

Cloning and Characterization of Rat Spermatid Protein SSP411: A Thioredoxin-Like Protein

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ABSTRACT: In an attempt to identify new sperm-specific genes that are involved in sperm maturation, fertilization, and embryo development, such as the mammalian ortholog of the sperm-supplied protein gene, *spe-11*, in *Caenorhabditis elegans*, we cloned and characterized a new spermatid-specific protein gene, *ssp411*, from adult rat testes. The *ssp411* cDNA shared >85% sequence identity with an unnamed human protein, FLJ21347, and an uncharacterized mouse testicular protein called transcript increased in spermiogenesis 78 (TISP78). A 2.8-kb *ssp411* mRNA was expressed in a testis-specific and age-dependent manner; the mRNA was evident at 28 days and remained at high levels throughout adulthood. An SSP411 protein of molecular weight 88 000 was detected in testicular extracts by Western blot analysis. *Ssp411* mRNA and SSP411 protein, as analyzed by in situ hybridization and immunohistochemistry, were both expressed in a stage-dependent fashion during the cycle of the seminiferous epithelium. The *ssp411* mRNA was predominantly lo-

calized to round and elongated spermatids, with maximal expression at stages VII–XII. The SSP411 protein was mainly observed in elongated spermatids and reached its highest levels during stages V–VI. A conserved thioredoxin-like domain was detected in the N-terminal region of SSP411 and its orthologs. An analysis of the predicted 3-dimensional structural modeling and folding pattern further suggested that SSP411 is identifiable as a member of thioredoxin family. In summary, we have identified a new rat spermatid protein gene, *ssp411*, and its orthologs in human and mouse and demonstrated that SSP411 might belong to a testis-specific thioredoxin family. This suggests that SSP411 may play a role in sperm maturation, fertilization, and/or embryo development, as has been shown in thioredoxin family.

Key words: Thioredoxin, spermatogenesis, testis, gene cloning.

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The mechanisms involved in controlling mammalian sperm development, fertilization, and embryo development remain poorly understood. Although many sperm surface antigens and sperm-specific cytoplasmic and nuclear proteins have been isolated and studied (for reviews, see Naz and Vanek, 1998; Vacquier, 1998; Eddy, 2002), it is estimated that only 10%–20% of the sperm polypeptides have been identified. These sperm proteins are known to play roles in sperm-egg interactions, to function as enzymes in sperm activity, and to serve as nuclear proteins for chromosomal structure. For instance, ~60 *spe* genes are associated with spermatogenesis-defective phenotypes in *Caenorhabditis elegans* (for a review, see L'Hernault, 1997).

Many of the *spe* mutant genes identified in *C. elegans* affect developmental stages before the formation of sper-

matozoa (Varkey et al, 1995) or sperm-oocyte interactions (Singson et al, 1998). The *spe-26* gene encodes a cytoskeletal actin-associated protein and is necessary for spermatid formation (Varkey et al, 1995). The *spe-9* gene encodes a sperm transmembrane protein with epidermal growth factor-like repeats and has been suggested to play a role in fertilization by sperm-egg interactions (Singson et al, 1998). The *spe-11* gene was the first sperm protein gene demonstrated to directly contribute to the development of embryos from fertilized eggs (Hill et al, 1989; Browning and Strome, 1996). The SPE-11 protein was identified in mature sperm and is not detectable in the oocyte. Only short-lived single-celled embryos develop if eggs are fertilized by mutant sperm that lack SPE-11. The *spe-11* gene is considered to be a paternal-effect embryonic-lethal gene (Hill et al, 1989), and its protein product, SPE-11, has been referred to as “sperm-supplied protein” (Browning and Strome, 1996).

Our general hypothesis is that the mammalian ortholog of *spe-11* is a sperm-specific gene that is involved in early embryo development. To test this hypothesis, we used degenerate oligonucleotides prepared from conserved amino acid sequences of SPE-11 from 3 nematodes to

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isolate new cDNAs from adult rat testes that exhibited sequence similarity with *C. elegans spe-11*. We describe the cloning and characterization of the newly identified *ssp411* cDNA, an analysis of a thioredoxin-like domain in SSP411, and an examination of the age- and stage-dependent expression of both *ssp411* mRNA and protein in the rat testis.

Materials and Methods

Tissue Collection

Sprague-Dawley rats aged 7–90 days were purchased from Charles River Breeding Laboratories (Wilmington, Mass). The use of animals was approved by the Rockefeller University Animal Care and Use Committee. Testes and other tissues were collected for the analysis of *ssp411* mRNA and protein as described elsewhere (Feng et al, 1995; Zhang et al, 2002).

Isolation of SPE-11-Related cDNAs from Rat Testis

To identify the mammalian ortholog of *spe-11*, degenerate oligonucleotides were prepared from conserved amino acid sequences of SPE-11 from 3 nematodes; these were used to isolate new cDNAs from adult rat testes that exhibit sequence similarity with *C. elegans spe-11* (Browning and Strome, 1996). Clone 24 was isolated, by reverse-transcription polymerase chain reaction (RT-PCR), from adult rat testicular RNA using a series of degenerated oligonucleotides prepared from the amino acid sequences WKRYLRKSWD and HYKKWLEKK in SPE-11, which are conserved in 3 nematodes (Browning and Strome, 1996). The RT-PCR-generated cDNA products that contained ~500 bp were analyzed by hybridization with the *C. elegans spe-11* cDNA (provided by Dr Susan Strome, Indiana University, Bloomington, Ind). Clone 24, which showed strong positive hybridization to *spe-11* cDNA, was subcloned into pGEM-3Z vector and further analyzed.

Clone 411 was isolated by screening a 5' stretch plus cDNA library of rat testis (BD Biosciences Clontech, Palo Alto, Calif), using a radiolabeled clone 24 cDNA fragment as the hybridization probe. Approximately $0.8\text{--}1.0 \times 10^6$ phage plaques were screened. Filters were hybridized overnight at 42°C in a solution that contained $5\times$ SSPE (750 mM NaCl, 50 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, and 5 mM EDTA [pH 7.4]), $5\times$ Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 $\mu\text{g}/\text{mL}$ single-stranded salmon sperm DNA. The filters were washed twice in $2\times$ standard saline citrate (SSC) and 1% SDS at room temperature for 15 minutes, once at 42°C for 30 minutes, and then in $1\times$ SSC and 1% SDS at 42°C for 15–30 minutes. Phage DNA was prepared from purified positive phage clones (Feng et al, 1995) and subjected to Southern blot analysis. Phage DNA isolated from clone 411 was hybridized to both clone 24 and *spe-11* cDNA and was subcloned into pGEM-3Z vector for further analysis. Nucleotide sequences of the isolated cDNA clones were analyzed by the DNA Sequencing Laboratory at the Protein/DNA Technology Center of Rockefeller University.

5'- and 3'-RACE-PCR for the Generation of Full-Length *ssp411* cDNA

The 5' end of the *ssp411* cDNA was generated by 5'-rapid amplification of cDNA ends (RACE)-PCR (Kressler et al, 2002) using the GeneRacer Kit (Invitrogen Corp, Carlsbad, Calif). The first-strand cDNA was synthesized using 80-day-old rat testicular total RNA as a template. The *ssp411* cDNA was then amplified by PCR using a 44-mer oligonucleotide provided in the kit and a 26-mer oligonucleotide, GSP1 (5'-CTGTCGGTCCCGTGGAGTAGCGGTGAA-3'), derived from clone 411, which was complementary to the sequence at 1021–1046 of the *ssp411* cDNA. The PCR products were subcloned into pCR4-TOPO using the TOPO TA Cloning Kit (Invitrogen) and analyzed by nucleotide sequencing.

Similarly, the 3' end of the *ssp411* cDNA was also prepared from adult rat testicular RNA using 3'-RACE-PCR with a 25-mer GeneRacer 3' primer provided in the kit and a 26-mer oligonucleotide, GSP2 (5'-GCCCTGCTGGCCCGATCTGAGATCAG-3') derived from clone 411 that was complementary to the sequence at 735–760 of the *ssp411* cDNA. The PCR products were subcloned into pCR4-TOPO vector and characterized by sequencing analysis. A pair of primers containing DNA sequences at positions 1–22 and 2559–2580 nt, which were obtained from the 5'- and 3'-end cDNA fragments, was used to isolate a full-length *ssp411* cDNA using RT-PCR.

Computer Analysis of the Amino Acid Sequence Deduced From *ssp411* cDNA

Protean software (DNASTAR, Inc, Madison, Wis) and SIM software (Expert Protein Analysis System [ExpPASy]) proteomics tools (available at: <http://us.expasy.org>) were used to analyze the alignment of the deduced amino acid sequence of SSP411 with human FLJ21347, mouse TISP78, and *C. elegans* SPE-11. The N-glycosylation and phosphorylation sites were predicted using ScanProsite software on the ExpPASy proteomics server. In addition, the NetNGlyc 1.0 and NetOGlyc 2.0 servers of Center for Biological Sequence Analysis (available at: <http://www.cbs.dtu.dk>) were also used for predicting the sites of N- and O-linked glycosylation, respectively. The theoretical molecular weight was calculated using ExpPASy proteomics tools.

3-Dimensional Structural Analysis of the Thioredoxin-Like Domain in SSP411

The 3-dimensional structure of the SSP411 protein was initially analyzed using the 3D-PSSM server (available at: <http://www.sbg.bio.ic.ac.uk/~3dpssm/>) by submitting the protein sequence directly (Kelley et al, 2000). A thioredoxin fold was found at the N-terminal region of the SSP411 protein. The 3-dimensional structure of the N-terminal domain modeling was next carried out using the PLOP program (kindly provided by Dr Richard A. Friesner, Department of Chemistry and Center for Biomolecular Simulation, Columbia University, New York; Eyrich et al, 1999). The 3-dimensional model of the thioredoxin domain in the SSP411 was further analyzed by comparing it with the available crystallographic structures of 2 thioredoxin proteins, thioredoxin (Eklund et al, 1991; Nicastro et al, 2000) and human thioredoxin-like protein (Jin et al, 2002), using the

ProSup server (available at: http://lore.came.sbg.ac.at:8080/CAME/CAME_EXTERN/PROSUP/index.html; Lackner et al, 2000). The root mean square deviation (RMSD) of structurally equivalent residues was also calculated, which is a common numerical measure of the difference between 2 protein structures.

Northern Blot Analysis

Total RNA was isolated from various tissues by extraction with TRIzol Reagent (Invitrogen), as described elsewhere (Zhang et al, 2002). Twenty micrograms each of total RNA isolated from rat testes and other tissues were subjected to Northern blot analysis. The RNA was denatured with 6% formaldehyde and 50% formamide, fractionated in 1.1% agarose gel, and transferred onto Nytran-Plus membranes (Schleicher & Schuell, Inc, Keene, NH; Feng et al, 1998, 2000). Newly isolated *ssp411* cDNA fragments were used to prepare radiolabeled probes for detecting *ssp411* mRNA on the RNA blots. Autoradiograms were obtained by exposing the RNA blots to x-ray films.

Semiquantitative RT-PCR

The *ssp411* mRNA in the rat testes ages 7–90 days and in various other tissues from adult rats was analyzed by RT-PCR using the ThermoScript RT/PCR System (Invitrogen; Feng et al, 1995; Zhang et al, 2002). The RT reaction was performed at 50°C for 60 minutes in a volume of 20 μ l that contained 2 μ g each of total RNA isolated from varied rat tissues, 1 mM deoxynucleotide triphosphate (dNTP), 50 μ M oligo (dT)₁₂ as a primer, 40 U of the RNase inhibitor RNaseOUT, and 15 U of ThermoScript reverse transcriptase. An aliquot of 1–5 μ l of cDNAs (0.1–0.5 μ g RNA equivalent), synthesized as described above, was used as templates for PCR analysis that contained 0.2 μ M each primer, 0.2 mM dNTPs, and 1 U of Platinum Taq DNA polymerase High Fidelity (Invitrogen). The cDNAs were amplified by PCR for 25–35 cycles at 94°C for 30 seconds, at 60°C for 30 seconds, and at 68°C for 2.5 minutes in each amplification cycle. Different amounts of RNA equivalents (0.1–0.5 μ g) and various numbers of PCR cycles (25–35) were tested for PCR analysis, to ensure that the results obtained were within the linear portion of the reaction.

A pair of *ssp411*-specific primers was used for amplification: upstream primer 5'-ATGAGCCACCATTCCCCACCAC-3' and downstream primer 5'-TCATTGGTGTAGCAGCTTTCGTA-3'. These were derived from nucleotide sequence at 63–84 and 2410–2432 of the *ssp411* cDNA, respectively. A single species of 2.4-kb RT-PCR product that contained the coding region of the *ssp411* cDNA was generated after 28–30 cycles of amplification. The levels of total RNA in each sample used for RT-PCR analysis were quantified by measurement of β -actin mRNA levels using upstream primer 5'-TTGTAACCAACTGGGACGATATGG-3' and downstream primer 5'-GATCTTGATCTTCATGGTGCTAGG-3', which were purchased from BD Biosciences Clontech. An expected 764-bp β -actin DNA fragment was obtained by RT-PCR with 20 cycles of amplification (Zhang et al, 2002).

Expression and Purification of Recombinant SSP411 Protein

The full-length *ssp411* cDNA was subcloned into pET-28a(+) (Novagen, Madison, Wis) bacterial expression plasmid, pET-

SSP411, which contained a tag protein of 6 histidine residues at the N-terminus (Studier et al, 1990; Apezteguia et al, 1994). The recombinant protein was produced by transforming the pET-SSP411 expression plasmid to *Escherichia coli* BL21 (DE3) (Novagen). An overnight bacterial culture that contained the pET-SSP411 expression plasmid was diluted to 1:100 in Luria broth medium and grown at 37°C until A₆₀₀ reached 0.4–0.6. Induction of the expression of the histidine-tagged SSP411 protein from T7 promoter was achieved by adding 1 mmol/L isopropyl β -D-thiogalactoside (IPTG) and a further 3–5 hours of induction. The induced bacterial cells were harvested by centrifugation, and the recombinant SSP411 protein was isolated with BugBuster protein extraction reagent (Novagen) and purified using a protein refolding kit (Novagen). The obtained soluble recombinant SSP411 protein, which contains a 1-kD histidine tag at the N terminus, was further purified by affinity chromatography using a His•Bind column (Novagen). The purified recombinant protein was dialyzed against 20 mM Tris-HCl (pH 8.5), concentrated by freeze-drying, and stored at –20°C until further use.

Production of Polyclonal Antibodies

A synthetic oligopeptide containing 17 amino acids (ERM-RRVPVALPEMVRAL) derived from the C-terminal region at amino acids 681–697 of the SSP411 protein was prepared and conjugated to Keyhole Limpet Hemocyanin (KLH) (SynPep Corporation, Dublin, Calif) as an immunogen. The high-performance liquid chromatography-purified KLH-conjugated SSP411 oligopeptide was used to immunize New Zealand White rabbits. Immunization was carried out by Covance Research Products, Inc (Denver, Penn): 5 injections of 250 μ g each, 3 test bleedings, and 2 ELISA tests to determine antibody concentrations. Antibody Ab1065 with high titers was further purified using Econo-Pac Serum IgG Purification Columns (Bio-Rad Laboratories, Hercules, Calif) and characterized by Western blot analysis. Recombinant SSP411 protein produced from full-length *ssp411* cDNA as described above was used as a positive control for analyzing the immunoreactivity of the newly generated antibody and for characterizing the SSP411 protein in the testis.

Western Blot Analysis

Proteins were extracted from various rat tissues, including testes, using CytoBuster protein extraction reagent (Novagen). An aliquot of 20 μ g of protein isolated from rat tissues was fractionated on a 10%–12% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Billerica, Mass). Antibody Ab1065 was used for detecting SSP411 protein in the testis at a dilution of 1:500 to 1:1000. After incubation with a secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ), the SSP411 protein on the Western blots was visualized using the enhanced chemiluminescence (ECL+plus) detection system (Amersham Pharmacia Biotech).

Tissue Fixation and In Situ Hybridization Analysis

Testicular tissues were collected from adult rats at 60–65 days of age, and paraffin sections were prepared using protocols described elsewhere (van Pelt et al, 1999; Pusch et al, 2000). Rats

were anesthetized with isoflurane (ABBOTT Laboratories, Abbott Park, Ill), and the testes were fixed by perfusion through the abdominal aorta with 4% paraformaldehyde (PFA). The testes were removed, further fixed in 4% PFA at 4°C overnight, dehydrated in graded ethanol, and embedded in paraffin. Paraffin sections of 7 μ m thickness were prepared for in situ hybridization analysis. After deproteination and acetylation, testicular sections were hybridized with antisense and sense RNA probes to detect *ssp411* mRNA in the testis. RNA probes were prepared with digoxigenin-UTP (DIG-UTP) by in vitro transcription with SP6 and T7 RNA polymerase using a DIG RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, Ind). A cDNA fragment containing the nucleotide sequence at 331–703 of the *ssp411* cDNA was used as a template to prepare antisense RNA for detection of the *ssp411* mRNA on testicular sections. The 331–703 fragment is contained within the 3 isolated cDNA clones and was not found in other genes. The primers used to prepare RNA probes from this DNA fragment were selected by computer analysis with PrimerSelect software (DNASStar). The sense RNA probe was also prepared as a negative control. Antisense and sense DIG-labeled *ssp411* RNA probes were applied onto testicular sections and incubated overnight at 55°C. The sections were then washed with high stringency buffer that contained 50% formamide and 2 \times SSC at 50°C for 30 minutes. The DIG-labeled RNA probe that bound to testicular sections was detected by color reaction with incubation of anti-DIG antibody overnight at 4°C, as described in the DIG Nucleic Acid Detection Kit (Roche). At least 10 testicular sections were evaluated for a group of stages.

Immunohistochemical Staining

Paraffin sections (7 μ m) were prepared from adult rat testes as described above and processed for immunostaining. After blocking with 10% normal goat serum, testicular sections were incubated with IgG-enriched antibody Ab1065 overnight at 4°C at a dilution of 1:100–1:1000. The sections were then incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Inc, Burlingame, Calif) at a dilution of 1:600 for 1 hour at room temperature. The visualization of positive signals was done using the Vectastain Elite Kit (Vector). As negative controls, antibody Ab1065 was preincubated with an excess of a synthetic oligopeptide of SSP411 at 681–697 for 2 hours at room temperature or overnight at 4°C before use in immunostaining. In general, 10–20 testicular sections were evaluated for each stage.

Results

Isolation and Characterization of *ssp411* cDNA From Adult Rat Testis

A series of degenerate oligonucleotides was synthesized from the regions in the SPE-11 protein that contained identical amino acid sequences that are shared among 3 species of nematodes, WKRYLRKWD and HYK-KWLEKK (Browning and Strome, 1996). Oligonucleotides prepared on the basis of these conserved peptides

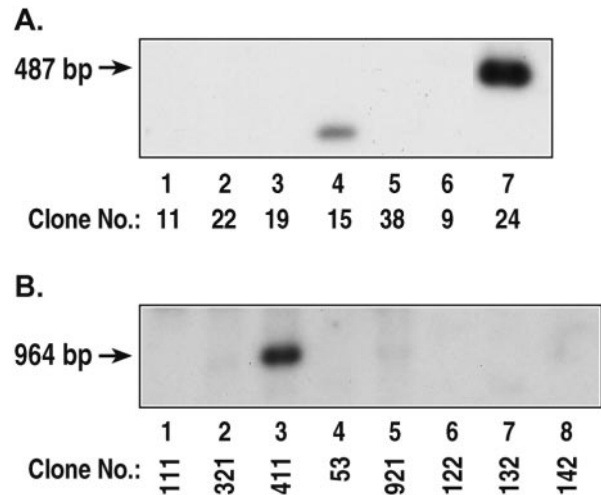


Figure 1. Southern blot analysis of clone 24 (A) and clone 411 (B) by hybridization with *C. elegans spe-11* cDNA. (A) The cDNA fragments of ~500 bp that were generated by RT-PCR from rat testicular RNA using degenerate oligonucleotides prepared from conserved amino acid sequences in *C. elegans* SPE-11 as primers. (B) The cDNA clones isolated from rat testicular cDNA library that showed positive hybridization with clone 24. The cDNAs shown in A and B were subjected to Southern blot analysis using *spe-11* cDNA as a hybridization probe. Both clone 24 and clone 411 showed strong positive hybridization signals with *spe-11*.

were used as forward and reverse primers, respectively, for the isolation of *spe-11*-related cDNAs by RT-PCR from 80-day-old rat testicular RNA. The RT-PCR-generated cDNA products of ~500 bp, as predicted from the *spe-11* cDNA sequence, were analyzed for their ability to hybridize the *C. elegans spe-11* cDNA. As shown in Figure 1A, clone 24 showed a strong positive hybridization signal to the *spe-11* cDNA; however, nucleotide sequence analysis revealed that clone 24 (487 bp in length; Figure 2A) did not contain the conserved amino acid sequences originally used for the preparation of degenerate nucleotides (indicated by underlining in Figure 3).

Because clone 24 demonstrated the strongest hybridization to the *spe-11* cDNA (Figure 1A), it was used as a hybridization probe for the isolation of longer cDNAs from a rat testicular cDNA library. Among the positive clones isolated from the cDNA library (Figure 1B), clone 411 (964 bp) showed the highest intensity of hybridization with clone 24. In addition, clone 411 hybridized to *C. elegans spe-11* cDNA (Figure 1B). This clone was thus further characterized by nucleotide sequence analysis. The positions of clones 24 and 411 corresponding to the full-length *ssp411* cDNA, which was generated by RACE-PCR, as described below, are indicated in Figure 2A.

The 5'- and 3'-RACE-PCR cloning method was used to generate 2 overlapping 5' and 3' *ssp411* cDNA fragments from adult rat testicular RNA (Figure 2). Using primers prepared from cDNA sequences obtained from clone 411 (Figure 2A), a 1.0-kb DNA fragment was gen-

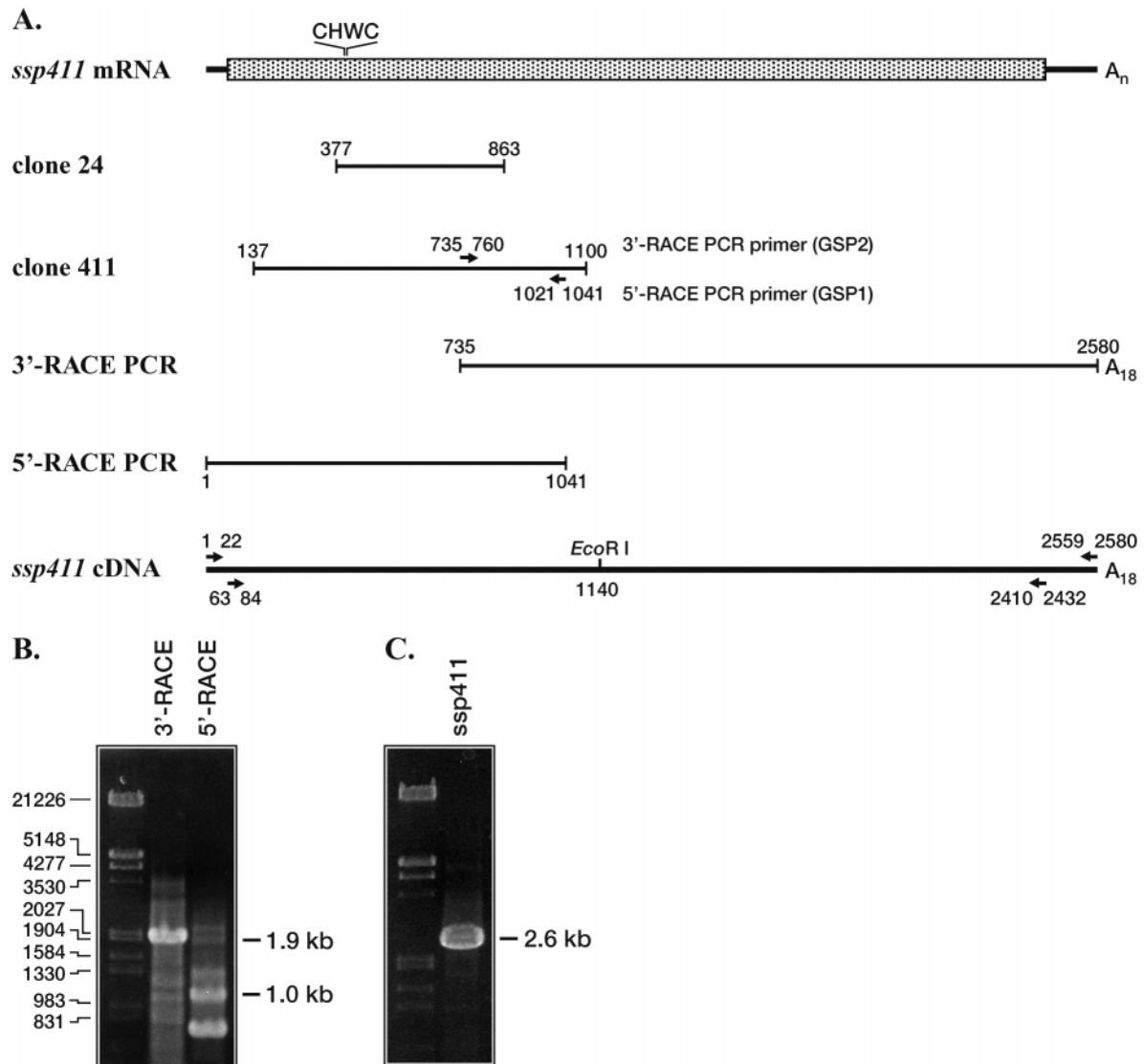


Figure 2. Isolation of a full-length cDNA coding for *ssp411* mRNA by RACE-PCR. The structure of *ssp411* mRNA is shown in **A**, the UTRs in lines and the coding region in stippled box. The thioredoxin-like domain, C-H-W-C, is indicated. The locations of clone 24 and clone 411 in the full-length *ssp411* cDNA are indicated. The primers used for 5' and 3' RACE-PCR and for RT-PCR analyses are also indicated in **A**, RACE-PCR-generated 5'- and 3'-end cDNA fragments are shown in **B**, and RT-PCR-generated 2.6-kb full-length *ssp411* cDNA is in **C**.

erated by 5' RACE-PCR that contained the 5' *ssp411* cDNA at positions 1–1046 nt, and a 1.9-kb DNA fragment was obtained by 3' RACE-PCR that contained the 3' *ssp411* cDNA from 735–2598 nt (Figure 2B). These RACE-PCR-generated overlapping DNA fragments were isolated and analyzed by nucleotide sequencing and used to prepare primers for the isolation of a 2.6-kb full-length *ssp411* cDNA from the rat testis (Figure 2C). An *EcoRI* restriction-enzyme site was detected at the full-length *ssp411* cDNA; both clone 24 and clone 411 are located within the 1.1-kb 5' *EcoRI* fragment (Figure 2A).

Analysis of the Newly Isolated Rat *ssp411* cDNA

The DNA sequence of a total of 2598 nt of the isolated rat *ssp411* cDNA was determined and scanned against the

database, and all sequences with significant relatedness to the new sequence were identified. Nucleotide and protein sequences of the *ssp411* cDNA were submitted to GenBank, and accession numbers AY438568 and AAR12892 were assigned, respectively. The *ssp411* cDNA consists of 62 bp of the 5'-untranslated region (UTR), 2367 bp of the coding region with an open-reading frame of 789 amino acids (Figure 3), and 151 bp of the 3' UTR (Figure 2A). The amino acid sequence of SSP411 protein can be accessed through the NCBI Protein Database under accession number AAR12892.

A computer analysis of amino acid sequences deduced from *ssp411* cDNA using ExPASy proteomics tools revealed that a putative N-linked glycosylation sequence,



Figure 3. Comparison of amino acid sequence derived from rat testicular *ssp411* cDNA with those from published uncharacterized mouse testicular cDNA (BC050788) and human hypothetical protein FLJ21347. The differences found in the 3 species are indicated in the shaded box. The thioredoxin-like domain, C-H-W-C, is shown in the open box. The amino acid sequences derived from mouse testicular cDNA (BC050788) and mouse TSIP78 (XM_354637) are identical. The amino acids that are identical to SSP411 and SPE-11, are indicated with an overlying *. The amino acid sequences corresponding to the regions used in preparation of degenerate oligonucleotides from SPE-11 for the isolation of clone 24 are underlined. The arrows indicate the location of clone 411 in SSP411.

Asn-Leu-Ser, at amino acids 374–377, 3 tyrosine kinase phosphorylation sites at 67–75, 147–154, and 397–404, and many potential phosphorylation sites for protein kinase C and casein kinase II had been identified. In addition to the N-linked glycosylation site, 4 potential O-linked glycosylation sites were observed at amino acids 5 and 176 for serine and 54 and 245 for threonine, as analyzed by using the NetNGlyc 1.0 and NetOGlyc 2.0 servers of the Center for Biological Sequence Analysis.

Sequence Similarity With a Human Hypothetical Protein FLJ21347 and Mouse TISP78

Although clone 411 (Figure 1B) hybridized with *spe-11* cDNA from *C. elegans* (Browning and Strome, 1996), the SSP411 and SPE-11 proteins shared only 21% identity in their amino acid sequences. The amino acid sequences that are identical to both SPE-11 and clone 411 or SSP411 were indicated in Figure 3; these were mainly located at the 1.1-kb 5' *EcoRI* DNA fragment (Figure 2A). Of interest, compared with the published draft hu-

man genome sequences from the NCBI, the *ssp411* cDNA shares 85% and 87% nucleotide and protein sequence identity, respectively, with a 2.6-kb cDNA that encoded a new, unnamed human hypothetical protein, FLJ21347 (GenBank accession number AK025000; Figure 3). In addition, human FLJ21347 cDNAs with varied lengths of the 5' end were identified in several human cancer/tumor cell lines, including pancreas epithelia carcinoma (AAH25255), hepatoma HepG2 (AK025622), and uterus leiomyosarcoma (BC017468).

The *ssp411* cDNA also shared 92% and 93% identity at the nucleotide and protein levels, respectively, with several uncharacterized cDNAs isolated from mouse testes, the 488-bp transcript increased in spermiogenesis 78 (TISP78) cDNA (AB045721), a 1986-bp cDNA (BC050807), and a 2705-bp cDNA (BC050788) (Figure 3). In addition, the nucleotide sequence of a 2589-bp TISP78 cDNA (XM_354637) was predicted by automated computational analysis of mouse chromosome 11. All of these uncharacterized mouse cDNAs shared 99% se-

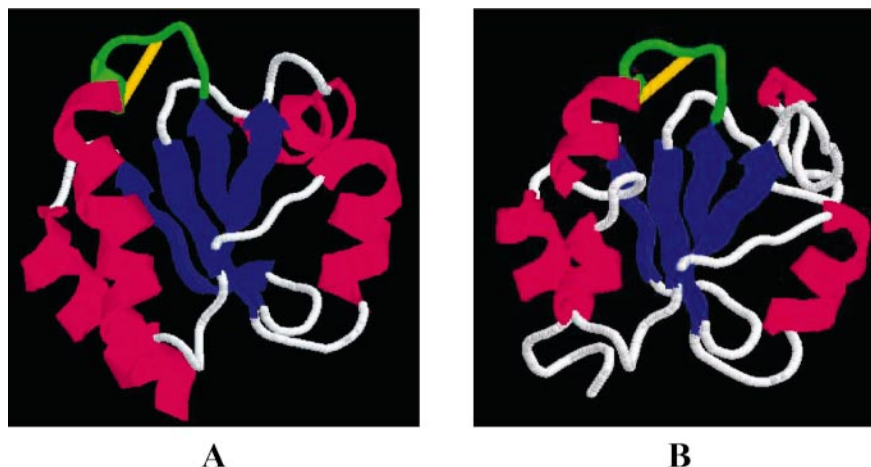


Figure 4. Comparison of the 3-dimensional structures between one typical thioredoxin domain (A) and the N-terminal region of SSP411 (1–357) (B) predicted by the PLOP program. The orange rod denotes a disulfide bond; green, residues involved in the active site; blue, β -sheets; red, α -helices; and white, loops between sheets and helices. The figure was drawn using the Protein Explorer Program.

quence identity with each other, which suggests that these cDNAs are derived from the same mouse gene. In addition, they showed 92%–93% identity with rat *ssp411*.

The comparison of amino acid sequences obtained from rat testicular SSP411, human FLJ21347 (AK025000), and mouse testis protein TISP78 (BC050788) is presented in Figure 3. The *ssp411* gene was mapped to chromosome 10 in rats, chromosome 17 in humans, and chromosome 11 in C57Bl/6 mice. All 3 genes contain 16 exons and 15 introns in a span of 8.6, 6.8, and 6.5 kb in human, rat, and mouse, respectively. We therefore suggest that FLJ21347 and TISP78 are the human and mouse orthologs, respectively, of the rat *ssp411* gene.

Analysis of a Thioredoxin-Like Domain in SSP411

A computer search for conserved domains revealed that SSP411 contained 2 cysteine residues in C-H-W-C located at amino acids 113–116. The C-X-X-C motif was shown to be the conserved domain for thioredoxin proteins and a potential active site for the reduction of proteins from their oxidized form (Holmgren, 1985, 1989; Whiteley et al, 1997; Arner and Holmgren, 2000; Shigenobu et al, 2000). The identified thioredoxin-like domain in SSP411 protein was conserved in the homologs, human protein FLJ21347 and mouse testicular protein TSIP78 (BC050788) (Figure 3). The C-H-W-C motif or other thioredoxin-like domain were not detected in *C. elegans* SPE-11.

To investigate the potential function of the thioredoxin-like domain in SSP411 protein at the secondary structure, the amino acid sequence of the SSP411 protein was submitted to the 3D-PSSM server (available at: <http://www.sbg.bio.ic.ac.uk/~3dpssm/>) for structural analysis.

A thioredoxin fold was identified at the N-terminal region of the SSP411 protein. A model for the 3-dimensional structure of N-terminal SSP411 was next built, using the PLOP program (Rapps and Friesner, 1999). Through the Protein Explorer Program, the 3-dimensional structure of the N-terminal SSP411 was compared with that of a typical thioredoxin protein, BacTrx (Protein Data Bank code, 1quw, 105 residues), which has a known tertiary crystallographic structure and function (Martin, 1995; Nicastro et al, 2000) (Figure 4). Like other members of the thioredoxin family, a typical thioredoxin protein has a common fold, the thioredoxin fold, which consists of central β -sheets (blue) flanked by α -helices (red) and an active site (green) with a catalytic disulfide bond (orange) between the sequence C-X-X-C (Eklund et al, 1991; Nicastro et al, 2000) (Figure 4A). In the predicted 3-dimensional structure of the SSP411 protein (Figure 4B), a similar thioredoxin fold was clearly identified at amino acids 85–202, and the potential active site with a disulfide bond was detected at amino acid 113–116, which contains a C-H-W-C thioredoxin-like domain.

The high conservation of the thioredoxin fold in SSP411 was also confirmed by the results obtained from protein structure alignment between these 2 structures using the ProSup program (Lackner et al, 2000). The ProSup program is a refined and frequently used tool for 3-dimensional protein structure comparison and alignment. It evaluates structure similarity among proteins, primarily by maximizing the number of structurally equivalent residues (Lackner et al, 2000) of 2 proteins while maintaining a certain RMSD cutoff such as 2 Å (Lackner et al, 2000). Between the typical thioredoxin fold and the protein fold identified in the N-terminal SSP411, 101 structurally equivalent residues were found, which ac-

counted for 96% of the residues (101 of 105) in the typical thioredoxin fold (>50% is significant). The RMSD of structurally equivalent residues between these 2 protein structures was 0.44 Å. These results demonstrate extensive structural similarity and fold conservation between SSP411 and a typical thioredoxin protein. Aligned with another thioredoxin-like protein from human, hTRXL (PDB code: 1gh2, 107 residues; Jin et al, 2002), 98 equivalent residues were detected by ProSup program with an RMSD of 1.72 Å. Therefore, the results obtained from structural modeling strongly suggest that SSP411 belongs to the thioredoxin family.

Testis-Specific Expression of the *ssp411* Gene

Primers containing nucleotide sequence at the positions of 63–84 and 2410–2432, as described in Figure 2A, were used for the analysis of *ssp411* mRNA in 63-day-old rats by semiquantitative RT-PCR. Among 11 tissues examined (including all of the male reproductive accessory organs), a single band of 2.4 kb *ssp411* cDNA was detected at high levels in the testis, and a faint band was occasionally found in the epididymis (Figure 5Aa). No other tissues tested expressed the *ssp411* gene. The β -actin mRNA level in each RNA sample was also measured by RT-PCR (Figure 5Ab), to ensure that the same amount of RNA was loaded for comparisons between sample lanes.

The tissue specificity of *ssp411* gene expression in adult rats was also examined by Northern blot analysis, using clone 411 cDNA as a hybridization probe (Figure 5B). A single species of 2.8-kb *ssp411* mRNA was identified exclusively in the rat testis and was absent in other tissues of adult male rats and in the ovaries of immature and adult females (Figure 5B). Identical observations were obtained when another *ssp411* cDNA fragment, such as the 1.5-kb 3'-*Eco*RI fragment shown in Figure 2A, was used for hybridization.

Age-Dependent Expression of the *ssp411* Gene

We studied the expression pattern of the *ssp411* mRNA in developing rat testes, using semiquantitative RT-PCR and Northern blot analyses (Figure 6). Total RNA was isolated from testes of different ages ranging from 7 to 90 days. The *ssp411* cDNA generated by RT-PCR was first evident at 28 days and remained at high levels throughout adulthood (Figure 6Aa). The levels of β -actin mRNA in the RNA samples prepared from different-aged animals were unchanged (Figure 6Ab), which confirmed that the expression of *ssp411* gene in the testis was age dependent. Similarly, the results of Northern blot analysis (Figure 6B) showed that *ssp411* mRNA was first detected at age 28 days and was most abundant at age \geq 63 days. The age-dependent expression of *ssp411* mRNA was also studied in mouse testes. A 2.8-kb mRNA that hybridized to clone 411 was detected in 28- and 75-day-old mouse

testes but was not observed at 21 days (data not shown). The developmental profile of *ssp411* mRNA expression in adult rat testes, with no expression at age \leq 21 days, suggested that this gene is expressed in spermatids and not in spermatocytes or spermatogonia of the germ cells.

Characterization of the SSP411 Protein in Rat Testicular Extracts

To characterize the protein coded by the isolated *ssp411* cDNA, a recombinant SSP411 protein was produced as a histidine-tagged protein by subcloning the *ssp411* cDNA into pET-28a(+) bacterial expression plasmid (Studier et al, 1990; Apezteguia et al, 1994). SDS-polyacrylamide gel electrophoresis analysis confirmed that an 89-kD protein, which contained a 1-kD histidine tag protein at the N terminus, was detected in the IPTG-treated bacterial culture. The purified 89-kD SSP411 recombinant protein was used as a positive control for the analysis of the SSP411 protein expressed in rat testis (Figure 7).

To identify the protein(s) derived from the newly identified *ssp411* mRNA in the testis, we generated a polyclonal antibody against a 17-mer oligopeptide derived from the C-terminal region of the SSP411 protein. The antibody, Ab1065, was shown to immunoreact with the 89-kD SSP411 recombinant protein produced in *E. coli* (Figure 7, lane 1). To examine further the specificity of the newly raised antibody, protein extracts prepared from various tissues—including testis, ovary, liver, colon, and heart—of 63-day-old rats were subjected to Western blot analysis using antibody Ab1065 for detection. Only testis contained an 88-kD protein that immunoreacted with antibody Ab1065 (Figure 7, lane 2). Because the theoretical molecular weight of SSP411 was calculated to be 88190.97, the 88-kD protein identified in the testis by Western blot analysis is a full-length SSP411 protein.

Localization of the *ssp411* mRNA in Rat Spermatids

In situ hybridization was performed to determine the testicular cell types that express *ssp411* mRNA (Figure 8A). DIG-labeled antisense and sense RNA probes were prepared using a cDNA fragment that contained nucleotides at 331–703 of the *ssp411* cDNA as a template. Paraffin sections of adult rat testes were hybridized either with an RNA probe that contained the antisense sequence of the *ssp411* mRNA (Figure 8Aa–c) or with a sense probe as a negative control (Figure 8Ad). Hybridization with the antisense probe demonstrated strong signals in the seminiferous epithelium. Intense staining for *ssp411* mRNA was observed predominantly in round spermatids located in the inner half-layer of the seminiferous epithelium (tubule a, Figure 8Ab–c) as well as in early elongated spermatids whose cytoplasm protruded into tubular lumen (tubule b, Figure 8Ab–c). Hybridization with a sense probe

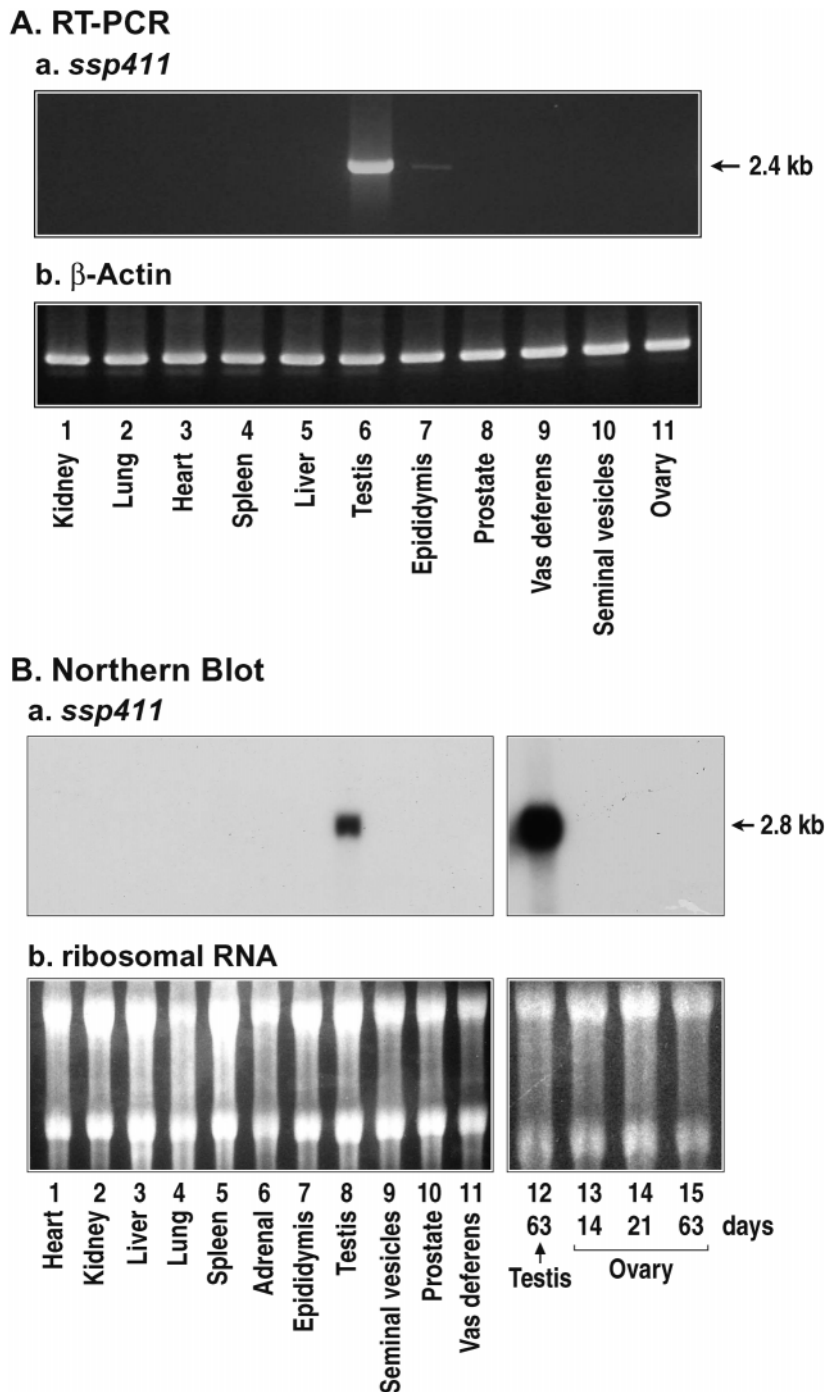


Figure 5. Testis-specific expression of the *ssp411* mRNA in adult rats. (A) Total RNA (2 μ g each) isolated from various tissues of adult rats was analyzed by RT-PCR for the presence of *ssp411* mRNA (a) using primers described in Figure 1A and β -actin mRNA (b) to monitor RNA loading. (B) total RNA (20 μ g each) isolated from various tissues of 63-day-old male rats (lanes 1–11) and ovaries of different ages (lanes 13–15) were subjected to Northern blot analysis of *ssp411* mRNA using clone 411 as a hybridization probe (a) and RNA loading by ethidium bromide staining (b).

for *ssp411* mRNA was negative (Figure 8Ad), which confirmed the specificity for the *ssp411* cDNA sequence.

The intensity of the hybridization signals varied markedly between the seminiferous tubules (Figure 8Aa), which suggests the stage-specific expression of

*ssp411*mRNA during the cycle of the seminiferous epithelium. The strongest hybridization was observed in round spermatids at stages VII–VIII and in elongated spermatids at stages IX–XI (Figure 8Ab–c). Weak hybridization signals were detected in round spermatids at

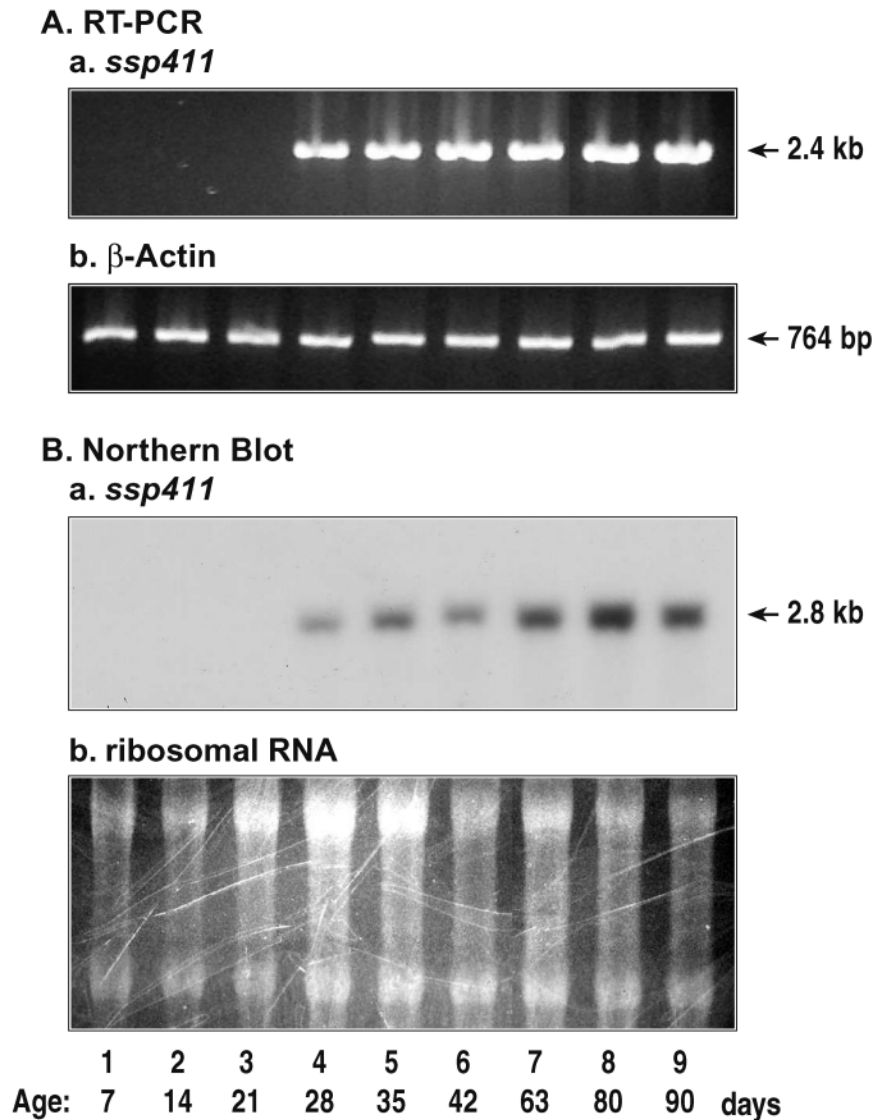


Figure 6. Age-dependent expression of the *ssp411* mRNA in rat testes examined by RT-PCR (A) and by Northern blot analysis (B). (A) Total RNA (2 μ g each) isolated from different-aged male rats was analyzed for the presence of *ssp411* (a) and β -actin (b) mRNA by RT-PCR, as described above. (B) total RNA (20 μ g each) isolated from male rats at age 7–90 days was subjected to Northern blot analysis of *ssp411* mRNA (a) and ethidium bromide staining of RNA samples (b).

stages II–VI and in elongated spermatids at stage XII. These data indicate that *ssp411* mRNA is expressed in the spermatids at specific stages of the cycle in the seminiferous epithelium.

Localization of SSP411 Protein in Adult Rat Testes

We next localized the SSP411 protein in the rat testis by immunohistochemistry using antibody Ab1065 for detection (Figure 8B). Strong immunostaining was mainly localized to elongated spermatids (Figure 8Ba, c, and d). No staining was detected when preimmune serum was used (data not shown). These observations suggest that the *ssp411* mRNA that is transcribed in round spermatids might not be translated into protein until later stages of

spermatogenesis, in elongated spermatids. The immunostaining for SSP411 protein on testicular sections was further confirmed by peptide neutralization (Figure 8Bb). No staining was detected if antiserum was preincubated with 5- to 8-fold excess of synthetic oligopeptide at 681–697.

Similar to those observed in *ssp411* mRNA by in situ hybridization (Figure 8A), varied intensities of immunostaining were observed in different regions of the seminiferous tubules (tubule a, Figure 8B). Maximal immunostaining of SSP411 was observed at stages V–VI (tubule a, Figure 8Bc). Strong staining was also observed in elongated spermatids at stages VII–VIII (tubule b, Figure 8Bd), with weak signals detected in some residual bodies at stage VIII. Immunostaining for the SSP411 protein was

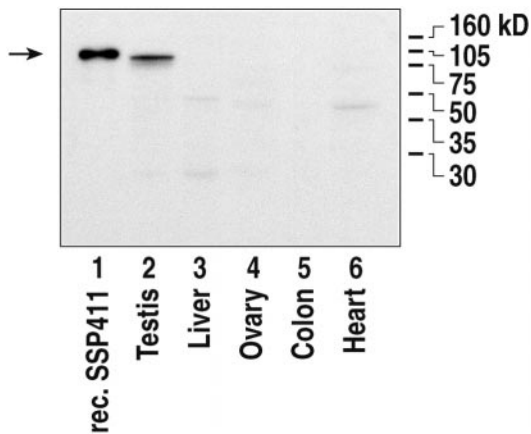


Figure 7. Western blot analyses of SSP411 protein in various tissues of 63-day-old rats, using newly generated antibody Ab1065 for detection. Bacterially expressed recombinant SSP411 protein (6.5 μ g) is shown in lane 1. Protein extracts prepared from testis, liver, ovary, colon, and heart (20 μ g each) (lane 2–6) of adult rats were subjected to Western blot analysis. The 89-kD SSP411 recombinant protein (indicated by an arrow) and the 88-kD SSP411 protein in the testis were shown to immunoreact with antibody Ab1065.

decreased to undetectable levels at stages IX–XI (tubule c, Figure 8Be). Weak immunostaining of the SSP411 protein started to appear again at stages XII–I (tubule d, Figure 8Ba).

The observations shown in Figure 8A B indicate that the transcription and translation of the *ssp411* mRNA and SSP411 protein probably occur at different stages in the cycle of the seminiferous epithelium. The transcription of *ssp411* gene was most active in both round and elongated spermatids at stages VII–VIII and IX–XII, respectively, and declined after stage XII. On the other hand, the translation of *ssp411* mRNA into SSP411 protein in the elongated spermatids was not initiated until stages XII–I, and it continued to increase, reaching maximal levels at stages V and VI. Our observations thus suggested that the *ssp411* mRNA transcribed at stages VII–XII might be stored until translational activation occurred at stages XII–I, similar to those reported for protamines (Kleene et al, 1984; Zhong et al, 2001; Giorgini et al, 2002).

Discussion

We isolated a novel *ssp411* cDNA that encodes a 2.8-kb mRNA and an 88-kD protein in adult rat testes. The *ssp411* cDNA shares >85% nucleotide and protein sequence identity with a human hypothetical protein, FLJ21347 (AK025000), and an uncharacterized mouse testicular protein, TISP78 (BC050788). All 3 genes contain 16 exons and 15 introns. We therefore suggest that the human FLJ21347 and mouse testicular protein TISP78 are the SSP411 homologs in human and mouse, respec-

tively. The expression of human FLJ21347 and mouse TISP78 in the testis has not been investigated, except that a 488-bp TISP78 cDNA was identified as a transcript that increased during spermiogenesis in mouse testes (AB045721). Our data indicate that, similar to those observed in the rat *ssp411* gene, the mouse *ssp411* gene is also expressed in an age-dependent manner in the testis, with its highest expression in adulthood. Although all 3 proteins contain a thioredoxin-like domain, their functions remain to be determined.

The *ssp411* cDNA was isolated on the basis of its hybridization to the *C. elegans spe-11* cDNA. The expression of *ssp411* mRNA and protein in the rat testis are similar in pattern to *C. elegans spe-11* (Browning and Strome, 1996): both are expressed in a testis- and stage-specific manner and are predominantly localized to spermatids. The SSP411 protein, however, shares only 21% amino acid sequence identity with that of SPE-11 in *C. elegans*. Of interest, the SPE-11 observed in 3 *Caenorhabditis* species, *elegans*, *briggsae*, and *vulgaris*, also had a low protein sequence identity of 55%. Although 60% of the amino acids in SSP411 that shared identity with SPE-11 in *C. elegans* also showed identity with those in 2 other nematodes, we infer that the newly isolated rat testicular SSP411 is not likely to be a mammalian SPE-11 ortholog. In view of the fact that HCH-1, which is a BMP-1-related protein in *C. elegans* (NP_510440), shares 20.7% identity in amino acid sequence with rat BMP-3 (NP_058801), we thus suggest that SSP411 is a SPE-11-related protein rather than an ortholog.

The transcriptional and translational profiles, as analyzed by in situ hybridization and immunohistochemistry, respectively, were obtained from observations of at least 10 testicular sections for each stage. We showed that both *ssp411* mRNA and the SSP411 protein were present in testicular germ cells in a cell- and stage-specific manner. The *ssp411* mRNA is expressed predominantly in round and elongated spermatids at specific stages of the cycle in the seminiferous epithelium of the rat testis. Weak expression of the *ssp411* mRNA was first detected in round spermatids at stages II–VI. The expression of the *ssp411* mRNA gradually increased and reached highest levels at stages VII and VIII in round spermatids and stages IX–XI in early elongated spermatids. The *ssp411* mRNA levels in elongated spermatids declined after stage XII. Immunoreactive SSP411 protein was, however, localized mainly to the elongated spermatids and not in round spermatids, which suggests that the production of SSP411 protein occurred during a later stage of spermatogenesis. Weak immunostaining of SSP411 protein started to appear at stages XII–I. The staining intensity gradually increased through stages II–IV, reached maximal levels at stages V–VI, and continued to stay at high levels at stages VII and VIII. SSP411 was not detectable at stages IX–

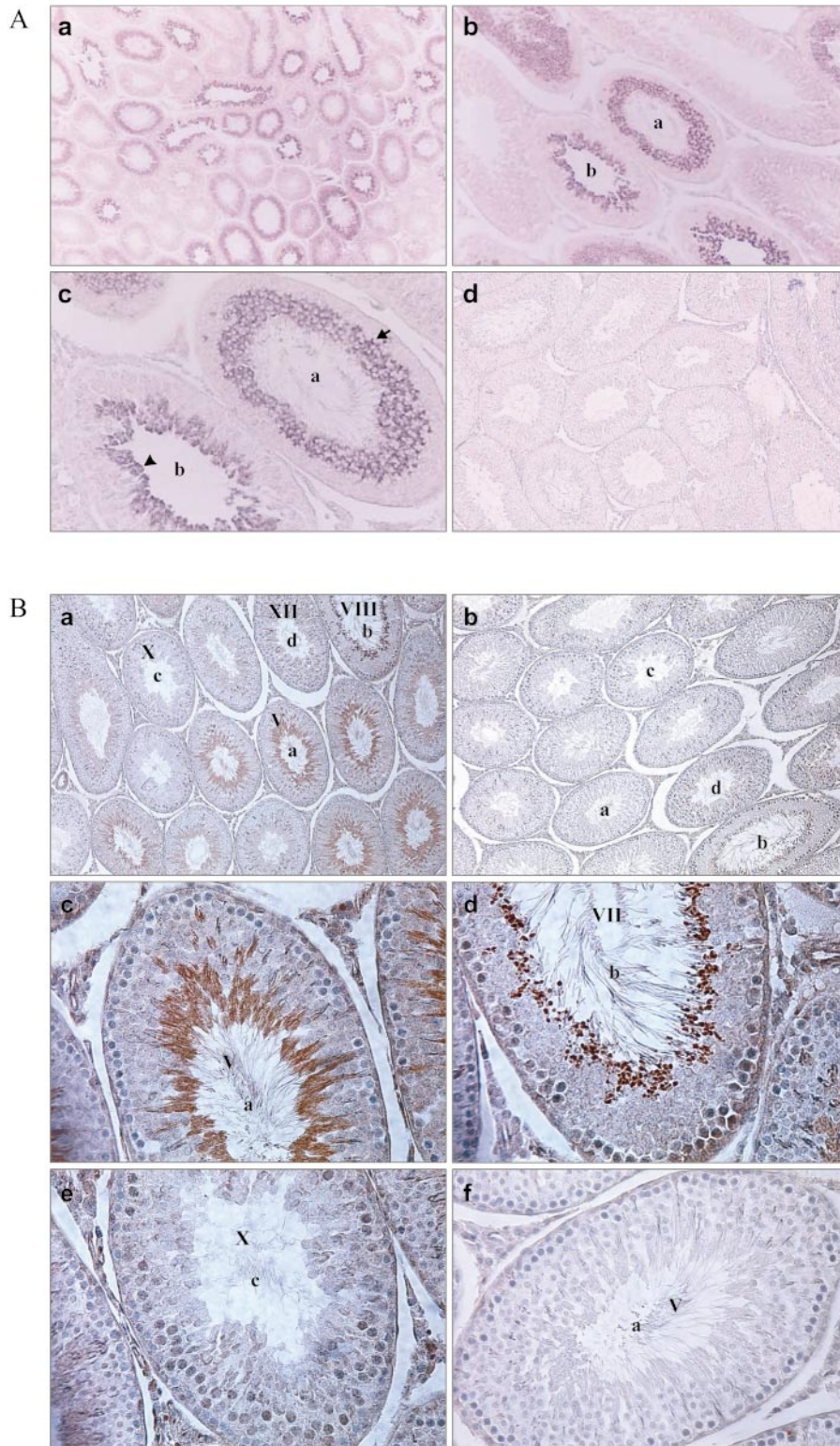


Figure 8. **(A)** In situ localization of the *ssp411* mRNA in the adult rat testis. Paraffin sections obtained from 60-day-old rat testes were hybridized with digoxigenin-labeled cRNA probe (a-c) or with a sense probe (d). No signal was detected after hybridization with the sense probe (d). Hybridization with the antisense probe demonstrated strong signals in the seminiferous epithelium in a stage-specific manner (a-b). At higher magnification (c), *ssp411* mRNA was found in round spermatids (arrow) at stages VII–VIII, located in the inner half-layer of the seminiferous epithelium (tubule a) and in early elongated spermatids (arrowhead) at stages IX–XI, having cytoplasmic protrusions into the tubular lumen (tubule b). Ten testicular sections were analyzed for each group of stages. Magnification, 40 \times in a, 100 \times in b and d, and 200 \times in c. **(B)** Localization of SSP411 protein in adult rat testes by immunohistochemistry. IgG-enriched antibody Ab1065 (1:200 dilution) (a, c–e) and serum preabsorbed with synthetic peptide (b, f) were

XI, after spermiation. Our observations suggest that the transcription and translation of the *ssp411* mRNA and SSP411 protein occur at different stages in the cycle of the seminiferous epithelium.

The posttranscriptional regulation of sperm protein production has been shown to play a role in gametogenesis. In haploid spermatids, the proteins required for morphogenesis are synthesized from the stored mRNAs that are packaged as ribonucleoprotein particles (mRNPs; Giorgini et al, 2002). For instance, the protamine genes were first transcribed in haploid round spermatids, and the protamine mRNAs were then stored in translationally inert mRNPs for up to 10 days, until translational activation in elongated spermatids (Kleene et al, 1984; Zhong et al, 2001; Giorgini et al, 2002). Similar observations have also been reported for sperm fibrous sheath (FS) protein genes, including spermatid-specific thioredoxin-2, Sptrx-2 (Miranda-Vizuete et al, 2003), FS39 (El-Alfy et al, 1999), and FS75 (Catalano et al, 2001). The FS mRNAs are expressed in round spermatids, stored for several days, and translated in elongating spermatids after the end of transcription, which occurs during nuclear condensation (Padma et al, 2001). As with protamines (Kleene et al, 1984; Zhong et al, 2001; Giorgini et al, 2002) and FS proteins (El-Alfy et al, 1999; Catalano et al, 2001; Miranda-Vizuete et al, 2003), *ssp411* mRNA transcribed at stages VII–XII might be stored in translationally inert mRNP particles until translational activation at stages XII–I.

A low level of *ssp411* mRNA was detected in the epididymis by RT-PCR but not by Northern blot analysis. Whether *ssp411* mRNA and SSP411 protein are present in the epididymis is currently under investigation by using in situ hybridization and immunocytochemistry, respectively. In addition, the nature of the SSP411 protein in the epididymis and mature sperm is also being characterized.

SSP411 contains a thioredoxin-like domain that consists of 2 cysteine residues in C-H-W-C at amino acids 113–116. This sequence was also observed in human protein FLJ21347 and mouse testicular protein BC050788. In addition to the highly conserved sequence W-C-G-P-C (Holmgren, 1985, 1989) in thioredoxin, various thioredoxin-like domains containing a C-X-X-C motif were identified. For instance, C-G-H-C in the protein disulfide isomerase (PDI; Whiteley et al, 1997), C-P-P-C in thiol: disulfide isomerase, and C-V-Y-C and C-P-Y-C in thiol:

disulfide interchange protein (Shigenobu et al, 2000) have been demonstrated to exhibit thioredoxin activity. PDI, which contains 2 regions that exhibit sequence homology to thioredoxin (Edman et al, 1985), has been shown to be a substrate for thioredoxin reductase, which indicates that the C-G-H-C domains in PDI are folded and recognized as thioredoxins (Lundstrom and Holmgren, 1990). The SSP411 protein contains a thioredoxin-like domain C-H-W-C at amino acids 113–116. A comparison of the predicted 3-dimensional structure of the SSP411 protein and a known thioredoxin protein suggested that SSP411 is also folded and recognized as a member of thioredoxin family.

Two spermatid-specific thioredoxin proteins, Sptrx-1 and Sptrx-2, identified in human and murine sperm have been shown to be predominantly expressed in round and elongated spermatids, and their proteins were localized to the tail of spermatozoa (Miranda-Vizuete et al, 2001, 2003; Sadek et al, 2001; Jimenez et al, 2002b). Sptrx-1 and Sptrx-2 incorporate to the sperm tail during different steps of sperm maturation. Sptrx-1 transiently associates to the FS during sperm tail assembly but does not remain as a permanent component of the FS in the mature sperm (Yu et al, 2002). In contrast, Sptrx-2 incorporates into the FS during the last steps of spermatid development and remains as an integral FS component in mature epididymal spermatozoa (Miranda-Vizuete et al, 2003). Another thioredoxin protein found in spermatids, thioredoxin-like 2 (Tx1-2) is apparent at the spermatid nucleus and tail during the spermatid elongation phase (Sadek et al, 2003). Further studies using immunofluorescence microscopy and confocal microscopy will be used to determine whether SSP411 protein is localized to the sperm nucleus, as are Tr1-2 (Sadek et al, 2003) and *C. elegans* SPE-11 (Browning and Strome, 1996), and/or in the tail of mature sperm, as are Sptrx-1 and Sptrx-2 (Miranda-Vizuete et al, 2001, 2003; Sadek et al, 2001; Jimenez et al, 2002a,b) and Tr1-2 (Sadek et al, 2003).

The number of functions assigned to the different thioredoxins is increasing as new members of the family are discovered. Thioredoxins catalyze the reduction of protein disulfide bonds and are involved in various biological functions, including posttranslational modification, protein turnover, chaperones, cell growth and differentiation, and the immune response (Holmgren and Bjornstedt, 1995; Arner and Holmgren, 2000; Tanaka et al, 2000; Sadek et al, 2003). In addition, thioredoxins serve as elec-

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applied onto testicular sections for the localization of the SSP411 protein. Intense staining was detected mainly in elongated spermatids. Maximal immunostaining was observed at stages V–VI (c, tubule a); strong staining was also observed at stages VII–VIII. (d, tubule b). Immunostaining was found in some residual bodies. The SSP411 could not be detected at stages IX–XI (e, tubule c). Weak staining reappeared in the tubules at stage XII (a, tubule d) and continued to increase and reach highest levels at stages V–VI (c). In general, 10–20 testicular sections were examined for each stage. Magnification, 100× in a–b and 400× in c–f.

tron donors for essential enzymes such as ribonucleotide reductase, regulators of transcription factor DNA binding activity, modulators of apoptosis, antioxidant defense, and participants in the regulation of the protein folding process (for reviews, see Arner and Holmgren, 2000; Nordberg and Arner, 2001; Powis and Montfort, 2001). The 3 thioredoxin proteins—Sptrx-1, Sptrx-2, and Tx1-2—found in the sperm tail have been suggested to play a role in the regulation of flagellar movement (Yu et al, 2002; Miranda-Vizuete et al, 2003; Sadek et al, 2003), and Tx1-2 has been shown to have microtubule-binding activity (Sadek et al, 2003). A thioredoxin homolog in *Drosophila* has been demonstrated to be involved in female meiosis and early embryo development (Salz et al, 1994). Moreover, a targeted disruption of the mouse thioredoxin (*Trx*) gene was lethal for the embryo; homozygous mouse embryos die immediately after implantation. The early lethality was caused by impaired DNA replication resulting from thioredoxin depletion (Matsui et al, 1996). The addition of thioredoxin to human sperm incubation media before in vitro fertilization increased the rate of blastocyst formation during early embryo development (Kuribayashi and Gagnon, 1996). A time-dependent increase in sperm membrane sulfhydryl groups exposed to the extracellular space was observed during the first hour of capacitation, which indicates that an important rearrangement of sulfhydryl-containing proteins occurs during the initiation of capacitation (de Lamirande and Gagnon, 1998, 2003). The protein sulfhydryl-disulfide status might be important for the regulation of human sperm capacitation and early embryo development (Kuribayashi and Gagnon, 1996; de Lamirande and Gagnon, 1998, 2003).

In summary, we have isolated a novel spermatid-expressed gene, *ssp411*, in rats, and we have identified 2 ortholog proteins in human and mouse. We also demonstrated that the expression of *ssp411* mRNA and protein in the testis is tissue specific and age dependent. Moreover, SSP411 was present in a stage-dependent manner during the cycle of the seminiferous tubules. Because the proteins involved in fertilization are expressed during late stages of spermatogenesis, the expression of the SSP411 protein in late elongated spermatids is indicative of a function in fertility regulation. From our analysis of the SSP411 protein, we conclude that this protein is a member of a new spermatid-specific thioredoxin family.

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