somatic promoter. A second set of 5 *Rfx1* clones initiated some 471 bp upstream of the *Rfx1* reference sequence and contained two short initial exons that were spliced to exon 2 (Fig. 2). This transcript has three upstream out-of-frame AUG codons. The abundance of transcripts with this 5'-end was estimated by real-time PCR using a downstream primer in exon 2. The novel exon was not detected in brain and only at trace levels in spermatogonia. It accounted for only about 2 percent and 4% of the transcripts in spermatocytes and spermatids, respectively. Thus, use of this promoter may be testis-specific, but it can only account for a small fraction of the increase in *Rfx1* mRNA.

Rfx2 also has a non-coding exon 1 (NM_009056). Seven RACE clones revealed only a range of starts within the normal exon 1, creating lengths of 33 to 152 bp (data not shown). Similarly, no novel cap sites were identified for *Rfx3* (NM_011265), which again has a non-coding exon 1. Seven *Rfx3* clones had variants of exon 1 measuring between 97 and 176 bp. One clone contained an additional small exon matching database clone AK041120.

In summary, results from these RACE clones indicates that accumulation of *Rfx2* transcripts in spermatocytes and of *Rfx1–3* in spermatids is apparently not due to activation of distinctly novel promoters. None of these promoters involves a TATA box, and it is not surprising that they generate a cluster of start sites (Juven-Gershon et al., 2008).

1.3 5'-RACE clones for Rfx4 identify a novel promoter site

Rfx4 has the most complex expression pattern of the gene family, with variations in both transcriptional start and polyadenylation sites (Fig. 3) (reviewed in Zhang et al., 2007). Because its only certain functional role is in brain, where the most upstream promoter is used (Blackshear et al., 2003), we have numbered the exons based on the brain transcript. Testis variant exons are shown slightly elevated. *Rfx4* is distinct in that brain exon1 establishes the *Rfx4* reading frame. Multiple transcript variants have been isolated from testis with different cap sites (Ex1Tb and Ex1Tc, Fig 3) as well as premature termination points (Ex14T and Ex15T, Fig. 3). We sequenced 10 5'-RACE clones. All initiated within a few nucleotides of one another at a novel location in the first intron (Ex 1Ta, Fig. 3). This particular cap site has not been previously identified in publications but is represented by a single mouse testis EST (CF1055749) in the nucleic acid databases. The clones with the most upstream sequence included a potential methionine start codon that generates the correct reading frame (Fig. 4). However, this AUG is located only 15 bp from the presumed cap site and also lies in an extremely poor Kozak consensus (CCCTACATGT, Fig. 3) (Kozak, 1991). With two strikes against it, it is not likely to be an efficient translational initiation point. In some RACE clones this start codon was at the extreme 5' end or missing (as is true for EST CF1055749). If translational initiation fails at this AUG, it must then bypass 7 out-of-frame AUG codons before finding an in-frame methionine codon at the very downstream end of exon 4. Upstream open reading frames generally but not invariably suppress use of subsequent ones (Morris and Geballe, 2000, 2006). A protein that did manage to initiate at the correct exon 4 met codon

would begin one third of the way through the 75-AA DNA binding domain. It would be similar to the protein generated from the 1Tc mRNA (Fig. 3). One RACE clone contained a 3'-extended 1Ta exon that splices 61 bp farther downstream, and joins exon 2 in the wrong reading frame (not shown). Full length R*fx4* transcripts beginning with exon 1Ta could be amplified from testis cDNA (but not brain) using PCR and a reverse primer just downstream of the stop codon in exon 18. This shows that exon 1Ta does generate at least some full length transcripts (not shown). In summary, the most prevalent testis *Rfx4* transcript begins at a novel site and could encode a functional protein, but appears unlikely to serve as an efficient mRNA.

1.4 Exon-specific real-time PCR shows that exon 1Tc accounts for Rfx4 transcripts in pachytene spermatocytes while exon 1Ta accounts for the bulk of transcripts in spermatids

We then used primer pairs to estimate the contributions of different potential *Rfx4* cap sites to the *Rfx4* transcript pool in separate germ cell populations. As already described, primers for exon 18 showed that expression is markedly up-regulated in spermatocytes relative to spermatogonia and that expression is yet 3 fold higher in round spermatids (Fig. 5). A primer pair specific for brain-expressed exon 1 found no target in any of the testis cell fractions, confirming prior studies. Primers for exon 2 to 4 will amplify from any transcript that includes the full DNA binding domain. This primer pair detected only traces of R*fx4* in spermatocytes but did identify elevated transcripts in round spermatids (Fig. 5). The primer pair for exon 1Ta to 4 gave essentially the same results as the exon 2 – 4 primer pair, indicating that exon 1Ta is not expressed significantly in spermatocytes but is responsible for much of the total *Rfx4* transcript pool in spermatids. Potential exon 1Tb, which has been reported in databases among human testis clones, was detected at no more than 1% the frequency of exon 1Ta (results not shown). Finally, exon 1Tc (located within intron 5 and responsible for approximately 100 human EST clones) accounts for most or all of the *Rfx4* detected in spermatocytes. Exon 1Tc is less prevalent in round spermatids.

In summary, we confirmed that expression from brain-specific exon 1 is undetectable in testis. *Rfx4* expression in mouse testis originates primarily from two alternate promoters, depending on the stage of germ cell maturation. Transcripts begin in intron 5 (ex1Tc) in spermatocytes and from intron 1 (ex1Ta) in round spermatids. The spermatocyte transcripts generate a protein that lacks the DNA binding domain and is of uncertain functional significance. While some spermatid transcripts encode a full length protein, they depend on a very poorly situated translational start codon, suggesting poor translational efficiency.

1.5 Western blotting confirms that RFX2 is the family member present at the highest level in spermatocytes

Our final experiment was to use western blotting to confirm that RFX2 is the only RFX protein that increases during pachytene. We obtained antibodies for RFX1, −2, and −4. We tested a commercial and an investigator-supplied antibody to RFX3 but did not find either one useful for blots from mouse testis. We probed blots made from isolated cell fractions, using an extract from the SCN region of mouse brain as a positive source for functional RFX4 (Araki et al., 2004). RFX1 levels were roughly comparable in the three cell fractions, though somewhat reduced in spermatogonia (Fig 6A). RFX2 was not detected in spermatogonia but strikingly up-regulated in spermatocytes, and retained in spermatids. RFX4 was readily detected in the SCN by two antisera. However, it was detected at much reduced levels in other germ cells by antiserum a, and with antiserum b was more evident in spermatids than in spermatocytes or spermatogonia (Fig. 6A). The different developmental profiles characterizing RFX1 and −2 were also documented using whole testis extracts made from a series of immature mice (Fig. 6B). RFX1 showed little change from the age at which spermatogonia are the most prevalent germ cells (7 days) through to adulthood. In contrast, RFX2 was present in but trace levels at

7 days, but began to increase at the known time of appearance of pachytene spermatocytes, about 14 days of age (Bellve et al., 1977).

The goal of the present study was to determine which RFX transcription factors are the most likely to influence changes in gene expression during the progress of spermatogenesis and to determine if they are expressed from novel promoters. We ignored RFX5 because it does not dimerize with the other members of the family and appears likely to be functionally restricted to changes in gene expression that relate to the constitutive or induced expression of the MHCII family (Reith and Mach, 2001; Drozina et al., 2005). During the latter half of meiosis, in pachytene spermatocytes, transcripts for both *Rfx2* and *Rfx4* are elevated. However, *Rfx*4 is expressed from a distinct promoter that is located within inton 5 and specifies a protein that lacks the conserved Rfx DNA binding domain (Rfx-A or variant 1, Zhang et al., 2007). All of the remaining *Rfx* family are up-regulated in the haploid phase of spermatogenesis. Once again only *Rfx4* undergoes a major shift in promoter use associated with this up-regulation. Oddly, the 1Ta promoter within the first intron generates transcripts that either lack a functional in-frame start codon, or have this codon in such a poor location as to suggest its very inefficient function. While this was the only intron 1 promoter detected by RACE cloning in mouse, a different intron 1 cap site (1Tb, Fig. 3) appears to be the major equivalent used in human spermatogenesis (RFX-B, variant 2, Morotomi-Yano et al., 2002). Western blotting confirmed that RFX2 is greatly up-regulated during pachytene and onward into early haploid cells, while RFX4 as a full length protein was not detected in spermatocytes. Taken together our results point to RFX2 as the most likely protein to influence changes in gene expression during meiosis and indicates that RFX1–3 must be considered for genes undergoing expression changes in the haploid cells. Rfx4 expression remains yet another oddity of spermatogenesis where transcript changes are not always easy to rationalize with functional consequences.

2. Experimental procedures

2.1 Mice

Hsd:ICR outbred mice were obtained from Harlan Sprague Dawley. The use and care of animals were approved by the University of South Carolina Animal Care and Use Committee.

2.2 Preparation of purified germ cells

Preparation of enriched populations of mid to late pachytene spermatocytes and round spermatids from adult mice by elutriation has been described (Fantz et al., 2001). Cells used for RNA isolation and protein extracts for western blotting were purified additionally by Percoll gradient centrifugation as described (Ma et al., 2008). Spermatogonia were isolated as non-adherent cells from 8-day-old mice testes as described previously (Ma et al., 2008).

2.3 5'-Rapid amplification of cDNA ends

The 5'-RACE was carried out using mouse testis RACE-ready cDNA from Swiss-Webster mice (catalog no. 3206, Ambion) following the protocols supplied. Ligation of the 5'-RNA adapter containing the forward primer site used a protocol that depends on the presence of the cap-specific pyrophosphate linkage, which should ensure that 5'-ends are genuine. A set of outer and inner reverse primers was designed for each *Rfx* family member (Supplement Table I) with a buried *Hind*III site at the 5' end of the inner primer to facilitate cloning. PCR products were purified (QIAquick PCR purification kit, Qiagen), cloned into pBluescript (Stratagene) and sequenced.

2.4 Reverse transcriptase dependent PCR and real-time PCR

Real-time PCR was carried out in an iCycler (Bio-Rad) using a SYBR Green real-time master mix (Bio-Rad) by procedures described previously (Ma et al., 2008). Primer pairs (Supplement Table I) were selected using the Primer 3 program (http://frodo.wi.mit.edu/) (Rosen et al., 2000) or a program at the supplier's web site. Individual mRNAs were normalized to 18S rRNA by differences in cycle thresholds (Ct) (Bustin et al., 2005) and results expressed as copies per 10^6 18S rRNA copies.

2.5 Western blot analysis

Extract preparation, electrophoresis in 8% gels, blotting conditions, and antibody incubations and detection have been described previously (Ma et al., 2008). Protein in modified SDS sample buffer (Ma et al., 2008) was determined after quenching mercaptoethanol with iodoacetamide (Hill and Straka, 1988). Antibodies used were: RFX1 (Santa Cruz I-19, sc-10652); RFX2 (Santa Cruz C-15, sc-10657); RFX4a made to AA 107–128; RFX4b, made to amino acids 126 to 451, both provided by Ryoko Araki (Araki et al, 2004); actin, Sigma A2066.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

3. Acknowledgments

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Appendix A. Supplementary data

Table of PCR Primers

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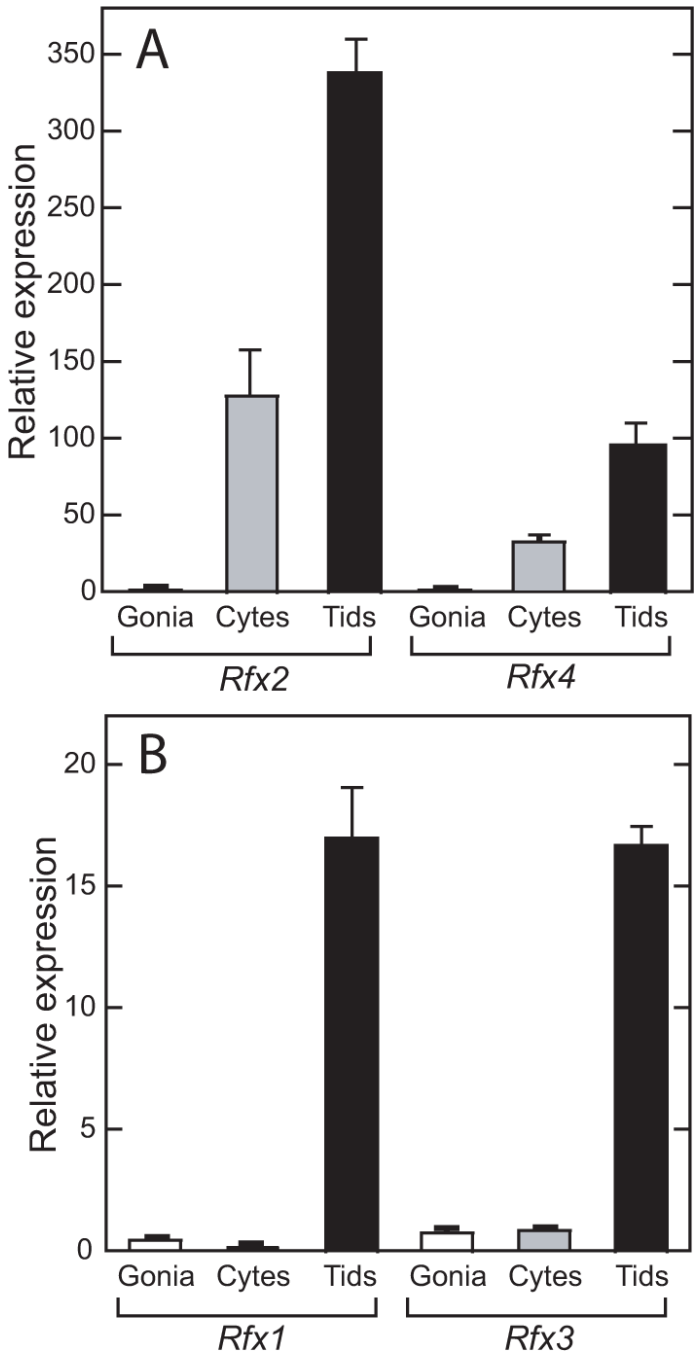

Fig. 1.

Relative levels of total RNA transcripts for *Rfx1* to *−4* in purified germ cell fractions determined by real-time PCR. Primer pairs were chosen to lie in the last exon of each gene, either in or near to the carboxyl end of the encoded protein. These pairs are expected to amplify all transcripts for functional proteins, although in the case of *Rfx4* there are known testis transcript variants that terminate farther upstream. Note that different scales are used for *Rfx2* and *−4* (more prevalent) and *Rfx1* and *−3*.

Fig. 2.

The 5'end of testis alternative *Rfx1* transcript. This RACE sequence contains two novel upstream exons that derive from intron 1 fused to exon 2. Exons are shown in alternating upper and lower case. The reading frame begins in exon 2 and is indicated at the end of the sequence. Upstream ATG and temination codons are boxed. Truncated versions of this transcript were also identified, beginning at the first upstream ATG codon (shown as shaded nucleotides).

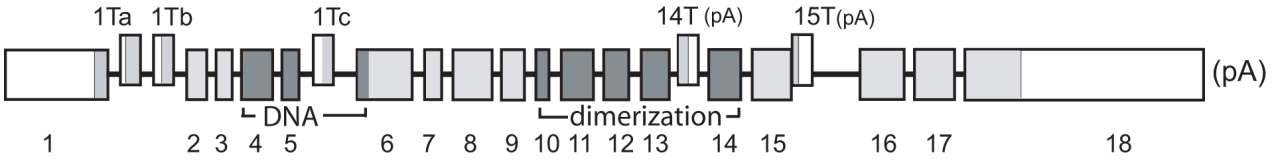

Fig. 3.

Exons of the mouse *Rfx4* gene. Exons are indicated approximately according to size; introns (thin line) are not. Exons are numbered in reference to the brain-specific transcript. Alternative exons used in testis transcripts are elevated. Alternative cap-site exons are designated 1Ta, 1Tb, and 1Tc. Alternative downstream exons with poly(A) sites are designated similarly. Coding regions are lightly shaded and both the DNA binding and dimerization domains are darkly shaded. These exons are associated with the following RFX4 transcript variants: exon 1, v. 3/D (NM_213594); exon 1Ta, identified in this report (Accession in progress); exon 1Tb, v. 2/B (NM_002920) and v. 4/C (AB095365); exon 1Tc, v. 1/A (NM_032491; exon 14T, (AK016791); exon 15T, v. 2/B (NM_002920).

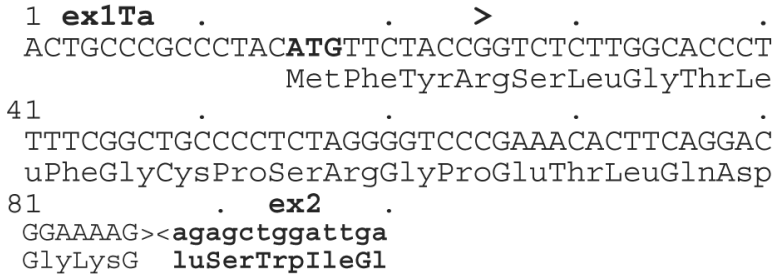

Fig. 4.

RFX4 testis exon 1Ta. The single family of 5-RACE clones isolated for *Rfx4* begin with this exon. It corresponds to a single spliced mouse EST clone, CF105749, derived from a mouse spermatid library, although that clone begins just downstream of the initiation codon (arrowhead at +25). Although the methionine start codon is very near the 5' end and does not lie is a good Kozak initiation site, it establishes the correct reading frame. If translated through to the stop codon in exon 18, this protein would contain an additional 9 amino acids at the amino terminal end compared to the one derived from the brain transcript. One RACE clone contained a longer version of this exon in which splicing does not occur for another 61 nucleotides. In that case9 case, the reading frame does not connect to the correct frame in exon 2.

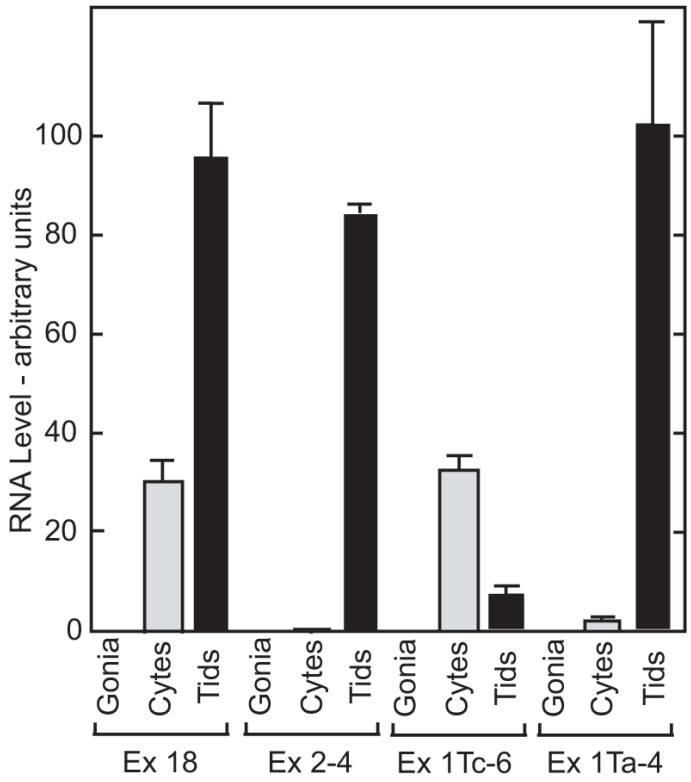

Fig. 5.
Prevalence of *Rfx4* transcripts in testis germ cell populations determined by real-time PCR.

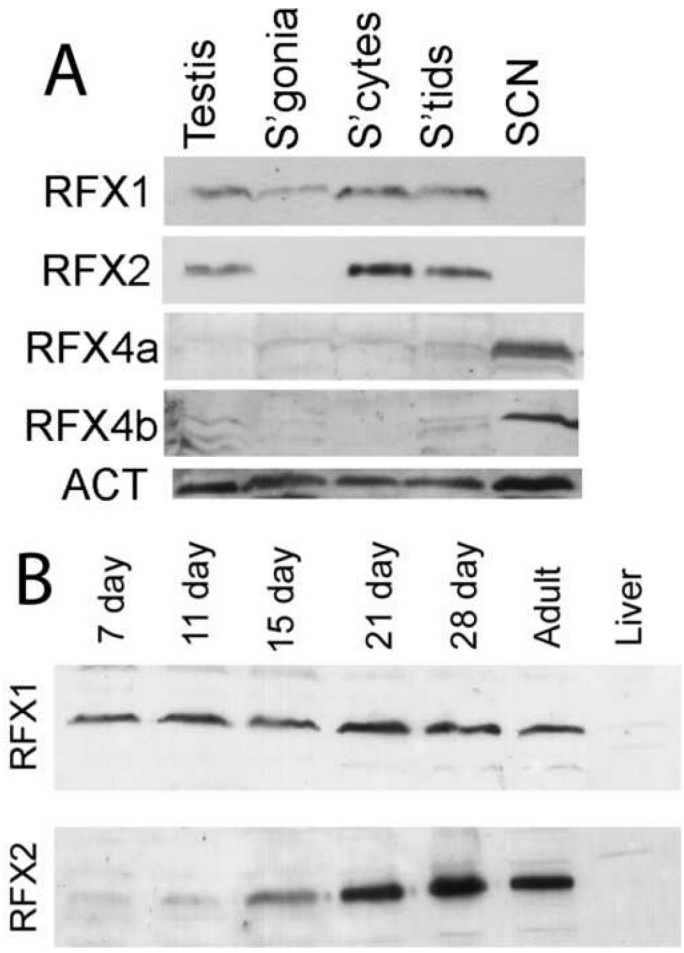

Fig. 6.

Prevalence of RFX factors during germ cell development assayed by western blot. A) Extracts (50 ug) prepared from adult, decapsulated testis and from isolated germ cell populations. The SCN region of adult brain was used as a source of full length RFX4 (50 ug). B) Whole testis extracts prepared from testes during development of the first wave of spermatogenesis (25 ug). Spermatogonia are the most advanced cells at 7 days. Pachytene spermatocytes first appear at about 14 days and round spermatids between 18 and 21 days (Bellve et al., 1977). Results are representative of at least three independent blots.