Absolute mRNA levels and transcriptional regulation of the mouse testis-specific thioredoxins

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ABSTRACT

Thioredoxins function as general protein disulphide reductases. Mammalian male germ cells are equipped with a set of three testis-specific thioredoxins named Sptrx-1, -2 and –3, respectively. Sptrx proteins are expressed either in different structures within the sperm cell or at different stages of sperm development, suggesting a refined regulation in the timing and levels of expression for each *Sptrx* gene. Previous studies based on qualitative northern-blot and *in situ* hybridization analyses restricted the presence of Sptrx mRNAs to adult testis, but nothing is known about their transcriptional regulation or relative expression levels in this tissue.

In this report we investigate the transcriptional profiles of the mouse *Sptrx* genes in terms of the germ cell-specific regulation by promoter analysis in GC-2spd(ts) cells. Besides, we perform a comprehensive quantification of the *Sptrx* mRNA molecules by real-time PCR in whole-animal experiments. By these means, we show that transcription is differentially regulated for each *Sptrx* gene and identify the 5'-flanking regions anticipated to contain the cis-regulatory elements responsible, at least in part, for the transcriptional silencing and/or activation of the *Sptrx* genes. In addition we show remarkable age-associated variations between the *Sptrx* mRNA expression patterns. Finally we corroborate the testis-specific expression pattern of *Sptrx-2* and *Sptrx-3*, but surprisingly we find a wider expression pattern for *Sptrx-1*

Keywords: Thioredoxin, sperm, testis, promoter, gene expression, real-time PCR

1. INTRODUCTION

Cellular redox balance is maintained in living organisms by various non-enzymatic as well as enzyme-based systems [1]. Among them, thioredoxin (Trx) and related proteins have emerged as one of the most important thiol-based systems being involved in many physiological as well as pathophysiological processes [2]. The mammalian thioredoxin system consists of the hydrogen donor NADPH and the selenoprotein thioredoxin reductase (TrxR) coupled to the redox active protein thioredoxin [3, 4]. Eukaryotic organisms are equipped with two ubiquitous thioredoxin systems: a cytoplasmic one composed of Trx-1 and TrxR-1, and a mitochondrial one formed by Trx-2 and TrxR-2 [3, 5]. Moreover, different forms of thioredoxins and thioredoxin reductases with unique properties such as organelle- or tissue-specific localization have been reported [6-11]. In this regard, male germ cells are endowed with three testis-specific thioredoxins named Sptrx-1 [12, 13], Sptrx-2 [14, 15] and Sptrx-3 [16], respectively, thus reflecting a key role of this family of proteins in spermatogenesis.

Sptrx-1 comprises one N-terminal repetitive domain of a 15 amino acid motif and one C-terminal thioredoxin domain [12, 13] displaying both reducing and oxidizing activities [17]. Sptrx-1 is located in the developing tail of elongating spermatids, transiently associated with the longitudinal columns of the fibrous sheath. This transient association during sperm tail formation strongly supports a regulatory role of Sptrx-1 in sperm development [12, 13]. Sptrx-2 is also a multi-domain protein composed by one N-terminal thioredoxin domain followed by three NDP-kinase domains [14, 15]. Sptrx-2 is associated with the longitudinal columns of the sperm fibrous sheath and also with the ribs that connect these two columns. Unlike Sptrx-1, Sptrx-2 becomes a structural part of the mature sperm tail and can be detected in ejaculated spermatozoa. Sptrx-2 could be involved in the reduction of disulfide bonds within the sperm fibrous sheath components [14, 15]. In contrast to *Sptrx-1* and *Sptrx-2*, the *Sptrx-3* gene codes for a unique thioredoxin domain and was originated as a genomic duplication of the *Trx-1* gene [16]. Sptrx-3 is a Golgi apparatus-associated thioredoxin showing a transient association with the developing spermatid acrosome. Its function might be related to the post-translational modification of proteins required for acrosomal biogenesis [16].

Spermatogenesis is a complex process leading to the formation of one haploid motile cell and the specific gene expression occurring through spermatogenesis must be tightly regulated and coordinated [18, 19]. Transcription initiation is a key regulatory step in the modulation of gene expression and involves the interaction of many transcription factors and cis-regulatory DNA elements [20]. During spermatogenesis, spermatogenic cell-specific genes are expressed as well as spermatogenic cell-altered transcripts due to different transcription start sites usage, alternative polyadenylation or splicing and mRNA shortening or degradation [21]. As mentioned above, a rigorous regulation in the timing and levels of gene expression is required and likewise transcriptional initiation regulatory mechanisms, post-transcriptional regulatory pathways are crucial for spermatogenesis [22]. For example, transcriptional machinery is inactive in elongating spermatids due to the changes occurring in the chromatin structure and nuclear elongation; in contrast, pachytene spermatocytes and round spermatids are transcriptionally active and display very similar transcription rates [21]. Thus, some mRNAs are transcribed during pachytene spermatocyte or round spermatid stages and then stored as transcriptionally repressed free messenger ribonucleoprotein particles to be actively translated in elongated spermatids [21].

Here we report the first analysis of the mouse *Sptrx-1, Sptrx-2* and *Sptrx-3* gene transcription by characterizing their corresponding promoters and also by performing a meticulous quantification of their steady-state mRNA copy numbers.

2. MATERIALS AND METHODS

2.1. Luciferase reporter constructs

BAC clones RP23-291E22, RP23-332H6 and RP23-332H16 containing mouse *Sptrx-1*, *Sptrx-2* and *Sptrx-3* genomic regions respectively were obtained from BACPAC Resources Center (http://bacpac.chori.org/). All the *Sptrx* 5'-flanking regions were amplified by PCR using combinations of the mutagenic primers listed in Supplemental Data Table 1, which introduced unique restriction sites (*Mlu*I/*Xho*I for *Sptrx-1* and *Sptrx-3* and *Mlu*I/*Bgl*II for *Sptrx-2*) at the 5' and 3' ends, respectively. All the PCR products were first cloned into pGEM-Teasy (Promega) and subsequently verified by sequencing and comparison with sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov/genome//guide/mouse/). Next, the *Sptrx* 5´-flanking regions were removed from the pGEM-Teasy constructs by restriction enzyme digestion and cloned into the promoterless firefly luciferase reporter vector pGL-3 basic (Promega).

2.2. Cell Culture, transfections and luciferase assay

GC-2spd(ts) (ATCC no. CRL-2196), Hepa1-6 (ATCC no. CRL-1830) and Y-1 (ATCC no. CCL-79) cell lines were obtained from the American Type Culture Collection. All media and supplements were purchased from Life Technologies. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) at 37ºC or 32ºC in an atmosphere of 5% CO_2. GC-2spd(ts) cells were supplemented with 1mM non-essential amino-acids. All media contained 10% fetal bovine serum (FBS) and 2mM L-glutamine.

Transient transfections were performed in 24-well plates using Lipofectamine™2000 (Life Technologies) according to the manufacturer's instructions. A total of 0.5µg to 1µg of DNA was used in each reaction. Cells were incubated for 48 hours before lysis and preparation of cellular extracts. Independent parallel transfections using exclusively the pEGFP-N3 vector (Clontech) were always included as additional control for transfection efficiency, measured as number of cells expressing GFP.

Enzyme activity assays were performed to measure luciferase expression levels (Luciferase Assay Kit, Biothema AB, Sweden) using a Berthold FB12 luminometer (Berthold detection systems, Germany).

2.3. Animals

Male BALB/c mice of 7 weeks of age were purchased from Charles River Laboratories (Spain). Mice of other ages were obtained through the "Servicio de Animales de Experimentación of Córdoba University". Animals were individually killed by cervical dislocation. Testes were then removed and immediately frozen in liquid nitrogen. Mice were handled according to the policies and procedures stipulated by the European Community. The investigation was approved by the Ethical Committee of Córdoba University.

2.4. RNA Preparations and Reverse Transcription

RNA extraction and synthesis of standard RNA and cDNA were performed as described [23]. Commercial RNAs were purchased from Clontech, except for ovary total RNA that was obtained from Ambion. RNA sample quality was checked electrophoretically and quantification was performed spectrophotometrically. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples without previous cDNA synthesis.

2.5. Primer design for real-time PCR

Primers were designed *in silico* using Oligo 6.1.1/98 software (Molecular Biology Insights). To obtain the highest specificity and performance, primers were required to have high Tm ($\geq$ 80°C) and optimal 3' ΔG ($\geq$ -6.7 kcal/mol) values. Several pairs of primers were designed for each *Sptrx* gene (Table 2). All primer pairs generated specific PCR products of the desired length. PCR products were further verified by nucleotide sequencing.

2.6. Real-time PCR

Real-time PCR conditions were as detailed [23]. PCR reactions were performed in quadruplicate. No primer-dimers were detected, and investigated transcripts showed optimal PCR efficiencies. An absolute standard curve was constructed with an external standard in the range of 10^2 to 10^9 RNA molecules. The number of mRNA molecules was calculated from the linear regression of the standard curve, as previously described [23].

3. RESULTS

3.1. Sptrx mRNA and protein expression levels in GC-spd(ts) cell line

Promoter analysis studies offer valuable information about transcriptional regulation during spermatogenesis. The identification of the cis-acting and trans-acting elements directing the spermatogenic-specific transcription of *Sptrx genes* is essential to shed more light onto their role in sperm physiology. However, a major limitation to this approach is the availability of adequate cell lines that can recapitulate different aspects of spermatogenesis. To our knowledge, GC-spd(ts) is the only spermatogenic mouse cell line available, which is able to undergo meiosis *in vitro* and develop a primordial tail and acrosome when cultured at 32ºC [24]. To test whether the *Sptrx* genes are expressed in GC-2spd(ts) cells, we performed western-blot analysis on crude protein extracts from these cells grown at 37ºC and 32ºC, but we were unable to detect any of the three proteins (data not shown). As this result may only reflect the translational inactivity of the *Sptrx* transcripts, we followed to measure the actual numbers of *Sptrx* mRNA molecules in GC-2spd(ts) cells. In agreement with their protein levels, the *Sptrx-2* and *Sptrx-3* mRNAs were virtually absent (≤ 0.001 molecules/pg total RNA); however, although at very low levels, we succeeded to detect and quantify the *Sptrx-1* transcript at both 37ºC (0.083 molecules/pg) and 32ºC (0.457 molecules/pg).

Overall, these data indicate that the GC-spd(ts) cell line has very low or undetectable *Sptrx* mRNA and protein levels suggesting that their expression is repressed or silenced.

3.2. Promoter activity of the 5'-flanking region of the Sptrx genes

Genomic sequences corresponding to the 5'-flanking regions of mouse *Sptrx-1*, *Sptrx-2* and *Sptrx-3* genes were identified and cloned into the promoterless pGL-3 basic vector (Fig. 1). These 5'-flanking regions ranged from –1 to –4kb upstream the initiation codon of each *Sptrx* gene. Reverse orientations of the –4kb fragments were also included as negative controls. Next, the ability of each fragment to promote transcription was evaluated by means of a luciferase reporter gene assay in GC-2spd(ts) cells. The experiments were initially carried out at both 37ºC and 32ºC. However, as no difference was found in the promoter activity (data not shown), we decided to perform additional experiments only at 37ºC.

The results presented in Fig. 2 indicate considerable differences among the promoter activities of the 5'-flanking regions of the three *Sptrx* genes. Thus, while the *Sptrx-1* –1kb region did not display any promoter activity compared with the empty vector, the promoter activities of the *Sptrx-2* and *Sptrx-3* –1kb regions were significantly higher than the control, being maximal in the case of *Sptrx-2*. Further differences appeared when we expanded the analysis to –2kb regions. Hence, the *Sptrx-1* –2kb fragment resulted in maximal promoter activity while, conversely, the *Sptrx-2* –2kb fragment significantly reduced that obtained with its corresponding –1kb region. In contrast, the *Sptrx-3* –2kb region did not display any statistically significant change of the promoter activity obtained with the –1kb alone. Noteworthy, the –4kb region of *Sptrx-3* displayed the maximal promoter activity, while the equivalent region of both *Sptrx-1* and *Sptrx-2* 5'-flanking regions showed no significant luciferase activity compared with the empty vector.

In addition to GC-2spd(ts), we transfected two other non-related mouse cell lines, Hepa1-6 (hepatoma) and Y-1 (adrenal gland). In these two non-spermatogenic mouse cell lines, the luciferase activities of the *Sptx-1*, *Sptrx-2* and *Sptrx-3* constructs were essentially the same as in the GC-2spd(ts) cells (Supplemental Data Fig. 1). Only the *Sptrx-3* –4kb construct revealed a significant difference showing as low promoter activity as those of the –2kb and –1kb regions in these two cell lines (Supplemental Data Fig. 1). Collectively, the results obtained in Hepa1-6 and Y-1 cells are mostly consistent with the *Sptrx* null expression profiles observed in GC-2spd(ts) since we did not find any clear differences among the three cell lines. On the other hand, the –2kb distal regions (between –2kb and –4kb) of both *Sptrx-1* and *Sptrx-2* 5'-flanking regions seem to account for a transcriptional silencing of these genes in the cell lines used in the study. The situation is reversed for the *Sptrx-3* promoter in GC-2spd(ts) cells, as the distal sequence present in the –4kb construct resulted in the highest promoter activity.

To gain more insight into the molecular mechanisms influencing the *Sptrx* transcriptional activities, we decided to analyze more in detail the distal 5'-flanking regions of *Sptrx-1*, *Sptrx-2* and *Sptrx-3* genes. Therefore, we made new constructs with the 2kb distal regions (from –2kb to –4kb from ATG) of *Sptrx-1* and *Sptrx-2* (Fig. 1) to investigate their ability to abolish any transcriptional activity. Also, we included as additional negative control the reverse orientations of those sequences that rendered the highest activity in the previous experiments (–2kb reverse for *Sptrx-1* and –1kb reverse for *Sptrx-2,* Fig. 2). Finally, we designed a new –3kb construct for *Sptrx-3* (Fig. 1) to check whether promoter activity of its 5'-flanking region increases with sequences longer than –2kb. To shorten the analysis we restricted the study to GC-2spd(ts) cell line due to the lack of clear differences with Hepa1-6 and Y-1 cell lines in the luciferase assays.

As shown in Fig. 2, *Sptrx-1* and *Sptrx-2* –2kb distal regions exhibited null promoter activity, being even lower than that of the empty pGL-3 basic vector. Interestingly, while the reverse orientation of the –2kb and –4kb regions of *Sptrx-1* showed no activity as expected, the reverse –1kb and –4kb regions of *Sptrx-2* displayed significant promoter activities (Fig. 2). Surprisingly, the –3kb region of *Sptrx-3* did not show any promoter activity (Fig. 2) suggesting the presence of a silencing regulatory element in the –2kb to –3kb fragment.

Taken together, these results indicate the existence of different mechanisms of transcriptional regulation for each *Sptrx* gene. First, the –2kb distal region of *Sptrx-1* appears to be responsible for a strict transcriptional repression; however the lack of promoter activity within the –1kb region may imply other regulatory mechanisms in the proximal –2kb region, which may be considered to contain the core promoter for *Sptrx-1* (Fig. 2). Second, the –1kb fragment of *Sptrx-2* displayed the maximal promoter activity and therefore we propose this region to be the core promoter for *Sptrx-2*; furthermore, although the –2kb distal region seems to play a role in the transcriptional repression, we cannot exclude other regions between –1kb to –2kb participating in this negative regulation (Fig. 2). Third, since all the *Sptrx-3* 5'-flanking regions displayed significant promoter activity except for the –3kb construct, it is plausible to consider the core promoter for *Sptrx-3* to be located in the –1kb fragment; the existence of potential cis-regulatory elements in the –2kb to –3kb interval as well as distal enhancer sequences in the –3kb to –4kb interval are also suggest by our results (Fig. 2).

3.3. Sequence analysis of the 5'-flanking region of the Sptrx genes

The 5'-flanking regions of the *Sptrx* genes were used to localize putative promoter sequences, cis-regulatory modules and other significant elements using the *Eldorado* program included in the GenomatixSuite 3.1.1 software (http://www.genomatix.de/). The output forms from this analysis are summarized in Fig. 3. Core promoters are predicted to be localized within regions of about 600 bp in the first 1.5kb from ATG where different regulatory modules are located: EGRF/NFAT (-1091 to –1113) and glucocorticoid responsive element/Nuclear Factor 1 (-767 to –804) in the *Sptrx-1* promoter; interferon regulatory factor/ETS1 (-655 to –678) in the *Sptrx-2* promoter and BRAC/HOX (-407 to –437), HOX/mouse Krueppel-like factor (-404 to –429) and enhancer CCAAT binding factor/SREBP1 (-229 to –259) in the *Sptrx-3* promoter. Furthermore, two repetitive regions were found: one of 1206 bp in the *Sptrx-2* 5'-flanking region and another one of 4394 bp in the *Sptrx-3* 5'-flanking region.

In addition, we used the *MatInspector* program included in GenomatixSuite 3.1.1 software (http://www.genomatix.de/) to look for putative transcription factor binding sites in the –1kb region from the ATG of each *Sptrx* 5'-flanking region. The most significant binding sites are listed in Table 1 and it is noteworthy that the analysis did not retrieve any TATA-box within the –1kb region of *Sptrx-1*. The fact that *Sptrx-1* –1kb construct does not show any promoter activity (Fig. 1) may be related to the lack of any TATA-box within this region or the presence of the EGRF/NFAT regulatory module outside the –1kb region.

3.4. Testicular Sptrx mRNA molecule numbers

Initial studies on mouse *Sptrx* mRNA expression patterns were based on qualitative multiple-tissue northern blots and *in situ* hybridization analyses [13, 15, 16]. Here, we used a quantitatively rigorous approach based on reverse transcription followed by real-time PCR amplification [23] to provide for the first time a valuable new information on the steady-state copy numbers of the three murine *Sptrx-1, Sprtx-2* and *Sptrx-3* transcripts.

We first wanted to know how the mouse male fine-tunes the number of testicular mRNA molecules during aging. Therefore, two primer pairs were initially designed per each *Sptrx* gene. These primers amplify a proximal or distal sequence away from the corresponding 5´-UTR (Table 2). For comparison, transcript levels for the novel form of thioredoxin/glutathione reductase (TGR) and for the cytosolic thioredoxin system (Trx1 and TrxR1) were quantified in parallel.

As shown in Table 3, vast differences in transcript abundance depending on the primer pair were found for two (*Sptrx-1* and *Sptrx-3*) out of the three investigated *Sptrx* genes. Remarkably, in 7-week-old animals, SPT1.5 that amplifies part of the sequence encoding the C-terminal thioredoxin domain, quantified only 9.4 % (82 *vs* 876 molecules/pg) of the *Sptrx-1* mRNA molecules given by SPT1.2, which amplifies the fragment containing the proposed start codon [13]. Regarding the primer pair used for quantification of *Sptrx-3* mRNA, SPT3.6 located in exons 5 and 6 quantified only 4.2 % (4.7 *vs* 111 molecules/pg) of the molecules determined by means of SPT3.1 that is located in exons 1 and 2 [16]. In clear contrast to what occurs in *Sptrx-1* and *Sptrx-3* mRNA quantification, SPT2.1 that amplifies most of the sequence encoding the N-terminal thioredoxin domain of Sptrx-2, yielded the same number of mRNA molecules as SPT2.2 (294 *vs* 273 molecules/pg), which amplifies part of the sequence encoding its first complete NDP-kinase domain.

With respect to the temporal expression pattern of the *Sptrx* mRNAs (Table 3), outstanding findings are as follows: (i) While the *Sptrx-1* transcripts were at appreciable levels in testes from animals of all examined ages [from pre-pubertal (1/2-week-old) to adult (4- to 7-week-old) mice], *Sptrx-2* and *Sptrx-3* mRNAs were basically restricted to the post-pubertal testis. (ii) Nonetheless, the *Sptrx-1* mRNA was infrequently expressed in pre-pubertal testis, standing for < 0.3 % of the adult amounts. (iii) Adult testes of different ages displayed significant different levels of *Sptrx-1*, *Sptrx-2* and *Sptrx-3* mRNAs (data from SPT1.2, SPT2.1 and SPT3.1 primer pairs, respectively); the amounts of mRNA in 4/5-week-old testes accounted for about 50% of the mRNA molecules quantified in 7-week-old testes. (iv) Primer-associated differences in *Sptrx-1* and *Sptrx-3* mRNA levels increased as the animals aged. For instance, SPT1.5

quantified most of the *Sptrx-1* mRNA molecules given by SPT1.2 in pre-pubertal testes, whereas in adult mice, 4/5 and 7-weeks of age, SPT1.5 quantified only 18% and 9.4%, respectively, of the molecules determined by means of SPT1.2.

Conversely to the age-associated differences in testicular *Sptrx* mRNA levels, *Trx1* and *TrxR1* transcript amounts remained basically unchanged as the animals aged. Interestingly, however, *TGR* mRNA abundance was reduced in young animals, particularly in pre-pubertal 1/2-week-old mice, similar to the situation with *Sptrx* mRNAs. Although *Trx1*, *TrxR1* and *TGR* transcripts are all present in testis, only *TGR* mRNA is highly overproduced in this organ as compared to other mouse tissues [25].

3.5. Primer-Associated Differences in Testicular mRNA Levels

The unforeseen differences found in association with the primer pair used for quantification of *Sptrx-1* and *Sptrx-3* mRNAs (Table 3), were further investigated by designing additional primer pairs (Table 2). For comparison, a new SPT2.3 primer pair was also designed for quantification of *Sptrx-2* mRNA molecules. To shorten the analysis, quantifications were made in a unique sample of testicular RNA pooled from 9 out of the 12 mice of 7 weeks of age.

As shown in Table 4, the highest number of *Sptrx-1* mRNA molecules (1226 molecules/pg) was determined by means of SPT1.2. Primer pairs located either up- or down-stream of SPT1.2 quantified only part of the *Sptrx-1* transcripts. Thus, SPT1.1 detected 66 % (814 molecules/pg) of the maximal *Sptrx-1* mRNA molecules given by SPT1.2. This finding indicates that, while methionine 4, chosen as the start site [13], is encoded by all *Sptrx-1* transcripts, 44 % of them lack codons for the alternative potential starts methionine 2 and 3. With respect to those primer pairs (SPT1.3 to SPT1.7) located downstream of SPT1.2, an outstanding progressive reduction was detected in *Sptrx-1* mRNA abundance, running in the 5´- to 3´ direction. This finding indicates that < 10 % of all *Sptrx-1* transcripts quantified by means of SPT1.2, contain the sequence encoding the C-terminal thioredoxin domain, where SPT1.5 to SPT1.7 are located (see Table 2). Such a striking accumulation of truncated *Sptrx-1* transcripts is consistent with premature transcription termination and/or transcript degradation, and it may be related to the extremely short 3´-UTR exhibited by both mouse and human *Sptrx-1* mRNAs [12, 13].

With regard to *Sptrx-3* mRNA, data in Table 4 indicate that whereas exons 1 to 4 are present in all transcript molecules (SPT3.1, SPT3.2 and SPT3.3 data), exon 5 (SPT3.4) or exon 6 (SPT3.5) are present in a rather small percentage (12 % each) of total molecules. In agreement with data in Table 3, exons 5 and 6 (SPT3.6) were simultaneously present only in 4 % of *Sptrx-3* mRNAs.

In contrast to the above referred findings on *Sptrx-1* and *Sptrx-3* mRNAs, similar number of transcript copies (at about 270 molecules/pg) was determined by the three (SPT2.1, SPT2.2 and SPT2.3) primer pairs designed for quantification of *Sptrx-2* mRNA.

3.6. Tissue Distribution of Sptrx mRNA

As previously mentioned, initial multiple-tissue northern blots identified adult testis as the only organ expressing *Sptrx-1, Sptrx-2* and *Sptrx-3* mRNAs [6], with no hybridization signal being obtained in any other tissue despite the use of low stringency conditions and extended exposure time. Given the sensitivity and reproducibility of real-time PCR for absolute quantification of *Sptrx* mRNA levels *in vivo* (Table 3), we further quantified the actual copy numbers of *Sprtx-1, Sprtx-2* and *Sprtx-3* mRNAs in a variety of adult (7-week-old) tissues other than testis. Total *Sptrx-2* and *Sptrx-3* mRNAs were quantified by means of SPT2.1 and SPT3.1 pair primers, respectively. With regard to *Sptrx-1*, maximal transcript amounts were quantified by means of SPT1.2, and the specific percentage retaining the sequence for the C-terminal thioredoxin domain by SPT1.5. We confirmed the testis-specific expression pattern of *Sptrx-2* and *Sptrx-3* genes, since their transcripts were nearly absent (≤ 0.001 molecules/pg) in lung, heart, brain, liver, kidney, spleen and ovary. However, in contrast to previous findings, *Sptxr-1* transcripts were present not only in testis (876 molecules/pg in Table 3) but also in lung, heart and brain (although at very low levels of about 0.07 molecules/pg), and to a higher extent in ovary (6.3 molecules/pg). Interestingly, when only considering those *Sptrx-1* transcripts containing the sequence for the C-terminal domain, the ovary level (3.3 molecules/pg) constituted 4% of the amount quantified in testis (82 molecules/pg in Table 3). In agreement with this wider expression pattern of *Sptrx-1*, examination of EST sequences revealed its presence in cDNA from unfertilized egg and embryo, corresponding approximately to 11% of all available mouse *Sptrx-1* EST. In contrast, *Sptrx-2* and *Sptrx-3* sequences were detected in EST exclusively from testes.

4. DISCUSSION

Regulation of gene expression during spermatogenesis is exerted according to a complex and coordinated scheme where different cis-acting and trans-acting elements converge to either activate, modify or silence the expression of a particular group of specific genes responsible for the formation of a specialized cell as the spermatozoon. Among those gene products participating in the spermatogenic process we have recently identified three sperm-specific thioredoxins [6]. Since control of redox imbalance has been reported to be important in both the physiology of normal spermatogenesis and the aetiology of some spermatogenic abnormalities [26], regulation of *Sptrx* gene expression is likely to be of great importance.

Sptrx proteins are expressed either in different structures within the sperm cell or at different stages of sperm development [6] and, therefore, the regulation of their specific expression pattern should differ for each *Sptrx* gene. We first investigated the transcription initiation of the *Sptrx* genes by promoter analysis of their respective 5'-flanking regions, ranging from –1kb to –4kb, because distal regulatory regions are frequently responsible of cell type-specific expression [27, 28]. Experiments were carried out in the mouse spermatogenic GC-2spd(ts) cell line, where a recent study has identified the regulatory factor X2 to activate transcription of the testis-specific histone *Ht1* gene [29]. Although, neither Sptrx proteins nor mRNAs are found in GC-2spd(ts) cells we completed the promoter analysis as a first approach to establish which fragments in the 5'-flanking regions are responsible for the transcriptional silencing of *Sptrx* genes in GC-2spd(ts) cells and somatic cell lines.

In silico analyses and luciferase assays indicate that the putative promoters for all *Sptrx* genes must be contained in the –1.5kb proximal region from their respective ATG codons, where different regulatory elements are predicted to be located. However, the *Sptrx-1* –1kb region, lacking any TATA-box, does not show promoter activity. One could speculate that the EGRF/NFAT module (located at –1091 to –1113) is essential for the activity because the *Sptrx-1* –2kb region, which contains this module, indeed exhibits the highest activity. Besides, the –1-2kb region, apart from harbouring three predicted TATA-boxes at –1163, –1294 and –1752, contains several transcription binding-sites (data not shown), which may be functional to activate *Sptrx-1* transcription. Also, the presence of a 525 bp intron within the 5'-UTR of *Sptrx-1* [13] suggests that the transcription initiation site, and therefore other cis-regulating sequences necessary for transcriptional initiation of *Sptrx-1*, should be located upstream of this intron (–639bp from the ATG codon).

Importantly, the *Sptrx-1* and *Sptrx-2* –2kb distal regions may function as transcriptional silencers since –4kb constructs lack any promoter activity being even lower than pGL-3 basic empty vector basal activity. Since it is known that methylation status of CpG dinucleotides in CpG-poor promoters is important for transcriptional silencing [30-32], we made a prediction for CpG islands in the *Sptrx* 5'-flanking regions using the *CpG island finder* from EMBOSS tools (http://www.ebi.ac.uk/emboss/). One CpG island in the *Sptrx-1* and three in the *Sptrx-2* (but none in *Sptrx-3*) –2kb distal regions were found, suggesting that the status of these predicted CpG islands might play a role on the somatic transcriptional silencing of *Sptrx-1* and *Sptrx-2* genes.

The *Sptrx-3* transcriptional repression seems to be regulated in a completely different way than that of *Sptrx-1* and *Sptrx-2*. The fact that neither *Sptrx-3* mRNA nor protein are found in GC-2spd(ts) cells while *Sptrx-3* constructs (except the –3kb construct) exhibit promoter activity, raises an apparent contradiction. One explanation can be a silencing sequence located in the 3'-flanking region as occurs in other genes such as the murine CD46 [33]; however, additional experiments are required to support such a notion. On the other hand, the *Sptrx-3* 1kb distal region (–3-4kb from ATG) is likely to contain an upstream activating sequence from where spermatogenic-specific factors may enhance *Sptrx-3* transcription. In fact, the *Sptrx-3* 1kb distal region alone does not show any promoter activity (data not shown) and therefore is not able to direct *Sptrx-3* transcription by itself. Nonetheless, our results indicate that the presence of this region in –4kb *Sptrx-3* constructs can increase the promoter activity in GC-2spd(ts) cells, but not in Y-1 or Hepa1-6 cells.

Apart of transcription initiation regulation, we have also investigated how the steady-state *Sptrx* mRNA amounts change during mouse development and whether post-transcriptional mechanisms can affect *Sptrx* gene expression. We performed real-time PCR in whole-animal experiments, which revealed interesting new features about *Sptrx* transcription patterns. *Sptrx-1* mRNA is detected in testes from pre-pubertal animals (1-2 weeks) although at very low levels. In contrast, *Sptrx-2* and *Sptrx-3* mRNA are first identified after puberty (4-5 weeks and onwards) as described before [15, 16]. In addition, *Sptrx-1, Sptrx-2* and *Sptrx-3* mRNA levels in adult testes are significantly different and increase when animals age.

We also found vast primer-associated differences for expression of *Sptrx-1* and *Sptrx-3*, which increased with age as well. Several mechanisms have been described to operate during spermatogenesis such as different transcription start site usage, mRNA degradation and alternative splicing [21] and it is possible that some of these mechanisms may explain these disparities.

For *Sptrx-1*, differences in results with SPT1.1 and SPT1.2 primer pairs may be explained by the existence of several transcription start sites upstream and downstream the SPT1.1 annealing sequence. As SPT1.1 amplifies about 2/3 of the total transcripts that SPT1.2 does, major transcription start site/s should be located downstream of the SPT1.1 annealing sequence. On the other hand, a main feature of the *Sptrx-1* gene is the presence of only one intron in the 5′-UTR region, between potential start methionine 3 and 4. Regulatory sequences within this intron might also explain the lower amount of *Sprtx-1* transcript molecules quantified by SPT1.1 as compared to SPT1.2. Conversely, differences between SPT1.2 and SPT1.3 compared to SPT1.7 may be related to mRNA degradation or shortening at the 3'-end, a mechanism that has been proposed to play a key role in the control of gene expression [34]. This degradation mechanism may be associated to the particularly short 3'-UTR of 17bp of mouse *Sptrx-1* [17]. Interestingly, we found that only <10% of all *Sptrx-1* transcripts retain the C-terminal thioredoxin domain and thereby their functionality.

With regard to the *Sptrx-3* primer-associated differences, we hypothesize that two major mechanisms may operate in this case: mRNA degradation at the 3'-end and alternative splicing within *Sptrx-3* exon 5. Although Δ5-*Sptrx-3* splicing variant has not been identified in humans [16], we cloned this splicing variant from a mouse testis cDNA library (Clontech). Thus, the primer pair SPT3.4 can detect full-length transcripts and also partially degraded mRNAs, whereas SPT3.5 amplifies full-length transcripts and Δ5-*Sptrx-3* spliced variants and SPT3.6 detecting all the full-length non-degraded *Sptrx-3* mRNAs. Taken together, and considering as functional only those mRNAs amplified by the SPT3.5 pair (full-length and Δ5-*Sptrx-3*), it seems that on an average of about 125 *Sptrx-3* transcripts per pg of total RNA, no more than 11.9% are functional, corresponding 8.2% to the more abundant Δ5-*Sptrx-3* transcripts and only 3.7% to full-length transcripts.

In conclusion, these results indicate that transcription of *Sptrx* genes is exquisitely regulated and that the developing spermatozoon needs to fine-tune the expression of these genes to complete its development. This work constitutes an initial analysis of *Sptrx* genes transcription regulation and additional experiments in a more physiological context such as promoter analyses by *in vivo* electroporation [35] are envisaged to complement the present work.

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REFERENCES

[1] J. Nordberg, and E. S. Arner, Reactive oxygen species, antioxidants, and the mammalian thioredoxin system, Free Radic Biol Med 31 (2001) 1287-1312.

[2] S. Gromer, S. Urig, and K. Becker, The thioredoxin system-From science to clinic, Med Res Rev 24 (2004) 40-89.

[3] K. Hirota, H. Nakamura, H. Masutani, and J. Yodoi, Thioredoxin superfamily and thioredoxin-inducing agents, Ann N Y Acad Sci 957 (2002) 189-199.

[4] A. Holmgren, Antioxidant function of thioredoxin and glutaredoxin systems, Antioxid Redox Signal 2 (2000) 811-820.

[5] A. Miranda-Vizuete, A. E. Damdimopoulos, and G. Spyrou, The mitochondrial thioredoxin system, Antioxid Redox Signal 2 (2000) 801-810.

[6] A. Miranda-Vizuete, C. M. Sadek, A. Jimenez, W. J. Krause, P. Sutovsky, and R. Oko, The Mammalian testis-specific thioredoxin system, Antioxid Redox Signal 6 (2004) 25-40.

[7] P. M. Cunnea, A. Miranda-Vizuete, G. Bertoli, T. Simmen, A. E. Damdimopoulos, S. Hermann, S. Leinonen, M. P. Huikko, J.-Å. Gustafsson, R. Sitia, and G. Spyrou, ERdj5, an endoplasmic reticulum (ER)-resident protein containing DnaJ and thioredoxin domains, is expressed in secretory cells or following ER stress, J Biol Chem 278 (2003) 1059-1066.

[8] A. Hosoda, Y. Kimata, A. Tsuru, and K. Kohno, JPDI, a novel endoplasmic reticulum-resident protein containing both a BiP-interacting J-domain and thioredoxin-like motifs, J Biol Chem 278 (2003) 2669-2676.

[9] Q. A. Sun, L. Kirnarsky, S. Sherman, and V. N. Gladyshev, Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems, Proc Natl Acad Sci U S A 98 (2001) 3673-3678.

[10] A. K. Rundlöf, M. Jarnard, A. Miranda-Vizuete, and E. S. Arnér, Evidence for intriguingly complex transcription of human thioredoxin reductase 1, Free Radic Biol Med 36 (2004) 641-656.

[11] A. K. Rundlöf, and E. S. Arnér, Regulation of the Mammalian Selenoprotein Thioredoxin Reductase 1 in Relation to Cellular Phenotype, Growth, and Signaling Events, Antioxid Redox Signal 6 (2004) 41-52.

[12] A. Miranda-Vizuete, J. Ljung, A. E. Damdimopoulos, J.-Å. Gustafsson, R. Oko, M. Pelto-Huikko, and G. Spyrou, Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa, J Biol Chem 276 (2001) 31567-31574.

[13] A. Jiménez, R. Oko, J.-Å. Gustafsson, G. Spyrou, M. Pelto-Huikko, and A. Miranda-Vizuete, Cloning, expression and characterization of mouse spermatid specific thioredoxin-1 gene and protein, Mol Hum Reprod 8 (2002) 710-718.

[14] C. M. Sadek, A. E. Damdimopoulos, M. Pelto-Huikko, J.-Å. Gustafsson, G. Spyrou, and A. Miranda-Vizuete, Sptrx-2, a fusion protein composed of one thioredoxin and three tandemly repeated NDP-kinase domains is expressed in human testis germ cells, Genes Cells 6 (2001) 1077-1090.

[15] A. Miranda-Vizuete, K. Tsang, Y. Yu, A. Jiménez, M. Pelto-Huikko, C. J. Flickinger, P. Sutovsky, and R. Oko, Cloning and Developmental Analysis of Murid Spermatid-specific Thioredoxin-2 (SPTRX-2), a Novel Sperm Fibrous Sheath Protein and Autoantigen, J Biol Chem 278 (2003) 44874-44885.

[16] A. Jiménez, W. Zu, V. Y. Rawe, M. Pelto-Huikko, C. J. Flickinger, P. Sutovsky, J.-Å. Gustafsson, R. Oko, and A. Miranda-Vizuete, Spermatocyte/spermatid-specific thioredoxin-3, a novel Golgi apparatus-associated thioredoxin, is a specific marker of aberrant spermatogenesis, J Biol Chem 279 (2004) 34971-34982.

[17] A. Jiménez, C. Johansson, J. Ljung, J. Sagemark, K. D. Berndt, B. Ren, G. Tibbelin, R. Ladenstein, T. Kieselbach, A. Holmgren, J. A. Gustafsson, and A. Miranda-Vizuete, Human spermatid-specific thioredoxin-1 (Sptrx-1) is a two-domain protein with oxidizing activity, FEBS Lett 530 (2002) 79.
[18] K. Willison and A. Ashworth, Mammalian spermatogenic gene expression, Trends Genet 3 (1987) 351-355.
[19] R. P. Erickson, Post-meiotic gene expression, Trends Genet 6 (1990) 264-269.
[20] A. Hochheimer, and R. Tjian, Diversified transcription initiation complexes expand promoter selectivity and tissue-specific gene expression, Genes Dev 17 (2003) 1309-1320.
[21] K. C. Kleene, A possible meiotic function of the peculiar patterns of gene expression in mammalian spermatogenic cells, Mech Dev 106 (2001) 3-23.
[22] D. Elliott, Pathways of post-transcriptional gene regulation in mammalian germ cell development, Cytogenet Genome Res 103 (2003) 210-216.
[23] M. J. Prieto-Alamo, J. M. Cabrera-Luque, and C. Pueyo, Absolute quantitation of normal and ROS-induced patterns of gene expression: an in vivo real-time PCR study in mice, Gene Expr 11 (2003) 23-34.
[24] M. C. Hofmann, R. A. Hess, E. Goldberg, and J. L. Millan, Immortalized germ cells undergo meiosis in vitro, Proc Natl Acad Sci U S A 91 (1994) 5533-5537.
[25] J. Jurado, M. J. Prieto-Alamo, J. Madrid-Risquez, and C. Pueyo, Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse, J Biol Chem 278 (2003) 45546-45554.
[26] R. J. Aitken, A. L. Ryan, B. J. Curry, and M. A. Baker, Multiple forms of redox activity in populations of human spermatozoa, Mol Hum Reprod 9 (2003) 645-661.
[27] M. A. Yui, G. Hernandez-Hoyos, and E. V. Rothenberg, A new regulatory region of the IL-2 locus that confers position-independent transgene expression, J Immunol 166 (2001) 1730-1739.
[28] G. Lakshmanan, K. H. Lieuw, K. C. Lim, Y. Gu, F. Grosveld, J. D. Engel, and A. Karis, Localization of distant urogenital system-, central nervous system-, and endocardium-specific transcriptional regulatory elements in the GATA-3 locus, Mol Cell Biol 19 (1999) 1558-1568.
[29] S. A. Wolfe, D. C. Wilkerson, S. Prado, and S. R. Grimes, Regulatory factor X2 (RFX2) binds to the H1t/TE1 promoter element and activates transcription of the testis-specific histone H1t gene, J Cell Biochem 91 (2004) 375-383.
[30] S. Eden, and H. Cedar, Role of DNA methylation in the regulation of transcription, Curr Opin Genet Dev 4 (1994) 255-259.
[31] C. De Smet, A. Loriot, and T. Boon, Promoter-dependent mechanism leading to selective hypomethylation within the 5' region of gene MAGE-A1 in tumor cells, Mol Cell Biol 24 (2004) 4781-4790.
[32] Z. Siegfried, S. Eden, M. Mendelsohn, X. Feng, B. Z. Tsuberi, and H. Cedar, DNA methylation represses transcription in vivo, Nat Genet 22 (1999) 203-206.
[33] M. Nomura, A. Tsujimura, N. A. Begum, M. Matsumoto, H. Wabiko, K. Toyoshima, and T. Seya, Identification and characterization of a silencer regulatory element in the 3'-flanking region of the murine CD46 gene, Biochem J 351 Pt 2 (2000) 353-365.
[34] R. Parker, and H. Song, The enzymes and control of eukaryotic mRNA turnover, Nat Struct Mol Biol 11 (2004) 121-127.
[35] P. Bigey, M. F. Bureau, and D. Scherman, In vivo plasmid DNA electrotransfer, Curr Opin Biotechnol 13 (2002) 443-447.

FIGURE LEGENDS

Fig. 1. Diagram of the constructs used in the luciferase assays. 5'-flanking regions from ATG of *Sptrx* genes were inserted upstream of a firefly luciferase reporter gene. RO, reverse orientation; U, upstream.

Fig. 2. Promoter activity of *Sptrx* 5'-flanking regions in GC-2spd(ts) cells. Different 5'-flanking region constructs (Fig. 1) of *Sptrx-1*, *Sptrx-2* and *Sptrx-3* were used in a luciferase reporter gene assay in GC-2spd(ts) cells. "*n. t.*", non transfected. Bars indicate mean ± SEM with data expressed as percentage of promoter activity compared to that of the pGL-3 empty vector. Comparisons among constructs were performed using ANOVA followed by the Student-Newman-Keuls post-hoc test (N=3-6, $P<0.05$). Statistical significant differences with respect to pGL-3 empty vector are indicated with an asterisk.

Fig. 3. Schematic representation of *Sptrx* 5'-flanking regions. Predicted promoter sequences, regulatory modules and repetitive sequences are drawn for each *Sptrx* gene according to the output forms from GenomatixSuite 3.1.1 software (http://www.genomatix.de/).

Supplemental Data Fig 1. Promoter activity of *Sptrx* 5'-flanking regions in GC-2spd(ts), Hepa1-6 and Y-1 cell lines. Different constructs of *Sptrxs* 5'-flanking regions (Fig. 1) were used in a luciferase reporter gene assay in Hepa1-6 (white bars), Y-1 (grey bars) and GC-2spd(ts) (black bars) cells. "*n. t.*", non transfected. Bars indicate mean ± SEM with data expressed as percentage of promoter activity compared to that of the pGL-3 empty vector. Comparisons among constructs were performed using ANOVA followed by the Student-Newman-Keuls post-hoc test (N=3-6, $P<0.05$). Statistical significant differences with respect to pGL-3 empty vector are indicated with an asterisk. No significant differences are indicated as "*n. s.*"

Table 1

Table 1. Putative transcription binding sites in –1kb 5'-flanking regions of *Sptrx* genes

5'-flanking region	*Transcription binding site*	*nt position from ATG*
Sptrx-1	AP-4	-840
	GC-box	-611
	Basic Krueppel-like factor	-613
	AREB-6	-383
	c-Myc/Max	-349
	v-Myb	-323
	MEF-2	-226
	MSX-1 and MSX-2	-145, -137
	Retinoic acid receptor	-23
Sptrx-2	GC-box	-962
	MAZ binding site	-960, -758
	TATA-box	-518
	Octamer binding factor	-487
	Nuclear factor Y	-312
	Binding site for S8 type homeodomains	-309
	HOX-1.3	-274
	CCAAT/enhancer binding protein	-195
	Activating transcription factor	-161
Sptrx-3	SP-1 like transcription factor TIEG	-991
	BRN-2	-971, -935, -931, -809
	MEF-2	-957, -945
	TATA-box	-805, -563, -184
	Prostate-specific homeodomain protein NKX3.1	-799
	TG-interacting factor	-756
	GATA-3	-755, -616, -376
	GATA-1	-592, -270
	NFAT	-581
	BTE binding protein	-515
	NF-kappaβ	-513
	CCAAT/enhancer binding protein	-479
	Binding site for S8 type homeodomains	-435
	Nuclear factor Y	-128
	CCAAT-box	-128

Table 2

Table 2. Primers used for quantification of mRNA molecules

Target (GenBank)	Primers: Primer pair name	Primers: Sequence[a]	Primers: 5'-position	Primers: Exon[b]	Fragment size (bp)[c]
Sptrx-1	SPT1.1	5´-AAAGAATAAGGACAAGGGGCTGGACATGAAC-3' (F) 5´-CTTTCATTTGCTTTTCCTCCGTTGTTGAGTG-3' (R)	93 191	1 1	99
(AF196282)	SPT1.2	5´-TGAAAGGGGCAGCAACGAAAATCCATTAC-3' (F) 5´-GGACGGTATTCTCTGAAAACATGGGGGTG-3' (R)	186 385	1-2 2	200
	SPT1.3	5´-TCTAAATCCTCGGGCTACTCCAAGCAGAC-3' (F) 5´-CGTTGCAGGCTTGAGGGTGCTC-3' (R)	451 555	2 2	105
	SPT1.4	5´-GACAGCGTTCAGTCAAAGGAGAGTGAAGA-3' (F) 5´-TTCCTGGGACTGGATGGTGTCTTCTTC-3' (R)	1096 1293	2 2	198
	SPT1.5	5´-TTGGAGCAGAAATAGAGACCCTGGAGGAAG-3' (F) 5´-CCACCAGCTTCTCTCCAGCGTCTTTG-3' (R)	1355 1456	2 2	102
	SPT1.6	5´-TTGGAGCAGAAATAGAGACCCTGGAGGAAG-3' (F) 5´-GGGAGGTGAAAGATCTCACAGTCTTGCAC-3' (R)	1355 1610	2 2	256
	SPT1.7	5´-TTTGAAGCACGAGGATGTGATATTCTTGGAG-3' (F) 5´-ACAAGGGCACCAGAAAATTCACCCAC-3' (R)	1524 1667	2 2	144
Sptrx-2	SPT2.1	5´-CAAAAAGCGTGAAGTCCAGCTACAGTCAGTC-3' (F) 5´-TTCAGCAACGACGAAGTGAAGAATCTCATC-3' (R)	150 345	2-3 4-5	196
(AF548543)	SPT2.2	5´-GTGAGGAAGAGAAAGATGACGTGTTGAACG-3' (F) 5´-ATGGATCTTGCACACTGTTCTTGCTTCTTC-3' (R)	1112 1227	11-12 12	116
	SPT2.3	5´-AGCCTC ATGTGACACACAAAGAAAGAATGG-3' (F) 5´-ACCATGACTAGCGACATGCCCAAAGATAAG-3' (R)	1505 1697	14-15 15-16	193
Sptrx-3	SPT3.1	5´-CAGCAGCAAGTCCCAGATGTTAATCATGGT-3' (F) 5´-TTGTTTCCAGCATCGCTGAACAATTCTTTC-3' (R)	49 135	1 2	87
(AY495589)	SPT3.2	5´-TGAAAGAATTGTTCAGCGATGCTGGAAAC-3' (F) 5´-AGATGAGTCCACATCCACCTGAGCAAAC-3' (R)	105 259	2 3	155
	SPT3.3	5´-GCTTGTGGTGGTAGAGTTTTCAGCAAAGTG-3' (F) 5´-ATCTGGAATGTGGGTAGCATTGTGATGTC-3' (R)	136 309	2 4	174
	SPT3.4	5´-AAAATGTCACGTTTGCTCAGGTGGATGTG-3' (F) 5´-CTTCGGTCCACTTCTGAGGCAGCAC-3' (R)	222 385	3 5	164
	SPT3.5	5´-AAA ATGTCACGTTTGCTCAGGTGGATGTG-3' (F) 5´-TTTCCAGTTGTTTAGCATCAGCTCCATGACA-3' (R)	222 434	3 6	213/147
	SPT3.6	5´-GCTGCCTCAGAAGTGGACCGAAGAG-3' (F) 5´-CATGCAAGACACAGAGCATTAGCTCCTG-3' (R)	363 521	5 6	159

[a]Sequences of forward (F) and reverse (R) primers.
[b]Exon numbers were according to NCBI Evidence Viewer, except those for *Sptrx-3* that were according to [16].
[c]Each primer pair generates a single PCR fragment. The exception is SPT3.5 that generates two fragments, the shortest of 147 bp lacking exon 5.

Table 3

Table 3. Age-associated differences in testicular mRNA levels

Transcripts were quantitated in testes from *n* mice of different ages. Primers for amplification of *Sptrx-1, Sptrx-2* and *Sptrx-3* are given in Table 1, and those for *TGR, Trx1* and *TrxR1* in [25]. Data are the means ± S.E. of mRNA molecules per pg of total RNA. Comparisons among mouse groups were done using ANOVA followed by the Student-Newman-Keuls test. Comparisons between primers were done using the Student's *t* test. Average values with the same superscript are not statistically different ($P > 0.05$).

	Age		
Target Primer pair	**1/2 weeks** ($n = 6$)	**4/5 weeks** ($n = 6$)	**7 weeks** ($n = 12$)
Sptrx-1			
SPT1.2	0.22 ± 0.016[a]	369 ± 23	876 ± 38
SPT1.5	0.19 ± 0.016[a]	65 ± 8.7	82 ± 2.4
Sptrx-2			
SPT2.1	0.005 ± 0.001[b]	198 ± 4.5[c]	294 ± 15[d]
SPT2.2	0.007 ± 0.001[b]	211 ± 5.6[c]	273 ± 18[d]
Sptrx-3			
SPT3.1	≤ 0.001	60 ± 4.0	111 ± 4.2
SPT3.6	≤ 0.001	5.5 ± 0.70[e]	4.7 ± 0.68[e]
TGR	26 ± 2.2	382 ± 26	506 ± 13
Trx1	87 ± 1.7[f]	76 ± 13[f]	65 ± 3.1[f]
TrxR1	11 ± 0.56	28 ± 3.4[g]	26 ± 1.8[g]

Table 4

Table 4. Primer-associated differences in testicular mRNA levels

Transcripts were quantitated in a sample of total RNA pooled from testes of 9 out of the 12 mice of 7 weeks of age analyzed individually in Table 3. Primers are given in Table 1.

Sptrx-1		*Sptrx-3*	
Primer pair	molecules/pg	Primer pair	molecules/pg
SPT1.1	814	SPT3.1	114
SPT1.2	1226	SPT3.2	118
SPT1.3	698	SPT3.3	145
SPT1.4	192	SPT3.4	16
SPT1.5	79	SPT3.5	15
SPT1.6	61	SPT3.6	4.7
SPT1.7	48		

Supplementary Table

Click here to download Supplementary Material: 6 - Sup. Table

Figure 1

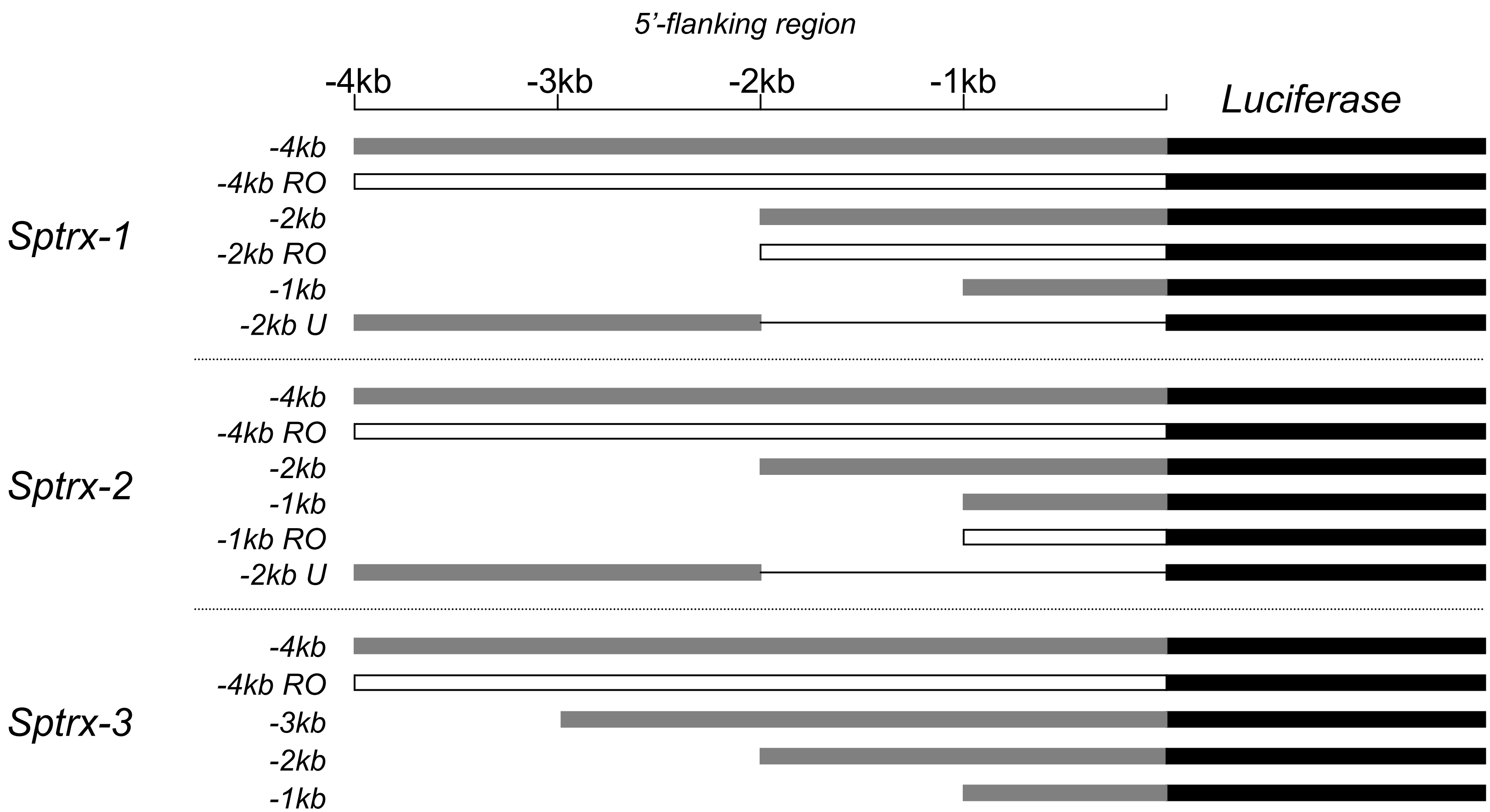

Figure 1

Figure 2

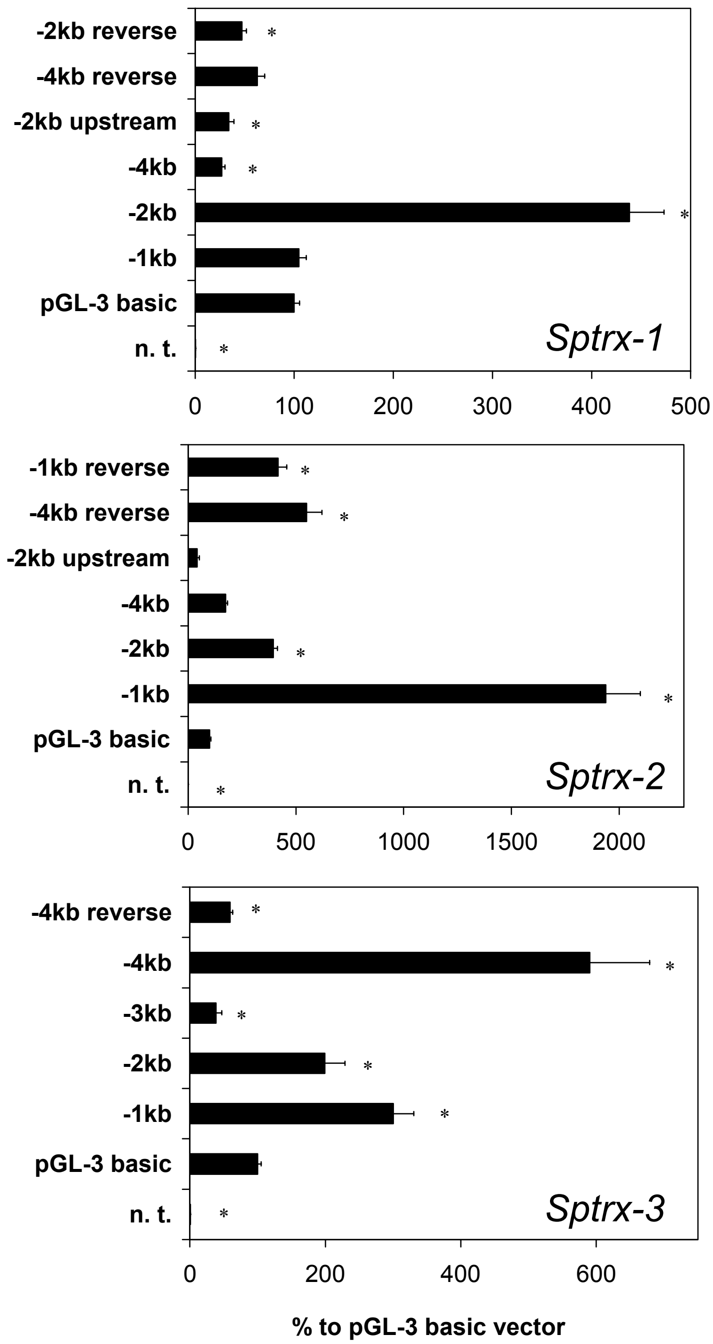

Figure 2

Figure 3

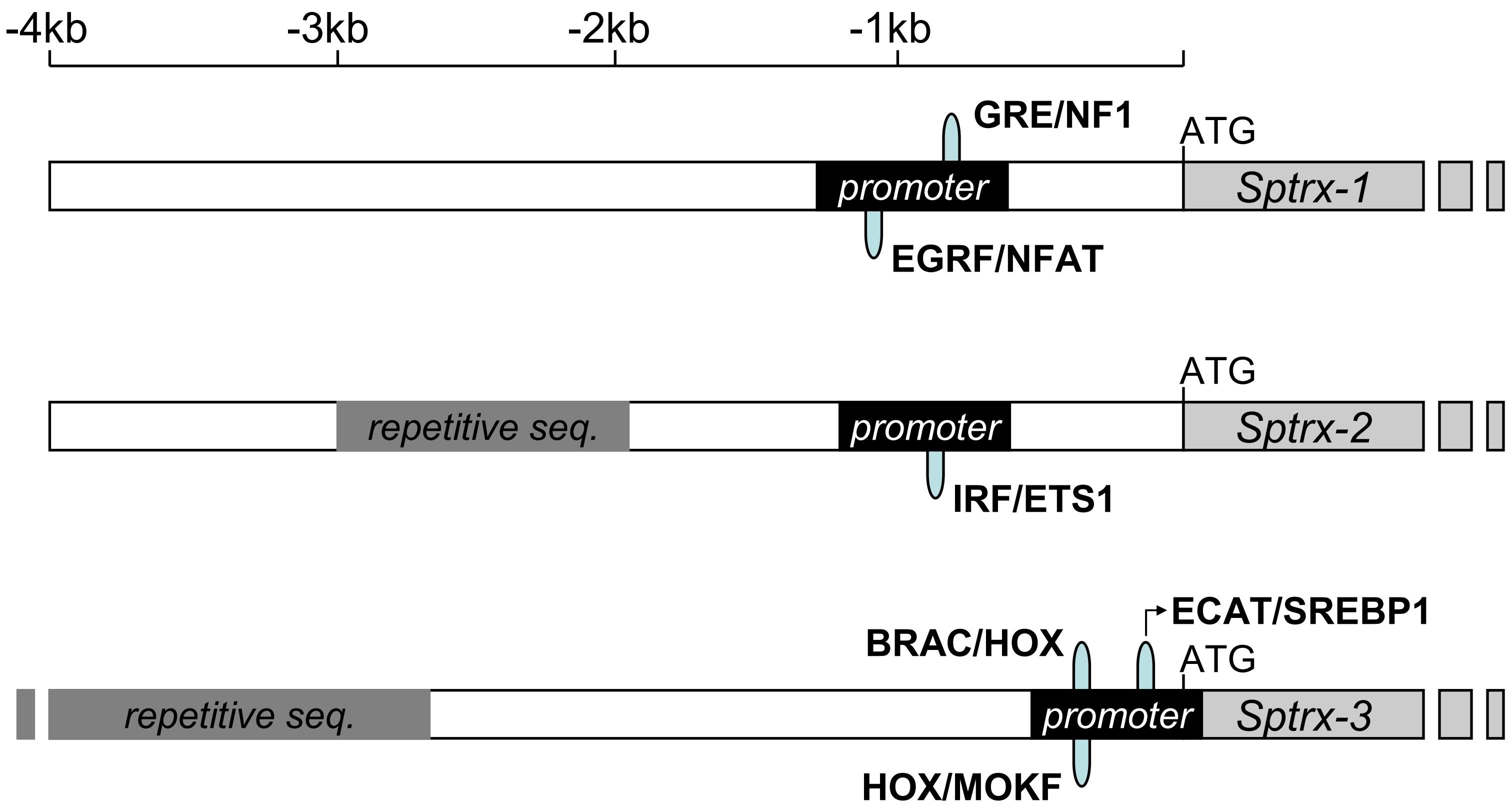

Figure 3

Supplementary Figure