Identification and expression profiling of 10 novel spermatid expressed CYPT genes

ARTICLE *in* MOLECULAR REPRODUCTION AND DEVELOPMENT · MAY 2006

Impact Factor: 2.53 · DOI: 10.1002/mrd.20463 · Source: PubMed

6 AUTHORS, INCLUDING:

John E Nielsen
Rigshospitalet
89 PUBLICATIONS **2,094** CITATIONS
SEE PROFILE

Kristian Almstrup
Rigshospitalet
66 PUBLICATIONS **1,483** CITATIONS
SEE PROFILE

Henrik Leffers
University of Copenhagen
162 PUBLICATIONS **9,592** CITATIONS
SEE PROFILE

Identification and Expression Profiling of 10 Novel Spermatid Expressed CYPT Genes

MARTIN A. HANSEN,[1]* JOHN E. NIELSEN,[1] MASAMI TANAKA,[2] KRISTIAN ALMSTRUP,[1] NIELS E. SKAKKEBæK,[1] AND HENRIK LEFFERS[1]

[1]*Department Growth and Reproduction, Rigshospitalet, Copenhagen University Hospital, GR5064, Blegdamsvej 9, DK-2100, Denmark*

[2]*Department of Pharmacology, Institute of Experimental Animals, St. Marianna University School of Medicine, Kawasaki, Japan*

ABSTRACT To identify candidate genes for poor sperm morphology, we have screened for genes expressed during spermiogenesis. We identified 10 new members of the cysteine-rich perinuclear theca (CYPT) family showing that this family contains at least 15 members, which also includes the casein kinase II target genes. Based on similarity the CYPT sequences could be divided into two groups, *Cypt1–10* and the novel members *Cypt12–15*. The 5′-end of the CYPT family is highly similar to exon1A and part of the first intron of *Zfy2*. Seven CYPT genes mapped to the X chromosome; six contained an intron and one was intron-less. One CYPT gene mapped to chromosome 3 and one mapped to chromosome 9 which were both intron-less. The upstream region of the CYPT family and *Zfy2* genes is conserved. For some the conservation extended over a large region, however, only about 150 nucleotides is conserved among all CYPT members and *Zfy2*. Nevertheless, the short conserved promoter leads to essentially identical expression profiles for the CYPT family members and *Zfy2*, which was clearly different from the profile of *Zfy1*. Expression of the CYPT family and *Zfy2* preceded the expression of other spermatid-specific genes such as the transition proteins and the protamines. In situ hybridization revealed a low expression in pachytene spermatocytes from stages IX–X followed by a strong upregulation in spermatids from stage VI with maximum expression in spermatids in stages VII–VIII. The CYPT family may function in the remodeling of the spermatid nucleus before condensation of the DNA. *Mol. Reprod. Dev.*

Key Words: testis; spermatogenesis; spermatids; gene expression

INTRODUCTION

Studies of global gene expression during mouse spermatogenesis have been reported by a several groups (Fujii et al., 2002; Schultz et al., 2003; Almstrup et al., 2004; Schlecht et al., 2004; Shima et al., 2004; Wrobel and Primig, 2005) and global gene expression in different animal models of infertility have been investigated and compared with respect to altered spermatogenesis (Ellis et al., 2004; Maratou et al., 2004). The studies have been conducted on the first wave of mouse spermatogenesis, where germ cells in different differentiation stages appear in succession on distinct days during postnatal development (Bellve et al., 1977; Russell et al., 1990). Gene expression in the distinct differentiation stages can therefore be determined as the cell types appear. Furthermore, if an mRNA is confined to germ cells, the cell types expressing the mRNA can be identified by the timing of the appearance of the mRNAs (Almstrup et al., 2004). The results from the different studies are in general agreement, however, studies of gene expression in isolated germ cells seems to differ from those performed on the first wave of spermatogenesis during postnatal development (Yu et al., 2003; Guo et al., 2004). The reason is not obvious, but may be caused by the relatively few genes analyzed in the studies on purified germ cells or by changes occurring during the tedious purification of the different germ cell types.

Investigations of the first wave of spermatogenesis showed that changes in gene expression occur in clusters where a large number of genes change expression at the same time and in the same cell types (Fujii et al., 2002; Schultz et al., 2003; Almstrup et al., 2004; Schlecht et al., 2004; Shima et al., 2004). We have previously reported that three main clusters and combinations of these can account for most of the genes up or downregulated in defined time-windows during testicular development (Almstrup et al., 2004). One of the clusters corresponded to genes expressed in spermatids, however, the function of most of the

Grant sponsor: Danish Research Council; Grant sponsor: The Japanese Science and Technology Corporation's CREST program.

Kristian Almstrup's present address is Molecular Genetics, Novo Nordisk, Novo Allé, DK-2880, Denmark.

*Correspondence to: Martin A. Hansen, Department of Growth and Reproduction, Rigshospitalet, Copenhagen University Hospital, GR5064, Blegdamsvej 9, DK-2100, Denmark.
E-mail: mail@maasha.dk

Received 2 November 2005; Accepted 26 December 2005
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20463

spermatid-specific genes was unknown. We believe that many of the genes that are uniquely expressed in spermatids could be involved in spermatid-specific functions, such as DNA condensation and re-modeling of the cell structure.

When studying human sperm, it is obvious that a large proportion of the spermatozoa have a poor morphology. Applying the strict criteria (WHO, 1999) most human semen samples have less than 10% spermatozoa with normal morphology (Jorgensen et al., 2002). In almost all cases, the reason for the high number of poor-morphology spermatozoa is unknown, but studies in mice have provided some clues to genes that may be involved (Balhorn et al., 1988; Belokopytova et al., 1993; de Yebra et al., 1993; Lee et al., 1995; Xu et al., 1999; Yu et al., 2000; Carrell and Liu, 2001; Tay and Richter, 2001; Zhao et al., 2001; Ashley et al., 2004). In order to gain more information on other genes that may affect sperm morphology, we have screened for genes that are specifically expressed in spermatids. These genes can be divided according to the timing of their upregulation. One cluster corresponds to genes that are specific for spermatids where the mRNAs first are detectable on days 28–34 pn, corresponding to initiation of expression in step 6 to 8 spermatids (Almstrup et al., 2004). However, genes that are exclusively expressed in spermatids will not be present in early spermatids because there is little or no transcription before spermatid step 5 or 6 (Almstrup et al., 2004). Thus, if an mRNA is required in early round spermatids (steps 1–5) they must be transcribed before the meiotic divisions. Consequently, another spermatid-expressed cluster is composed of genes that are upregulated in late pachytene or diplotene spermatocytes, appearing first on days 20–22 pn. For many of these genes the upregulation is followed by a gradual decline in round spermatids until they no longer are detectable typically in step 9–10 spermatids (Almstrup et al., 2004). However, some of these mRNAs show an increased level of transcription after the meiotic divisions is re-initiated (step 5–6 spermatids, corresponding to days 28–30 pn) at the same time as the truly spermatid-specific genes are expressed. These mRNAs probably encodes proteins that are required in round spermatids before the mRNAs can be transcribed in step 5–6 spermatids.

In this study, we report the identification of 10 novel members of the CYPT family (Kitamura et al., 2004), and we report the gene expression profile for the CYPT family, which is essentially spermatid-specific. The CYPT family includes at least 15 distinct but related genes; *Cypt1–10* are highly similar whereas *Cypt11–15* show a more distant relationship. Moreover, the 5′-end of the CYPT family members is highly similar to exon 1A of *Zfy2* and the similarity extends upstream from the transcription initiation site, to include the previously reported testis-specific *Zfy2* promoter (Zambrowicz et al., 1994; Kitamura et al., 2004). *Zfy2* is a paralog of *Zfy1*, who's human equivalent *ZFY* formerly was the primary candidate for the testis determining factor (TDF in human and Tdy in mouse). However, it has been proved that the *ZFY* and TDF are at different loci and that TDF is linked to *SRY* (Sinclair et al., 1988; Koopman et al., 1989; Berta et al., 1990; Koopman et al., 1990; Sinclair et al., 1990), and the function of *Zfy1* and *Zfy2* remains unknown.

MATERIALS AND METHODS

Animals

Animal studies were approved by The Japanese Pharmacological Society, and the animals were treated according to generally accepted guidelines for animal experimentation at St. Marianna University Graduate School of Medicine and guiding principles for the care and use of laboratory animals.

C3H/He mice strain and CD1 rats (Japan SLC, Shizuoka, Japan) were maintained under controlled conditions (22°C ± 2°C, 55% ± 5% humidity, 12 hr light/dark cycle) and were given laboratory chow (CE-2, Nippon CREA, Tokyo, Japan) ad libitum. Female mice were housed with males, and for both mice and rats the morning when the vaginal plug was found was designated day 1 of gestation. Pregnant females (mice and rats) were housed individually and gave birth to pups on gestational day 20 (mice) or day 23 (rats) and the day of birth was termed 'day 1 pn.' Testes were removed and used either for preparation of total RNA or fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C, and dehydrated in ethanol. Alternatively, fixation in Stieves fixative (Solution I: 90 g $HgCl_2$ in 1.5l H_20; Solution II: 400 g 40% formaldehyde and 80 g 98% acetic acid; just before use mix 38 ml Solution I and 12 ml Solution II) at 4°C for 24 hr to 96 hr. Mouse testes were prepared from day 1 pn, and every day until day 4 pn, and then on every second day until day 52 pn.

Differential Display Analysis

Total RNA was prepared using the NucleoSpin RNA II kit as described by the manufacturer (Macherey-Nagel, Düren, Germany) and cDNA was prepared as described previously (Jorgensen et al., 1999) using one-base-anchored (AAGCTTTTTTTTTTTV) downstream primers. All solutions were treated with diethylpyrocarbonate (DEPC) or made from DEPC-treated water. Differential display (DD) was subsequently performed using either one- or two-base-anchored primers (AAGCTTTTTTTTTTTVN) in combination with a range of upstream primers. The double band corresponding to a fragment derived from several CYPT family members (*Cypt1–10*) was displayed by the upstream primer CCAAGACTCAGAAGGAA in combination AAGCTTTTTTTTTTTG. The band corresponding to *Cypt12* was displayed by the upstream primer GTGCGGAATGAGA in combination with AAGCTTTTTTTTTTC. The transition protein 2 (*Tnp2*), and *protamine 1* (*Prm1*) mRNAs were displayed with the upstream primers GAAATGGTAGAATGGCT and CCTGCTCACAGGTTGGCTG, respectively, in combination with the downstream primer AAGCTTTTTTTTTTG. Bands were excised from the DD gels

and re-amplified using the same upstream primer and a downstream primer that was extended at the 5′-end with a T7-promoter sequence (taatacgactcactatagggAAGCTTTTTTTTTTTV; T7-promoter sequence in lower case), allowing direct sequencing with a Cy5-labeled T7-promoter complementary primer. The expression level on different days was quantified by phosphor imaging (semiquantitative analysis). All methods relating to DD have been described in details previously (Jorgensen et al., 1999).

Cloning of Additional CYPT Gene Family Members

A four-primer strategy was used to amplify additional members of the CYPT family; degenerate bases were inserted at positions with variable bases and to include as much of the sequences as possible the primers were partly overlapping. The primers were: first PCR: AGTCYTGGAGAGCTCYRCTAT and ACAGGACCACAGCTCAG; second PCR: AAACTCGAGAGCTCYRCTATGRCTC and AAAGAATTCTCAGCTCCCAGACCCGT, restriction sites for *XhoI* and *EcoRI*, respectively (underlined) were inserted to facilitate cloning. PCR conditions were: 2 min at 95°C; 40 cycles of: 30 sec at 95°C, 1 min at 60°C, 1 min at 72°C, and finally 5 min at 72°C. The resulting DNA fragments were purified on 2% low melting point agarose gels and cloned into pBS (Stratagene, San Diego, CA). Twenty clones were sequenced using standard M13- and M13-reverse sequencing, and a mixture of all plasmids were used for making biotin-labeled sense and anti-sense probes by linearizing the plasmids with either *XhoI* or *EcoRI*.

Preparation of Biotin Labeled Probes for In Situ Hybridization

The in situ hybridization (ISH) probes for the CYPT family members and probes for *Prm1* and *Tnp2* were made from the DD fragments by PCR re-amplification using the same T7-promoter tagged primer combined with a specific T3-promoter tagged primer. Two different probes were designed for the CYPT family, *Cypt1–10* matching *Cypt1* to *Cypt10*, and *Cypt12–15* matching *Cypt12* to *Cypt15*. *Cypt1–10*: aattaaccctcactaaaGGCTCCGAATGGAG, *Cypt12–15*: aattaaccctcactaaaGGAGCCAGTGAATGAG, *Prm1*: aattaaccctcactaaaGGGAGGCACCATCA, *Tnp2*: aattaaccctcactaaaGGGCGAAGATACAAG, T3-promoter sequence in lower case. PCR conditions were: 5 min at 95°C; 5 cycles of: 30 sec at 95°C, 1 min at 45°C, 1 min at 72°C; 20 cycles of 30 sec at 95°C, 1 min at 65°C, 1 min at 72°C, and finally 5 min at 72°C.

Two ISH probes, one probe matching *Zfy2* and *Cypt1–10* and one probe matching all ZFY family members (*Zfy1*, *Zfy2*, *Zfx*, and *Zfa*), were made by PCR directly on cDNA, using two sets of primers. The primers were: *Zfy2* (5′-end probe; exon 1A) first pair GTGGCCAAGAAAGTCCACTGGT and CTTTAATGTATTTCTGCTACGAGA, second pair aattaaccctcactaaaGGGTCTAGAGCAGCA and taatacgactcactatagGGAACCACAACAGCAA. ZFY family probe, first pair TTCCATTCAAGTGTAAGAGATGT and TCACTTGACAAATTCATGACATTG; second pair aattaaccctcactaaagGGCTTCAAACGCATATG and taatacgactcactataGGGATGCCTACTTTTAA (T3- and T7-promoter sequences are shown in lower case, respectively). PCR conditions were: first PCR: 5 min at 95°C; 30 cycles of: 30 sec at 95°C, 1 min at 62°C, 1 min at 72°C; and finally 5 min at 72°C. One microliter (out of 30 μl total volume) was transferred to a new PCR reaction and run as follows: 5 min at 95°C; 5 cycles of: 30 sec at 95°C, 1 min at 45°C, 1 min at 72°C; 30 cycles of 30 sec at 95°C, 2 min at 72°C, and finally 5 min at 72°C.

The resulting PCR product were purified on 2% low melting point agarose gels and sequenced from both ends, using Cy5-labeled primers complementary to the added T3- and T7-promoter tags. The positions of sequence ambiguities observed for the ZFY probe revealed a mixture of fragments derived from the 3′-end of *Zfy1*, *Zfy2*, *Zfx*, and *Zfa*. Aliquots of 200 ng were used for in vitro transcription labeling, using the MEGAscript-T3 (sense) or MEGAscript-T7 (anti-sense) kits, as described by the manufacturer (Ambion, Houston, TX). The composition of the 10x-nucleotide mix was: 7.5 mM ATP, GTP and CTP, 3.75 mM UTP, 1.5 mM biotin-labeled UTP. To estimate quantity and labeling efficiencies, aliquots of the labeled RNA product were analyzed by agarose gel electrophoresis, and dotted onto nitrocellulose filters and developed as described for ISH. The labeled probes were subsequently used for ISH as previously described (Nielsen et al., 2003).

RT-PCR

RNA was purified as described previously (Jorgensen et al., 1999). cDNA was synthesized using a HTTTTTTTTTTTTV primer mix and specific primers targeting each mRNA or group of mRNAs were designed: *Cypt1–10* AGTCYTGGAGAGCTCYRCTAT and ACAGGACCACAGCTCAG, *Cypt12* CTCTAGCATTAGTCATCCATGA and TGCCAATGACATCATGACCGA, *Cypt15* ATGCTGAAGAATTAACCGCCTA and GATTTTATTGTCATCATCCTATCT, *Zfy2* TGTTGTGGTTCTCGTAGCAGA and ATGTGTACTGCATCAGCTCCT, *Zfy1* TTCTGGGTTTTCAGGCGTTCT and ATGTGTACTGCATCAGCTCCT, *Zfx* CGTCCGGTGCGTATAACTGT and TGTGTGTAGCACCAGCTCCT. PCR was performed in 30 μl of (final concentrations): 12 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; 1.9 mmol/L MgCl; 0.1% Triton X-100; 0.005% gelatine; 250 μmol/L dNTP; 30 pmol of each primer. Cycle conditions: 2 min at 95°C; 40 cycles of: 30 sec at 95°C, 1 min at 62°C, 1 min at 72°C, and finally 5 min at 72°C. PCR products were run on 1.5% agarose gels and visualized by ethidium bromide staining. The marker was a 100 bp ladder (Amersham, Piscataway, NJ).

Bioinformatics

Multiple alignments were conducted with Muscle (Edgar, 2004). The mouse genome sequence used was the Build 33 assembly by NCBI.

The *cypt1–15* sequences were deposited in GenBank (acc. AM040451–60; BN000808–12).

RESULTS

Identification of Novel CYPT Family Members

In a DD analysis of mRNAs expressed during postnatal development of mouse testis, we observed a double band that was first detectable on day 18 pn and then gradually upregulated to reach the highest level on day 28 pn (Fig. 1). Sequencing of the bands showed that they were identical except for an insertion of four nucleotides (AAAG) in the upper band, however there were ambiguous sequence calls at several position, indicating that the bands could be derived from several highly similar mRNAs. Re-examining our database of previously identified DD-fragments revealed a related sequence derived from a band with an identical expression profile (Fig. 1).

The expression profiles suggested spermatid-specific expression with a two-step upregulation, first a slight increase from days 18–20 pn followed by a doubling on days 26–28 pn implying a mixed late pachytene and spermatid expression profile (Almstrup et al., 2004). We compared the expression profiles with the profiles of previously identified DD-bands corresponding to two well-characterized spermatid-specific genes: *Tnp2* (Shih and Kleene, 1992) and *Prm1* (Mali et al., 1989) (Fig. 1). The profiles showed that the expression of the novel genes preceded that of *Tnp2*, which in turn preceded *Prm1*.

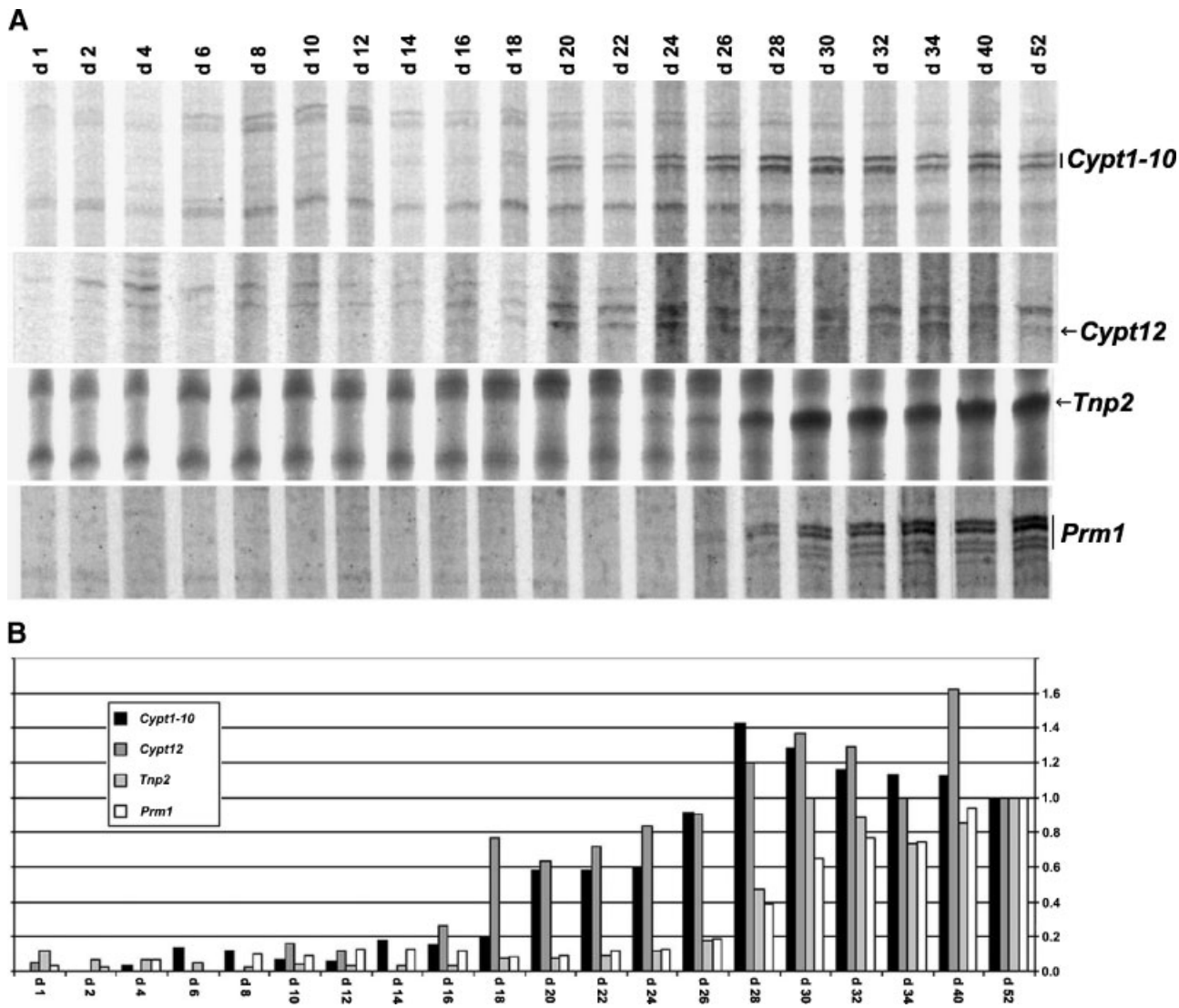

Fig. 1. Differential display (DD) expression analysis during postnatal development. **A**: DD gels showing the profiles of *Cypt1–10*, *Cypt12*, *Tnp2*, and *Prm1*, respectively. The multiple bands representing *Prm1* were caused by the use of alternative polyadenylation sites. **B**: Phosphorimager quantification of the bands shown in A. To allow comparison between the different displays, the expression level on day 52 pn was set to 1 for all the mRNAs.

A GenBank search revealed that one of these sequences was identical to *Cypt1*, which encodes a postacrosomal perinuclear theca protein (Kitamura et al., 2004). Further GenBank searches identified two additional related sequences: one sequence (GenBank acc. AK018908) along with the mRNAs represented in the double DD-band and the sequences detected by RT-PCR (see below) corresponded to a cluster of testis-specific mRNAs mapping to the X chromosome (UniGene Mm.45302). The other sequence (GenBank acc. AK005586) belonged to another testis-specific cluster (UniGene Mm.27023). Using these sequences to search the mouse genome revealed three additional members.

To identify additional members of this family, we performed PCR amplification on cDNA from testes using degenerate primers matching the 5′- and 3′-ends of all the sequences in UniGene cluster Mm.45302. The amplified fragments were cloned and sequencing of 20 clones identified 10 different, but closely related sequences. Thus, we have in total identified 15 sequences and an alignment showed that all were related to *Cypt1* (Fig. 2). The sequences of *Cypt1-15* have been deposited in GenBank (acc. AM040451–60; BN000808–12).

The CYPT family mRNAs differed in length because of short insertions and deletions, but all the sequences were clearly related, with the highest similarity in the 5′- and 3′-ends (Fig. 2). Close inspection revealed that the CYPT sequences were not splice variants since there were multiple sites of single nucleotide mutations between any two sequences—or very short insertions or deletions incompatible with any splice site. Based on the similarity, the CYPT sequences could be divided into two clusters; *Cypt1–10* and *Cypt12–15*, whereas *Cypt11* is more distantly related, but with similarity to both clusters (Fig. 3A).

Most of the CYPT sequences included an open reading frame, but the derived peptide sequences varied because of the different gaps and insertions, however, all gaps within the coding region were multiple of three nucleotides, thus maintaining the reading frames for all the CYPT sequences except *Cypt11* and *Cypt13*, which both probably are pseudogenes, since the reading frame was disrupted, and no matching EST sequences have been reported. The CYPT peptide sequences have a common composition with a basic N-terminal composed of three to four small repeats of mainly basic residues, an extremely cysteine-rich region, followed by another basic region, and an acidic, proline-rich C-terminal with also contained 1–3 Protein kinase casein kinase II phosphorylation sites ([S/T]XX[D/E]). In *Cypt4*, and *Cypt9–10* the N-terminal cysteine-rich region consisted of only four cysteines where *Cypt3* and *Cypt2* have an additional stretch of five cysteines interspersed with basic residues which was duplicated in *Cypt1* and *Cypt5–8*. The *Cypt3–4* and *Cypt9–12* peptide sequences harbors one or two bipartite nuclear localization signals according to PSORTII.

The CYPT Family Is Similar to the Non Coding *Zfy2* Exon 1A

Kitamura and colleagues (Kitamura et al., 2004) reported that *Cypt1* and the Ckt sequences (*Cypt2–5*) shared a high similarity to the non coding *Zfy2* exon 1A. This similarity extended to the entire CYPT family where the sequences shared up to 80% similarity with the non coding exon 1A and part of the first intron of *Zfy2* (Fig. 2). Interestingly, the putative start codon present in the 5′-end of all the CYPT sequences was changed to ATT in the *Zfy2* sequence (Mardon and Page, 1989), thus preventing translation of the CYPT related exon. Instead, the start codon of *Zfy2* is located in exon 5 in the nomenclature used by Mahaffey et al. (1997).

Genomic Organization of the CYPT Family: Highly Conserved Putative Promoter Sequences

Comparison with the genomic sequence showed that the 15 CYPT sequences only mapped to nine loci in the mouse genome (Fig. 3B): *Cypt1–3, Cypt11*, and *Cypt13–15* mapped to the X chromosome and all included an intron except *Cypt13* which was intron-less. Furthermore, *Cypt4* mapped to chromosome 9, and *Cypt12* mapped to chromosome 3, but neither genes included an intron. Genomic sequences corresponding to *Cypt5-10* were not present in the current assembly of the mouse genome. The genes encoding *Cypt14* and *Cypt15* were located within a relatively short region on the X chromosome arranged in opposite directions in an inverted repeat with *Cypt14* and *Cypt15* located in each repeat unit separated by approximately 500,000 nucleotides. The similarity between the inverted repeats was not restricted to the mRNAs, but extended over approximately 17,000 nucleotides with an overall similarity of 89% indicating a segmental duplication.

Alignment of the genomic sequences immediately upstream from the nine loci allowed comparison of the putative promoter sequences along with the promoter sequence of *Zfy2* (Fig. 4). In accordance with Kitamura and colleagues (Kitamura et al., 2004), we found a high sequence conservation between the upstream sequences of all the CYPT genes and the *Zfy2* gene. Based on the length of the upstream similarity, the CYPT genes that mapped to the mouse genome, could be compared to elucidate the origin of the genes. The *Cypt12–15* sequences were conserved for more than 1,000 nucleotides; *Zfy2* and *Cypt2* were almost identical for more than 3,000 nucleotides upstream from the start codon; *Cypt2*, *Cypt4*, and *Zfy2* shared upstream sequence for more than 800 nucleotides and *Cypt1*, *Cypt2*, *Cypt4*, and *Zfy2* shared upstream sequence similarity for more than 600 nucleotides. Interestingly, the putative pseudogene *Cypt11* shared more than 1,300 upstream nucleotides with *Cypt1*, eventhough these sequences are quite distant with respect to the coding sequence (Fig. 3). However, the upstream region shared among all the genes only extended for approximately 150 nucleotides upstream from the start codon (Fig. 4). A TATA box located 50 nucleotides upstream of the transcription start was reported for the *Zfy2* gene (Mardon and Page,

Fig. 2. Multiple alignment of the CYPT sequences and the 5′-end of *Zfy2*. The *Cypt1* and the Ckt sequences (which has been renamed to *Cypt2–5*) are shown together with the corresponding sequences cloned in this study (marked by asterisks; see text for discussion). The *Zfy2* sequence is composed of the cDNA sequence (GenBank acc. M24401) in upper case supplemented with the genomic sequence (derived from GenBank acc. AC007979) in lower case. 180 nucleotides from *Zfy2* at position 367 were removed to facilitate alignment. The putative start- and stop-codons are indicated with ATG and STOP respectively. Below each alignment block a consensus track is showed: 'O' = 100%, '0' = 75–100%, 'o' = 50–75%, '·' = 25–50%.

1989; Zambrowicz et al., 1994), but the TATA box motif was poorly conserved in the CYPT sequences (Fig. 4). However, a well-conserved Inr element was present and could function as an alternative transcription initiator (Smale and Baltimore, 1989) and a fairly well conserved CAAT box was also present. Finally, all the CYPT sequences encoded a cAMP response element (CRE) (Nantel et al., 1996; Kitamura et al., 2004).

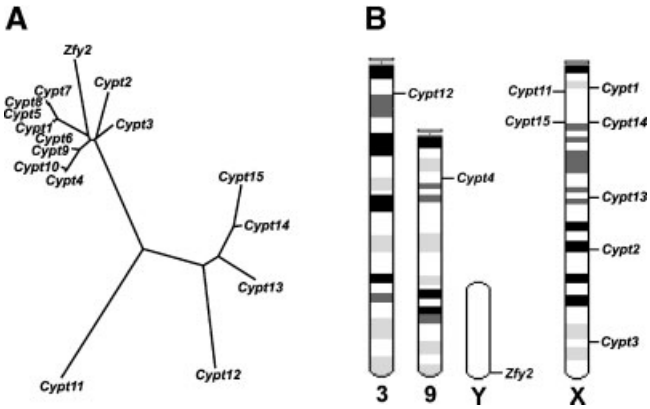

Fig. 3. Relationship of the *Zfy2* and CYPT sequences and their genomic location. **A**: Neighborhood-Joining tree generated with ClustalX showing the relationship between *Zfy2* and the CYPT sequences. **B**: Karyomap showing the genomic positions of *Zfy2* and the CYPT sequences that could be mapped to the mouse genome.

The CYPT Family and *Zfy2* Have Similar Expression Profiles

To determine if the relatively short conserved upstream sequence resulted in a common expression profile, we used RT-PCR to compare the expression of members of the CYPT family and the ZFY family members, using *Prm1* as a reference for spermatid expression (Fig. 5). The results showed that the CYPT family members and *Zfy2* had essentially identical expression profiles: all were weakly detectable on day 22 pn and strongly expressed from day 24 pn, whereas the *Prm1* profile showed weak expression from day 24 pn and was strongly expressed from day 26 pn. The apparent delay in the timing of the expression compared with profiles determined by DD is caused by the lower sensitivity of the RT-PCR method. Kitamura and colleagues (Kitamura et al., 2004) reported expression of *Cypt1* from day 29 pn using the Northern blot analysis, which is less sensitive than RT-PCR and therefore suggested a delayed expression. The primers for *Zfy1* were located in exon 1 and exon 2, respectively (Koopman et al., 1989) and should have resulted in a single band of 204 nucleotides. However, the *Zfy1* RT-PCR gave rise to several bands, most likely caused by alternative splicing of the mRNA previously reported (Koopman et al., 1989), which made it difficult to precisely determine the expression profile. Nevertheless, *Zfy1* expression was weakly detectable from day 12 pn and increased to reach a maximum on day 24 pn, suggesting that it mainly was expressed in spermatocytes, and thus clearly different from the *Zfy2* profile. *Zfx* expression was detectable on all days and the profile indicated that it was mainly expressed in somatic cells or expressed at a constant level in all cell types (Almstrup et al., 2004), which is in accordance with previously reported data (Mardon et al., 1990).

Cell Type Specific Expression

To precisely identify the expressing cell types we performed ISH with probes derived from the CYPT and ZFY families. In mice, the seminiferous epithelium can be divided into 12 distinct stages (designated I–XII; Russell et al., 1990) each containing three to four layers of specific germ cells in different differentiation stages. Thus, different germ cell differentiation types can be precisely identified by determining the stage of the seminiferous epithelium. Because of the high similarity between the 5′-end of *Cypt1–10* and exon 1A of *Zfy2* and the high similarity in the coding region among the ZFY members, ISH probes must be designed carefully to minimize cross-hybridization (see Materials and Methods). However, it is impossible to distinguish between all the mRNAs. Thus, the *Cypt1–10* and *Cypt12–15* probes cross-hybridized to some extent with each other and with *Zfy2* and visa versa, however, neither matched other ZFY family members. The ZFY probe matched all ZFY-family members, but not the CYPT family.

The *Cypt1–10* and *Cypt12–15* ISH probes showed essentially identical expression profiles. The mRNAs were weakly expressed in pachytene spermatocytes from stages IX–X. After meiosis the signal remained weakly detectable in round spermatids until a strong upregulation occurred in spermatids from stage VI with maximum expression in stages VII–VIII, followed by a rapid decline (Fig. 6A). The expression of *Tnp2* and *Prm1* was exclusive to spermatids, *Tnp2* was detectable from stages V–VI, and *Prm1* from stages VII–VIII. Thus, as suggested from the DD and RT-PCR results,

Fig. 4. Multiple alignment of the putative conserved promoter. The *Zfy2* and the CYPT sequences that could be identified in mouse genome were aligned. Putative CRE, CAAT, TATA and Inrelements along with the putative start codon for the CYPT sequences are boxed. Below each alignment block a consensus track is showed: 'O' = 100%, '0' = 75–100%, 'o' = 50–75%, '·' = 25–50%.

CYPT members showed maximum expression in round spermatids in stage VIII preceding the expression of *Tnp2*, which showed maximum expression in stage VIII, and *Prm1* with maximum expression in stages VIII–IX (Fig. 6A). In contrast to CYPT and *Tnp2*, the *Prm1* mRNA remained detectable in elongated spermatids until the transition from step 14 to 15 spermatids (data not shown). Thus, the expression patterns determined by ISH were, for all the mRNAs, in accordance with the DD and RT-PCR expression profiles.

The expression profiles for the *Zfy1* and *Zfy2* were also investigated by ISH (Fig. 6B). This showed that *Zfy2* was expressed in the same cell types as the CYPT genes, confirming their similar expression pattern. The expression pattern detected with the *ZFY* probe was clearly different from that observed for the *Zfy2* probe: weak expression was detected in all germ cells, albeit at very different levels and also weakly in somatic cells. The level was especially high in a few isolated germ cells along the edge of tubuli, especially in stages I and II, corresponding to expression in A-spermatogonia (probably mitotic A-paired or A-aligned spermatogonia) (Stage I and II are not shown in Fig. 6). The level was lower in A1 and B spermatogonia and in early spermatocytes, but increased significantly in pachytene spermatocytes from stage VII and reached a maximum in spermatocytes in stages X–XI. This was followed by a gradual decrease in round spermatids from stage II to V before it again increased slightly in spermatids in step VII to VIII. Comparison with rat, which does not have a gene corresponding to *Zfy2*, revealed a similar pattern, however, expression of the ZFY family in rats peaked in late pachytene- and diplotene spermatocytes, followed by a consistent decrease in spermatids (results not shown). Combined with the profiles determined by RT-PCR and the fact that the probe also recognized *Zfy2*, this suggested that *Zfy1* was expressed in all germ cells until the meiotic divisions in stage XII, whereas the increased expression in mouse spermatids was derived from expression of *Zfy2*. Thus, ISH confirmed that *Zfy2* and the CYPT family shared the same expression profile, which was different from the profile of the other ZFY family members.

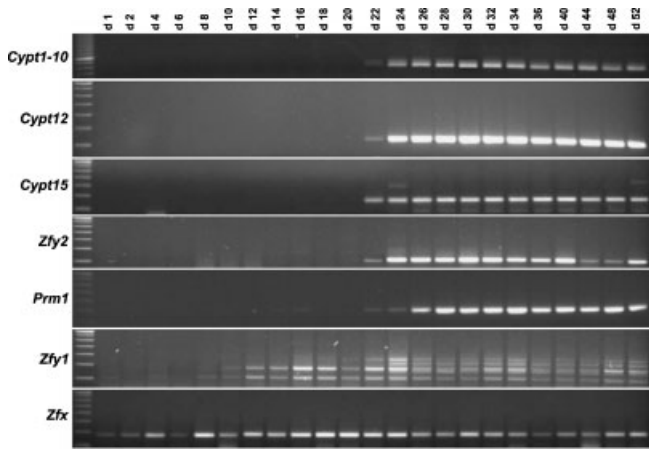

Fig. 5. RT-PCR expression analysis during postnatal development. The CYPT family was analyzed with two sets of primers specific for *Cypt1–10* and *Cypt12* (also matching *Cypt15*), respectively, *Zfy2*, *Zfy1*, and *Zfx* were amplified with primer sets specific for each mRNA (see Materials and Methods). The multiple bands observed for *Zfy1* was most likely caused by the presence of undescribed splice variants (Koopman et al., 1989). The *Zfy1* primers matched the 3′-end of *Zfy1* exon 1B and the 5′-end of exon 2 (exon 2 is conserved between *Zfy1* and *Zfy2*). The marker was a 100 bp ladder; the strong band corresponds to 800 bp (top band in all except *Cypt1–10*).

DISCUSSION

We here report a number of genes related to *Cypt1* previously discovered by Kitamura and colleagues (Kitamura et al., 2004), who also showed that *Cypt1* is identical to *Ckt1r3* which is a member of a previously reported, but unpublished family of casein kinase II targets (Ckt; Xu et al., unpublished; GenBank acc. AF463499–2). However, *Cypt1* and *Ckt1r3* are in fact different transcripts where *Ckt1r3* contains a gap of 15 nucleotides compared to *Cypt1* (Fig. 2). The sequence of one of our clones was identical to *Cypt1* (Fig. 2), which is a member of a family consisting of at least 15 members, including the Ckt family. Thus, we suggest to change the nomenclature of the CYPT family by renaming *Ckt1, Ckt1r1*, *Ckt1r2*, and *Ckt1r3* to *Cypt2–5*, respectively, and the remaining of our identified members have been named *Cypt6–15*.

The highly similar *Cypt1–10* genes appear to be mouse-specific with no similar genes present in other genome sequences, which is in accordance with negative results from ISH performed on rat testis with the probe matching *Cypt1–10* (data not shown). However, a gene with similarity to *Cypt12* is present in the rat genome and ISH showed that its expression in testis was similar to the expression of the mouse CYPT family (results not shown). This does not necessarily imply that proteins performing the function of the *Cypt1–10* are absent, since X and Y linked genes sometimes show a high evolutionary rate (e.g., the SPANX family (Kouprina et al., 2004)), which eventually results in divergence of the sequences to a level where the similarity is essentially absent in spite of functional similarities being conserved (see below).

We provide evidence suggesting that the origin of the mouse-specific *Zfy2* gene is the result of a duplication-event where a copy of the *Zfy1* gene was inserted into a gene belonging to the CYPT family, most likely *Cypt2* because *Cypt2* and *Zfy2* share more than 3,000 nucleotides of upstream sequence. This has resulted in a novel gene composed of the promoter and first exon from a CYPT member controlling the expression of a protein very similar to *Zfy1*. The promoter shared between the CYPT family and *Zfy2* directs mRNA expression early during spermatid differentiation, but initiated at a low level already in the late pachytene phase, possibly ensuring the presence of the mRNAs in the period after the meiotic divisions where transcription appear to be arrested (Almstrup et al., 2004). The highest activity directed by the common promoter was in step 5–6 spermatids, slightly before expression of most other

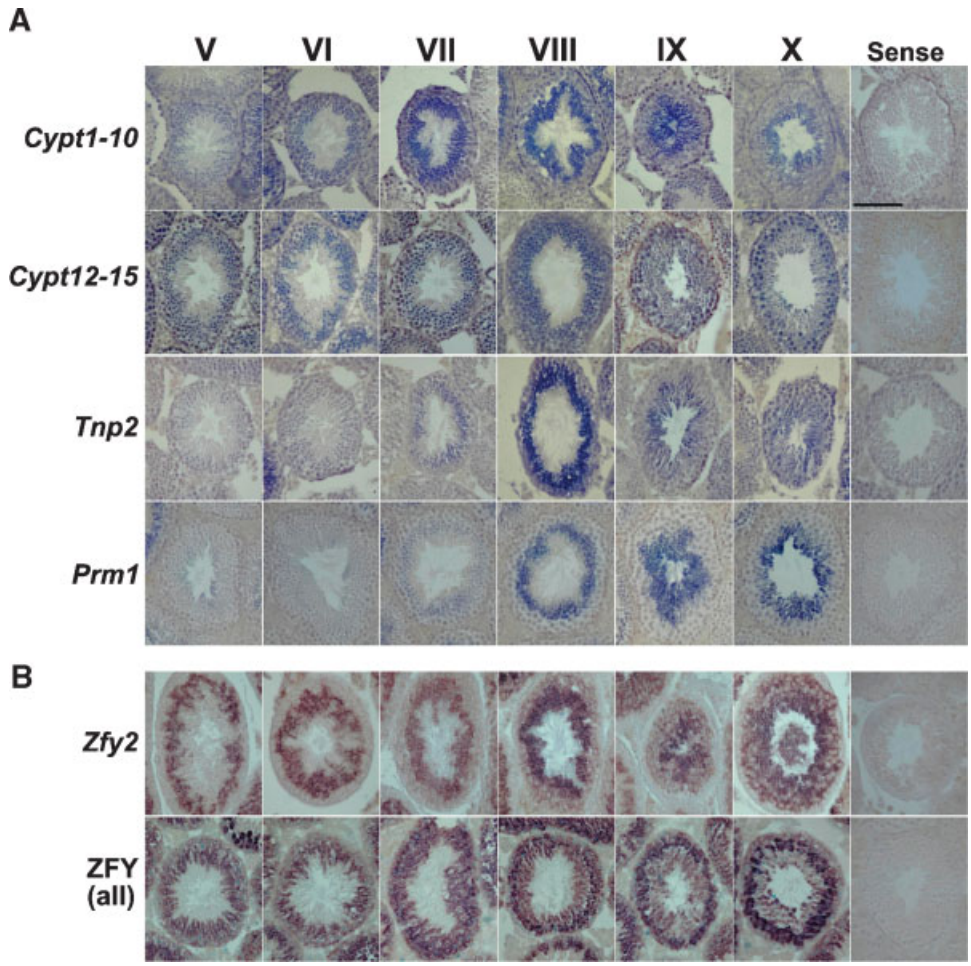

Fig. 6. Identification of the expressing cell types by in situ hybridization (ISH). Only selected stages of the seminiferous epithelium is shown. **A**: ISH with probes for *Cypt1–10*, *Cypt12–15*, *Tnp2*, and *Prm*1. Because of very similar sequences, the CYPT probes matched *Cypt1–10* and *Cypt12–15*, respectively, the *Tnp2* probe also matched *Tnp1* and the *Prm1* probe also hybridized to *Prm2*. Due to short development time, the ISH with the *Cypt1–10* probe essentially only showed hybridization to spermatids, however, longer development revealed weak signal from late pachytene and diplotene spermatocytes in stages IX, X, XI, and XII similar to the *Cypt12–15* probe. *Prm1* expression continued in elongating/elongated spermatids until steps 14–15, whereas CYPT family and *Tnp2* expression was only detectable until step 10–11 spermatids. The testes were all fixed in 4% paraformaldehyde. **B**: ISH with probes for *Zfy2* (which also matched *Cypt1–10*) and a ZFY probe matching *Zfy1*, *Zfy2*, *Zfx*, and *Zfa*. To optimize the ISH it was necessary to use different fixatives: ISH with probes matching *Cypt1–10*, *Cypt12–15*, *Prm1*, and *Tnp2* were made on testes fixed in 4% paraformaldehyde whereas ISH with the *Zfy2* and ZFY probes was performed on testes fixed in Stieves fixative. This results in differences in the morphology and in the appearance of the ISH signal (dark blue to black for paraformaldehyde and brownish for Stieves fixative). All images are in the same magnification and the bar in the *Cypt1–10* sense images corresponds to 100 μm.

spermatid-specific genes, which occur in step 7 or -8 spermatids. In fact, the expression of CYPT in the early spermiogenesis is, to our knowledge, the first transcripts to be expressed in spermatids after the meiotic divisions.

RT-PCR and ISH showed a clear difference in the expression of *Zfy1*, *Zfy2*, and *Zfx*. *Zfy2* expression was essentially confined to spermatids, whereas *Zfy1* was expressed in all germ cells albeit at different levels. *Zfy1* expression has previously been reported to start around days 10–12 post coitus (pc) with a peak at 13.5 pc coinciding with the proliferation of primordial germ cells (Mardon and Page, 1989), in accordance with the expression patterns observed by ISH. RT-PCR showed that *Zfx* was essentially constitutively expressed, in agreement with the expression previously reported by Mardon et al. (1990). The differential expression of *Zfy1* and *Zfy2* is also in accordance with reports showing that *Zfy1* and *Zfy2* utilize different promoters and non coding first exons (Zambrowicz et al., 1994; Mahaffey et al., 1997). Exon 1A is unique to *Zfy2* distinguishing *Zfy2* from the other ZFY family members, and this exon is spliced directly to exon 2, which is highly similar to exon 2 of *Zfy1* (Mahaffey et al., 1997), irrespectively of the presence of sequences corresponding exon 1B in the genomic sequence of *Zfy2*. Thus, the main difference between *Zfy1* and *Zfy2* is the use of different promoters, which results in the different expression profiles. Rat and other mammals only have the *Zfy1* gene and ISH performed on rat testes showed *Zfy* expression was

similar to mouse. However, the expression level in round spermatids in the rat showed a consistent decline without the increased level in step 6–8 spermatids observed in mouse. Thus, extended expression in spermatids is apparently not required for spermiogenesis, and it is unclear why mouse apparently have developed an expression strategy that leads to extended *Zfy* expression in spermatids.

A large number of genes are expressed during germ cell differentiation and many of these genes are unique to male germ cells (Schultz et al., 2003; Almstrup et al., 2004; Schlecht et al., 2004; Shima et al., 2004). Structural analysis of these genes showed that many have no introns (McCarrey and Thomas, 1987; Ashworth et al., 1990; Kleene et al., 1998; Yoshimura et al., 1999, 2001) suggesting that they may be retroposons (Vanin, 1985; Esnault et al., 2000; Mighell et al., 2000). Moreover, it has been suggested that the expression of large numbers of intron-less genes is a unique feature to male germ cells (Hisano et al., 2003). The observation that *Cypt4, Cypt12*, and *Cypt13* on chromosomes 9, 3, and X, respectively, are intron-less, but share the CYPT promoter excludes that these genes have arisen by simple retroposition unless the promoter region in fact is the remains of a now-lost exon as suggested by Kitamura and colleagues (Kitamura et al., 2004). Another possible explanation could be that the intron sequence at one point has been part of the coding region, which is supported by the fact that the introns (also the *Zfy2* intron) are highly conserved.

The expression profiles of the CYPT family showed that expression of the CYPT transcripts were initiated before the transition protein Tnp2 and the protamine Prm1, which both are involved in the condensation and shaping of the chromatin in the final maturation of the spermatozoa (Mali et al., 1989; Shih and Kleene, 1992). Kitamura and colleagues (Kitamura et al., 2004) determined that the expression of the CYPT1 protein was restricted to haploid spermatids at steps 9–16, however, since the mRNAs are present in all haploid spermatids, this suggests a delay in the translation due to translational arrest (Kleene, 1996) or that the antibody only binds the CYPT1 protein after phosphorylation by casein kinase II. Moreover, Kitamura and colleagues determined the precise expression localization to the postacrosomal perinuclear theca, and speculated that the function of the CYPT protein was involved in the nuclear shaping during spermiogenesis. Since the expression of the CYPT protein precedes that of the transition proteins and the protamines the function of the CYPT protein could be related to early re-modeling of the nucleus before the condensation of the DNA occur. Although the CYPT genes are not detectable in human, we have identified 10 human genes that share significant similarity to the CYPT promoter. All the human genes are reported expressed in the testis, and some exclusively in testis. Most of them have only been identified as ESTs or in silico, but a few known genes, VCY/X (Lahn and Page, 2000) and some of the SPANX genes (Westbrook et al., 2000, 2001; Zendman et al., 2003; Westbrook et al., 2004) also share the promoter. The SPANX proteins have been reported to be located in the nuclear membrane of a subset of spermatozoa, and their absence or presence may be a marker for sperm function (Westbrook et al., 2001; Salemi et al., 2004). However, a recent report showed that another murine gene has a weak similarity to the SPANX genes and may be the murine equivalents (Kouprina et al., 2004). Further analysis of the expression of the human genes and the localization of the peptides is in progress.

ACKNOWLEDGMENTS

Excellent technical help from Brian Vendelbo Hansen and Marlene Dalgaard was much appreciated.

REFERENCES

Almstrup K, Nielsen JE, Hansen MA, Tanaka M, Skakkebaek NE, Leffers H. 2004. Analysis of cell-type-specific gene expression during mouse spermatogenesis. Biol Reprod 70:1751–1761.

Ashley T, Gaeth AP, Creemers LB, Hack AM, de Rooij DG. 2004. Correlation of meiotic events in testis sections and microspreads of mouse spermatocytes relative to the mid-pachytene checkpoint. Chromosoma 113:126–136.

Ashworth A, Skene B, Swift S, Lovell-Badge R. 1990. Zfa is an expressed retroposon derived from an alternative transcript of the Zfx gene. EMBO J 9:1529–1534.

Balhorn R, Reed S, Tanphaichitr N. 1988. Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. Experientia 44:52–55.

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. 1977. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 74:68–85.

Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI. 1993. Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. Mol Reprod Dev 34:53–57.

Berta P, Hawkins JR, Sinclair AH, Taylor A, Griffiths BL, Goodfellow PN, Fellous M. 1990. Genetic evidence equating SRY and the testis-determining factor. Nature 348:448–450.

Carrell DT, Liu L. 2001. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 22:604–610.

de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. 1993. Complete selective absence of protamine P2 in humans. J Biol Chem 268: 10553–10557.

Edgar RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Ellis PJ, Furlong RA, Wilson A, Morris S, Carter D, Oliver G, Print C, Burgoyne PS, Loveland KL, Affara NA. 2004. Modulation of the mouse testis transcriptome during postnatal development and in selected models of male infertility. Mol Hum Reprod 10:271–281.

Esnault C, Maestre J, Heidmann T. 2000. Human LINE retrotransposons generate processed pseudogenes. Nat Genet 24:363–367.

Fujii T, Tamura K, Masai K, Tanaka H, Nishimune Y, Nojima H. 2002. Use of stepwise subtraction to comprehensively isolate mouse genes whose transcription is up-regulated during spermiogenesis. EMBO Rep 3:367–372.

Guo R, Yu Z, Guan J, Ge Y, Ma J, Li S, Wang S, Xue S, Han D. 2004. Stage-specific and tissue-specific expression characteristics of differentially expressed genes during mouse spermatogenesis. Mol Reprod Dev 67:264–272.

Hisano M, Yamada S, Tanaka H, Nishimune Y, Nozaki M. 2003. Genomic structure and promoter activity of the testis haploid germ

cell-specific intronless genes, Tact1 and Tact2. Mol Reprod Dev 65:148–156.

Jorgensen M, Bevort M, Kledal TS, Hansen BV, Dalgaard M, Leffers H. 1999. Differential display competitive polymerase chain reaction: An optimal tool for assaying gene expression. Electrophoresis 20:230–240.

Jorgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen AG, Andersson AM, Haugen TB, Horte A, Jensen TK, Magnus O, Petersen JH, Vierula M, Toppari J, Skakkebaek NE. 2002. East-West gradient in semen quality in the Nordic-Baltic area: A study of men from the general population in Denmark, Norway, Estonia and Finland. Hum Reprod 17:2199–2208.

Kitamura K, Iguchi N, Kaneko Y, Tanaka H, Nishimune Y. 2004. Characterization of a novel postacrosomal perinuclear theca-specific protein, CYPT1. Biol Reprod 71:1927–1935.

Kleene KC. 1996. Patterns of translational regulation in the mammalian testis. Mol Reprod Dev 43:268–281.

Kleene KC, Mulligan E, Steiger D, Donohue K, Mastrangelo MA. 1998. The mouse gene encoding the testis-specific isoform of Poly(A) binding protein (Pabp2) is an expressed retroposon: Intimations that gene expression in spermatogenic cells facilitates the creation of new genes. J Mol Evol 47:275–281.

Koopman P, Gubbay J, Collignon J, Lovell-Badge R. 1989. Zfy gene expression patterns are not compatible with a primary role in mouse sex determination. Nature 342:940–942.

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348:450–452.

Kouprina N, Mullokandov M, Rogozin IB, Collins NK, Solomon G, Otstot J, Risinger JI, Koonin EV, Barrett JC, Larionov V. 2004. The SPANX gene family of cancer/testis-specific antigens: Rapid evolution and amplification in African great apes and hominids. Proc Natl Acad Sci USA 101:3077–3082.

Lahn BT, Page DC. 2000. A human sex-chromosomal gene family expressed in male germ cells and encoding variably charged proteins. Hum Mol Genet 9:311–319.

Lee K, Haugen HS, Clegg CH, Braun RE. 1995. Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci USA 92:12451–12455.

Mahaffey CL, Bayleran JK, Yeh GY, Lee TC, Page DC, Simpson EM. 1997. Intron/exon structure confirms that mouse Zfy1 and Zfy2 are members of the ZFY gene family. Genomics 41:123–127.

Mali P, Kaipia A, Kangasniemi M, Toppari J, Sandberg M, Hecht NB, Parvinen M. 1989. Stage-specific expression of nucleoprotein mRNAs during rat and mouse spermiogenesis. Reprod Fertil Dev 1:369–382.

Maratou K, Forster T, Costa Y, Taggart M, Speed RM, Ireland J, Teague P, Roy D, Cooke HJ. 2004. Expression profiling of the developing testis in wild-type and Dazl knockout mice. Mol Reprod Dev 67:26–54.

Mardon G, Page DC. 1989. The sex-determining region of the mouse Y chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. Cell 56:765–770.

Mardon G, Luoh SW, Simpson EM, Gill G, Brown LG, Page DC. 1990. Mouse Zfx protein is similar to Zfy-2: Each contains an acidic activating domain and 13 zinc fingers. Mol Cell Biol 10:681–688.

McCarrey JR, Thomas K. 1987. Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. Nature 326:501–505.

Mighell AJ, Smith NR, Robinson PA, Markham AF. 2000. Vertebrate pseudogenes. FEBS Lett 468:109–114.

Nantel F, Monaco L, Foulkes NS, Masquilier D, LeMeur M, Henriksen K, Dierich A, Parvinen M, Sassone-Corsi P. 1996. Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. Nature 380:159–162.

Nielsen JE, Hansen MA, Jorgensen M, Tanaka M, Almstrup K, Skakkebaek NE, Leffers H. 2003. Germ cell differentiation-dependent and stage-specific expression of LANCL1 in rodent testis. Eur J Histochem 47:215–222.

Russell LD, Ettlin RA, Hikim APS, Clegg ED. 1990. Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press.

Salemi M, Calogero AE, Benedetto DD, Cosentino A, Barone N, Rappazzo G, Vicari E. 2004. Expression of SPANX proteins in human-ejaculated spermatozoa and sperm precursors. Int J Androl 27:134–139.

Schlecht U, Demougin P, Koch R, Hermida L, Wiederkehr C, Descombes P, Pineau C, Jegou B, Primig M. 2004. Expression profiling of mammalian male meiosis and gametogenesis identifies novel candidate genes for roles in the regulation of fertility. Mol Biol Cell 15:1031–1043.

Schultz N, Hamra FK, Garbers DL. 2003. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci USA 100:12201–12206.

Shih DM, Kleene KC. 1992. A study by in situ hybridization of the stage of appearance and disappearance of the transition protein 2 and the mitochondrial capsule seleno-protein mRNAs during spermatogenesis in the mouse. Mol Reprod Dev 33:222–227.

Shima JE, McLean DJ, McCarrey JR, Griswold MD. 2004. The murine testicular transcriptome: Characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71: 319–330.

Sinclair AH, Foster JW, Spencer JA, Page DC, Palmer M, Goodfellow PN, Graves JA. 1988. Sequences homologous to ZFY, a candidate human sex-determining gene, are autosomal in marsupials. Nature 336:780–783.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346: 240–244.

Smale ST, Baltimore D. 1989. The "initiator" as a transcription control element. Cell 57:103–113.

Tay J, Richter JD. 2001. Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. Dev Cell 1: 201–213.

Vanin EF. 1985. Processed pseudogenes: Characteristics and evolution. Annu Rev Genet 19:253–272.

Westbrook VA, Diekman AB, Klotz KL, Khole VV, von Kap-Herr C, Golden WL, Eddy RL, Shows TB, Stoler MH, Lee CY, Flickinger CJ, Herr JC. 2000. Spermatid-specific expression of the novel X-linked gene product SPAN-X localized to the nucleus of human spermatozoa. Biol Reprod 63:469–481.

Westbrook VA, Diekman AB, Naaby-Hansen S, Coonrod SA, Klotz KL, Thomas TS, Norton EJ, Flickinger CJ, Herr JC. 2001. Differential nuclear localization of the cancer/testis-associated protein, SPAN-X/CTp11, in transfected cells and in 50% of human spermatozoa. Biol Reprod 64:345–358.

Westbrook VA, Schoppee PD, Diekman AB, Klotz KL, Allietta M, Hogan KT, Slingluff CL, Patterson JW, Frierson HF, Irvin WP, Jr., Flickinger CJ, Coppola MA, Herr JC. 2004. Genomic organization, incidence, and localization of the SPAN-x family of cancer-testis antigens in melanoma tumors and cell lines. Clin Cancer Res 10:101–112.

WHO. 1999. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th edn.

Wrobel G, Primig M. 2005. Mammalian male germ cells are fertile ground for expression profiling of sexual reproduction. Reproduction 129:1–7.

Xu X, Toselli PA, Russell LD, Seldin DC. 1999. Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit. Nat Genet 23: 118–121.

Yoshimura Y, Tanaka H, Nozaki M, Yomogida K, Shimamura K, Yasunaga T, Nishimune Y. 1999. Genomic analysis of male germ cell-specific actin capping protein alpha. Gene 237:193–199.

Yoshimura Y, Tanaka H, Nozaki M, Yomogida K, Yasunaga T, Nishimune Y. 2001. Nested genomic structure of haploid germ cell specific haspin gene. Gene 267:49–54.

Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML. 2000. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc Natl Acad Sci USA 97:4683–4688.

Yu Z, Guo R, Ge Y, Ma J, Guan J, Li S, Sun X, Xue S, Han D. 2003. Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. Biol Reprod 69:37–47.

Zambrowicz BP, Findley SD, Simpson EM, Page DC, Palmiter RD. 1994. Characterization of the murine Zfy1 and Zfy2 promoters. Genomics 24:406–408.

Zendman AJ, Zschocke J, van Kraats AA, de Wit NJ, Kurpisz M, Weidle UH, Ruiter DJ, Weiss EH, van Muijen GN. 2003. The human SPANX multigene family: Genomic organization, alignment and expression in male germ cells and tumor cell lines. Gene 309:125–133.

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM, Russell LD, Behringer RR, Meistrich ML. 2001. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol 21:7243–7255.