Biochemical and Biophysical Research Communications **283,** 86–92 (2001)
doi:10.1006/bbrc.2001.4754, available online at http://www.idealibrary.com on IDEAL®

Evidence for FSH-Dependent Upregulation of SPATA2 (Spermatogenesis-Associated Protein 2)[1]

Onisto Maurizio,*[,2,3] Slongo M. Liliana,[2] Graziotto Romina,* Zotti Lorenza,* Negro Alessandro,† Merico Maurizio,‡ Moro Enrico,‡ and Foresta Carlo‡

**Department of Experimental Biomedical Sciences, †Department of Biochemistry, and ‡Department of Surgical and Medical Sciences, University of Padova, 35121 Padua, Italy*

Received March 22, 2001

Here we report the cloning and characterization of a novel cDNA named *spata* 2. SPATA2 is the ortholog of PD1, a human testicular protein which has been suggested to play a role in spermatogenesis. The *spata* 2 sequence reveals an open reading frame encoding a protein of 511 amino acids. Northern blot analysis with rat mRNA demonstrated two distinct transcripts of 2.2 and 4.0 kb. Tagging recombinant SPATA2 with the green fluorescent protein (GFP) and expressing the chimeric polypeptide in HLtat transfected cells indicated that SPATA2 is located in the nucleus. RT-PCR analysis revealed that *spata* 2 mRNA is expressed in the testis and to a lesser extent in the brain while skeletal muscle and kidney showed a barely visible signal. The same analysis demonstrated that isolated Sertoli cells express *spata* 2 mRNA. Treating Sertoli cells with FSH *in vitro* induced remarkable changes in the steady-state level of *spata* 2 mRNA in a time-dependent manner. In developing testis *spata* 2 transcripts were first detected 10 days post partum and expression levels increased steadily with age. The ability of FSH to stimulate *spata* 2 mRNA expression as well as its developmental expression suggests that this protein might play a role in regulating spermatogenesis and thus, according to the Gene Nomenclature Committee, we propose the name SPATA2 (Spermatogenesis associated protein 2) for this protein (or gene).

Key Words: **SPATA2; spermatogenesis; Sertoli cells; FSH; testis.**

The *pd* 1 gene sequence was isolated from human testis cDNA library and encodes a protein of 520 amino acids which has been proposed to play a role in human spermatogenesis (1).

Northern blot analysis for *pd* 1 mRNA from several human tissues demonstrated two distinct transcripts of 2.7 and 4.0 kb and revealed that PD1 protein is expressed moderately in testis and to a lesser extent also in the spleen, thymus, and prostate. The same sequence was also isolated from a human brain cDNA library and an mRNA expression level similar to that of testis was revealed in this tissue (2). Using the "radiation hybrids" technique, the human *pd* 1 gene has been mapped at 20q13.13, a chromosomal region where other spermatogenesis-associated genes have been recently localized (3, 4).

An immunohistochemical analysis of human testis showed that this protein is present in the Sertoli cells, but no reactivity was observed in any spermatogenic cells at any stage of maturity.

Western blot analysis detected the presence of a 60 kDa molecule in a crude extract of human testicular cells and showed different patterns of expression in various testiculopathies, demonstrating that PD1 is not only tissue-specific but it is also produced by Sertoli cells probably under the influence of spermatogenic cells (1).

To clarify further the role of this gene in the regulation of spermatogenesis we isolated and cloned the rat ortholog of *pd* 1, *spata* 2. In the present study we report a complete analysis of this new gene and on the basis of its expression pattern, stimulation by FSH and increase in expression during testicular development, we propose that *spata* 2 may be involved in the control of spermatogenesis.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats were used to isolate Sertoli cells and to obtain tissues for RNA extraction. Animals were housed and fed in accordance with guidelines for animal care of University of Padova and were killed humanely by asphyxiation with CO_2.

[1] The financial support of Telethon-Italy (Grant No. E.1065) and MURST (Italy) 1999 are gratefully acknowledged.

[2] These authors contributed equally.

[3] To whom correspondence should be addressed at Dipartimento di Scienze Biomediche Sperimentali, Viale G. Colombo 3, 35121 Padova, Italy. Fax: 0039 049 8276097. E-mail: onisto@civ.bio.unipd.it.

0006-291X/01 $35.00

Isolation of Sertoli cells and FSH stimulation. Sertoli cells were isolated from the testis of five 13 day-old Sprague–Dawley rats by the procedure of Scarpino *et al.* (5). The isolated Sertoli cells were plated at a concentration of 3.5×10^4 per well and cultured in Dulbecco's modified Eagle medium (Sigma, Milan, Italy) supplemented with glutamine (2 mM), Hepes (15 mM), nonessential amino acids, EGF (100 ng/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (40 μg/ml), and in the presence of hormones (2 μg/ml insulin, 10^{-9} M hydrocortisone, 10^{-7} M testosterone) at 32°C in a humidified atmosphere of 5% CO_2 in air. In duplicate time-course experiments, Sertoli cells were incubated for 1, 4, and 24 h in the presence of 100 ng/ml of FSH (Sigma). The purity of the Sertoli cells was assessed by measuring the synthesis and the release of 17-β Estradiol into the culture medium.

RNA isolation. Tissues or cells to be used for RNA extraction were isolated from rats and rapidly frozen in liquid nitrogen. Frozen tissue was disrupted and grinded to a fine powder using a mortar and pestle. Total RNA was isolated from about 30 mg of each tissue using the "RNeasy Mini kit" (Qiagen, Milan, Italy) according to manufacturer's instructions. Finally the RNA was eluted in 50 μl of sterile water by centrifugation at 8000*g* for 1 min. Yield and purity were checked by spectrophotometric determination at 260 and 280 nm.

cDNA cloning and sequencing of rat spata 2. Reverse transcription of 5 μg of total RNA using 200 ng of oligo(dT) primer (Roche Diagnostics, Milan, Italy) was performed for 1 h at 42°C in a final volume of 20 μl in the presence of 25 U of AMV reverse transcriptase (Roche), 1 × reaction buffer (50 mM Tris–HCl, pH 8; 75 mM KCl; 3 mM $MgCl_2$), 0.2 mM each of dNTPs, 1 U Rnase inhibitor (Roche), and 10 mM dithiothreitol (DTT).

An aliquot (5 μl) of the rat testis cDNA diluted 1:10 was used in PCR to amplify the encoding region of *spata 2* cDNA using the primers specific for human coding sequence: forward 5′-ATGGGGAAGCCCAGTTCAATG-3′ and reverse 5′-TCTATCTG-TACACGAGATGGGAG-3′. PCR reaction contained 1 U Taq DNA polymerase (Amersham-Pharmacia Biotech, Milan, Italy), 0.2 mM each of dNTPs (Amersham-Pharmacia), 0.2 μM of each primer in 1 × reaction buffer (10 mM Tris–HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl). Amplification was performed for 35 cycles as follows: 1 min denaturation at 94°C, 1 min primer annealing at 60°C and 2 min extension at 72°C. PCR product was electrophoresed on a 1.5% agarose gel in 1 × TAE buffer (50 mM Tris–HCl, pH 8; 20 mM sodium acetate; 2 mM Na_2EDTA) and stained in ethidium bromide. PCR products were cloned directly into pGEM-T Easy Vector (Promega–Italia, Milan, Italy) to determine their nucleotide sequence. cDNA sequences was obtained on both strands using the fluorescent-terminator method and run on the ABI 377 sequencer (Perkin-Elmer Applied Biosystems, Monza, Italy). Three independent PCR products have been analyzed to produce a consensus sequence. The RACE-PCR (Rapid Amplification of cDNA Ends-PCR) technique was used to amplify the *spata* 2 cDNA-ends: amplifications were performed using a gene specific primer and an anchor primer complementary to the adaptor, ligated to both ends of the ds cDNA, and according to Frohman *et al.* (6).

Analysis of protein and cDNA sequences. Hydrophobicity analysis (7) was performed using ProtScale software (ExPASy Molecular Biology WWW server, Swiss Institute of Bioinformatics, Geneva, Switzerland). Presence of a signal peptide was analysed by means of PSORT II algorithm (8). Sequence similarity searches were performed using the following software: BLASTN, BLASTP 2.0.5 (9) running on the NCBI BLAST server (Bethesda, MD); and FASTA3 (10) (EBI WWW server, Hinxton Cambridge, UK). Occurrence within the protein sequence of particular patterns of established or potential significance was inspected by searching PROSITE database (11).

Northern Blot analysis. Two micrograms of rat testicular mRNA were electrophoresed on 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon Hybond N+ membranes (Amersham-Pharmacia Biotech) following standard procedure. The filter was hybridized with a 439 bp radiolabeled (1×10^6 cpm/ml) cDNA probe of *spata* 2 (from base 1217 to base 1655) in 10 ml of "ExpressedHyb" hybridization solution (Clontech, Palo Alto, CA). Hybridization and washing conditions were as recommended by the manufacturer. Signal was visualized by autoradiography with Kodak X-Omatic film at −80°C using an intensifying screen for 3 days.

Expression of recombinant SPATA2 and intracellular distribution. To monitor the intracellular distribution of SPATA2, the coding sequence was cloned into the vector pEGFPN1(Clontech) to generate a plasmid termed pSPATA2-GFP that gave rise to a SPATA2-green fluorescent protein (GFP) fusion protein. For the transfections was used the HeLa-derived cell line HLtat, which constitutively expresses the HIV-1 Tat protein that confers a high transfection efficiency compared to the parent cell line (12). Cells were grown on glass coverslips in DMEM supplemented with 10% fetal calf serum and were transfected by calcium-phosphate coprecipitation. Forty eight hours later the cells were fixed with 3.7% formaldehyde in PBS and examined under fluorescent light (excitation 480 nm, emission 510 nm) using a Nikon E600 microscope.

Optimization of RT-PCR analysis. To determine the range of PCR cycles over which amplification was linear for each target molecule we performed preliminary linear range-finding experiments according to Ferre (13). Thirty nanograms of RNA from each sample were reverse-transcribed into cDNA at 42°C for 2 h in a mixture (20 μl) containing 200 ng oligo(dT) primer, 25 U of AMV-R reverse transcriptase (Roche), 1 × reaction buffer (50 mM Tris–HCl pH 8; 30 mM KCl; 8 mM MgC_2), 10 mM each of dNTPs, 1 U Rnase inhibitor (Roche), and 10 mM DTT. The cDNA was subsequently amplified (as above) using gene-specific primers for varying cycle numbers (from 18 to 30) including 30 s denaturation at 94°C, 30 s primer annealing at 60°C and 30 s extension at 72°C. Primers for the *spata* 2 gene were: forward 5′-ATGGATACGAAGTACAAGGATGAC-3′; reverse 5′-CTTCTCCAGCAACGTGGAC-3′. As an internal control for the amount of RNA used, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as above, but using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′. The predicted size of the PCR-amplified products was 452 bp for GAPDH and 367 bp for SPATA2. All primers were selected from 2 exons separated by one or more intronic sequences, to identify possible amplicons from contaminating genomic DNA. To exclude potential cross-contamination of cDNA in RT-PCR reactions, samples were run on PCR by using water as negative control as well as for each RNA sample a "no RT" control have been used. Aliquots (10 μl) of the PCR reactions were electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane (Hybond N+) via alkaline transfer method. The filters were then hybridized with ^{32}P-labeled SPATA2 and GAPDH probes in "ExpressHyb" hybridization solution (Clontech) as above. These filters were exposed to Kodak X-Omatic films at −80°C for 1 day.

Comparative analysis was performed in the linear range of amplification which, for the amount of totRNA (30 ng) used as starting material in RT-PCR, corresponds to the 18–22 and 24–28 cycles interval for GAPDH and SPATA2, respectively.

RESULTS

Cloning of spata 2 cDNA, Sequence Analysis and Intracellular Localization of SPATA2 Protein

The cDNA of *spata* 2 was isolated following a RACE-PCR strategy: the first PCR products were obtained using rat testis cDNA and a set of primers designed on the basis of human cDNA to amplify the entire coding sequence. These amplicons were about 1.5 kb long and their sequencing revealed that they were the rat ortholog of *pd* 1 (now *spata* 2). To complete the sequence

we performed both 3′ and 5′ RACE-PCR which added 533 bp to 3′-end and 102 bp to 5′-end. The resulting cDNA sequence (GenBank Accession No. AF123651) shown in Fig. 1 is 2189 bp long excluding the poly(A) tail. The translation start was assigned to the first in-frame ATG that appeared downstream of a nonsense TGA codon at bases 44 to 46. Sequence analysis reveals an open reading frame of 1533 bp, encoding a 511 amino-acid protein with a predicted molecular weight of 57.5 kDa and an estimated isoelectric point of 8.84. The *spata* 2 coding sequence is preceded by a 121 bp 5′ untranslated region and followed by a 3′ untranslated sequence of 534 bp that contains the not canonical polyadenylation signal ATTAAA 14 nucleotides from the start of the poly(A) tail. A comparison between the human PD1 and rat SPATA2 protein sequences using of BLASTP showed an 85% identity in 514 amino acids sequence overlap (data not shown).

Both nucleotide and amino acid sequence of the SPATA2 were compared with nonredundant database using BLASTN, BLASTP, and FASTA3 programs, but no significant homologies with other proteins or genes were found. However, the *D. melanogaster* database contains a gene (CG4057) whose protein product is similar to SPATA2 with an expect value of 1.2e-08 (32% identity in 129 amino acids sequence overlap).

Regarding SPATA2 aminoacid composition it is interesting to observe the presence and the distribution of the 17 Cys residues that are located mainly in the C-terminal domain of the protein.

A hydrophobicity analysis using the Kyte-Doolittle algorithm predicts that SPATA2 protein is highly hydrophylic (data not shown). Analysis performed by PSORT II software predicted no signal peptide; however a nuclear localization was predicted (52.2% of probability).

To investigate the intracellular localization, a SPATA2-GFP fusion protein was expressed in HLtat cells: as shown in Fig. 2 SPATA2-GFP was detected primarily in the nucleus while GFP alone (as control) was distributed throughout the cell in a diffuse pattern which corresponds to that described for EGFP from Clontech.

A Northern blot analysis performed on mRNA from testis detected two distinct transcripts of 2.2 and 4.0 kb for *spata* 2 (Fig. 3), as has been previously observed in the study of human *pd* 1 mRNA.

Expression of spata 2 mRNA in Adult Rat Tissues

To analyse the tissue specificity of the *spata* 2 transcript, an RT-PCR analysis was performed on different adult rat tissues. Preliminary experiments had revealed the conditions under which the PCR reaction remained in the exponential phase (data not shown), while the quantity and the quality of the mRNA samples from each tissue were normalised after amplification of GAPDH mRNA. The primers for RT-PCR were designed within the coding region of *spata* 2 gene. The length and the composition of the nucleotide sequence was verified by sequence analysis of PCR products. As shown in Fig. 4, the highest expression was detected in the testis and the brain, while kidney and skeletal muscle showed expression levels which were several fold lower. The *spata* 2 transcript could not be detected in the spleen, heart, liver, or intestine.

Regulation of spata 2 mRNA Expression

FSH is the most important regulator of Sertoli cell function and we therefore analysed its effect on the steady-state of *spata* 2 mRNA level in the rat Sertoli cells. In duplicate time-course experiments, isolated Sertoli cells were treated with FSH (100 ng/ml) *in vitro* for 1, 4, and 24 h. RT-PCR amplification of *spata* 2 and GAPDH mRNAs following FSH stimulation are illustrated in Fig. 5a: expression of *spata* 2 mRNA augmented slightly after 1 h of treatment which resulted in a marked increase after 4 h and some more after 24 h.

Since specific expression of *spata* 2 mRNA was found in adult rat testis, we next focused on analysis of its expression during testicular development. Total RNA isolated from rat testis at 2, 10, 14, 25, and 45 days post partum (dpp) were reverse transcribed into cDNA and amplified for *spata* 2. As shown in Fig. 5b the *spata* 2 transcripts were first detected between 10 and 14 days, they became clearly visible at 25 days, and produced a strong signal at 45 days.

DISCUSSION

PD1 (which in the future will be referred to as human SPATA2) is a novel protein expressed at the testicular level which has been proposed to play a role in the regulation of spermatogenesis (1). The cDNA was isolated from a human testis library while protein expression has been detected at the tubular compartment level with localization to the Sertoli cells. Due to the obvious limitation in isolating human samples we looked at the rat as the ideal animal model in the functional study of this protein. To determine the sequence of rat SPATA2 a full-length cDNA was isolated from a rat testis cDNA pool. Analysis of the nucleotide

FIG. 1. Nucleotide sequence of rat *spata* 2 cDNA. The predicted amino acid sequence is shown under the DNA sequence and the putative polyadenylation signal is underlined. The stop codon TAG is marked with an asterisk. The sequence data are available under GenBank Accession No. AF123651.

```
CGGAGCATGCGATGGTCCATGGGCACCATGCCAGTTCTTCATGTGATGTGCCAGCCTCCAGCCCCAGAGCCATGGGGGAGATGAGGTAGCCCTTGGGTTT  100
GATTTGGGGAAGCCCAGTTCAATGGATACGAAGTACAAGGATGACTTATTCCGGAAGTACGTGCAGTTCCATGAGGGCAAAGTGGACACCACCCCCGGCA  200
                      M  D  T  K  Y  K  D  D  L  F  R  K  Y  V  Q  F  H  E  G  K  V  D  T  T  P  G
AACAGTCTGGCAGCGATGAGTACCTGCGCGTGGCAGCTGCCACCCTGCTCAGCCTGCACAAGGTGGACCCTTTATATCGATTTCGGCTGATCCAGTTTTA  300
K  Q  S  G  S  D  E  Y  L  R  V  A  A  A  T  L  L  S  L  H  K  V  D  P  L  Y  R  F  R  L  I  Q  F  Y
TGAGGTGGTGGAGAGCTCCCTCCGCTCGCTGAGTAGCTCCAGCCTGAGCGCTCTGCACTGCGCCTTCAGCATGCTGGAGACAATGGCCATCAACCTCTTC  400
  E  V  V  E  S  S  L  R  S  L  S  S  S  S  L  S  A  L  H  C  A  F  S  M  L  E  T  M  A  I  N  L  F
CTGTTCCCCTGGAAGAAGGAGTTCCGCAGCATCAAGACCTACACGGGCCCTTTTGTTTACTATGTCAAGTCCACGTTGCTGGAGAAGGACATCCGAGCCA  500
 L  F  P  W  K  K  E  F  R  S  I  K  T  Y  T  G  P  F  V  Y  Y  V  K  S  T  L  L  E  K  D  I  R  A
TTCTAAAGTTCATGGGCTACGAACCTGAGTTGGGGACTGCATACAAACTCAAAGAGCTTGTGGAGTCCCTCCAGGTGAAGATGGTCTCCTTTGAGCTCTT  600
I  L  K  F  M  G  Y  E  P  E  L  G  T  A  Y  K  L  K  E  L  V  E  S  L  Q  V  K  M  V  S  F  E  L  F
CCTGGCCAAGGTCGAGTGTGAGCAGATGCTGGGAATGCACTCACAGGTAAAGGACAAGGGCTATTCAGAGCTGGATGTGGTGACTGAGCGCAAGAGCAGC  700
  L  A  K  V  E  C  E  Q  M  L  G  M  H  S  Q  V  K  D  K  G  Y  S  E  L  D  V  V  T  E  R  K  S  S
ACAGAGGATGCACGTGGGTGCTCAGATGCCCTGCGGAGGCGGGCTGAGAGTCGAGAGCACCTGACCACTTCCATGGCTCGTGTGGCACTCCAGAAGTCAG  800
 T  E  D  A  R  G  C  S  D  A  L  R  R  R  A  E  S  R  E  H  L  T  T  S  M  A  R  V  A  L  Q  K  S
CCAGTGAGCGCGCAGCCAAGGACTACTACAAGCCCCGAGTGACCAAACCCTCCAGGTCGGTGGATGCCTACGACAGCTACTGGGAAAGCAGGAAGCCCCC  900
A  S  E  R  A  A  K  D  Y  Y  K  P  R  V  T  K  P  S  R  S  V  D  A  Y  D  S  Y  W  E  S  R  K  P  P
CTCAAAGGCCTCACTGAGTCTGCGCAAGGAGCCCCTGGCTATGGATGTAGGGGAAGACTTGAAGGATGAGATCATCCGCCCATCCCCCTCATTGCTGACC  1000
  S  K  A  S  L  S  L  R  K  E  P  L  A  M  D  V  G  E  D  L  K  D  E  I  I  R  P  S  P  S  L  L  T
ATGTCCAGCTCCCCCCATGGTAGCCCTGATGACCTTCCCTCCATCTCCCCCATCAATGGCCTGGGCCTTCTTCGTAGCACGTACTTCCCCACTCAGGATG  1100
 M  S  S  S  P  H  G  S  P  D  D  L  P  S  I  S  P  I  N  G  L  G  L  L  R  S  T  Y  F  P  T  Q  D
ACGTGGACCTGTACACGGATTCCGAACCCAGGGCCACCTACCGGAGGCAAGATGCTCTGCGGTCAGATATATGGCTGGTCAAAAATGATGCCCACTCCAT  1200
D  V  D  L  Y  T  D  S  E  P  R  A  T  Y  R  R  Q  D  A  L  R  S  D  I  W  L  V  K  N  D  A  H  S  I
CTACCACAAGCGTTCACCCCCCACCAAAGAGTCTGCCCTCTCCAAGTGCCAAAACTGCGGCCTGTCCTGCAGCTCCTCCCTCTGTCAGCGCTGTGACAGT  1300
  Y  H  K  R  S  P  P  T  K  E  S  A  L  S  K  C  Q  N  C  G  L  S  C  S  S  S  L  C  Q  R  C  D  S
GTCTGTCCTTCGGCCTCCAAGCCCAGTGGTTTTCCCAGCAAGGCCTCTGCACACGACAGCCTGGTCCATGGGGCACCTATGCGGGAGAAATATGTGGGAC  1400
 V  C  P  S  A  S  K  P  S  G  F  P  S  K  A  S  A  H  D  S  L  V  H  G  A  P  M  R  E  K  Y  V  G
AGACTCAGGGCCTTGACCGGCTGGCAACTGTCCACTCAAAGCCCAAGCCCTCTACCACAGCCACCTCCCGCTGTGGCTTCTGTAACCGTGCAGGTGCCAC  1500
Q  T  Q  G  L  D  R  L  A  T  V  H  S  K  P  K  P  S  T  T  A  T  S  R  C  G  F  C  N  R  A  G  A  T
CAACACCTGCACCCAGTGTTCAAAAGTTTCCTGTGACCCCTGCCTCGGCGCCTACCACTACGACCCCTGCTGCAGAAAGAGTGAGCTGCACAAATTCATG  1600
  N  T  C  T  Q  C  S  K  V  S  C  D  P  C  L  G  A  Y  H  Y  V  P  C  C  R  K  S  E  L  H  K  F  M
CCCAACAATCAGCTGAATTACAAATCCACCCAGTTCTCCCATCCCGTGTACAGATAGGCCCCGCCCCCCATCTTCTGCTACAAGGGCTATACACCACTGA  1700
 P  N  N  Q  L  N  Y  K  S  T  Q  F  S  H  L  V  Y  R  *
CCTGGCTTGGCAGCAGAGCTGTGCTGGAAGGGAACAGAGACTTGAGCTTGACCATGGAAGCGCCAGAGCTAGCTGCTCTGTCACACAGCTCACTTGGCAC  1800
ACCACATCCCAGCCGGCAGCTGCTGTGTGATCTGAAGGACAGGATGGCACTTCCAGTCACTTTGCTGACCTCTCCACTCCCTGCTCAGCCGGTTTGGGTT  1900
TGGGTTCTGGTTCTGGTTCCCTTGAAATAAGGTCTCTGTCTCGGGACTCTTGCTACACCTGCCCCCCACTGCAAAGCCAGCTTGGACTGGGAAGGAGCCT  2000
TTGGGGTCACTTTCAAATCCTCATCAGGAGTGGTGAGCTAGAGGCCTGCTGGCTTGTGAAACGAGCCCTCCTGCCACACCCGGCCTCTGATGGCTCGCCT  2100
CTCTTTCTGCTCCCTTGATACAAGGATCCCTAGGCAGATGTCCCCACCCTTTGTCTCCCTCCAACTGGTATTAAAGGGGAGCCAAGTCC-polyA
```

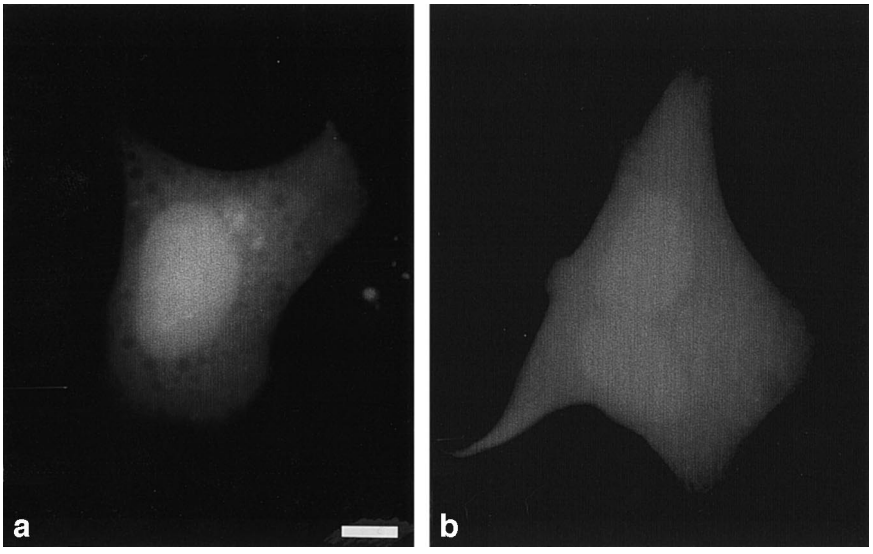

FIG. 2. Nuclear localization of SPATA2. As shown in (a), HeLatat cell transfected with plasmids expressing SPATA2 fused to GFP demonstrated a nuclear localization for this protein while GFP alone (b) was distributed throughout the cell in a diffuse pattern. Bar, 5 μm.

sequence revealed an open reading frame of 1533 bp encoding a 511-amino-acid protein with a predicted molecular weight of 57.5 kDa. The translation start is not the same as in human cDNA, but is 21 bp downstream and thus generates a protein that is 9 amino acids shorter on the whole. The first ATG in the *spata* 2 open reading frame is in reasonable agreement with the consensus for the nucleotide context of the initiation site (14). The sequence identity with human PD1 is 85% in 514 amino acid overlap revealing that SPATA2 protein has been highly conserved during mammalian evolution.

Northern blot analysis for *spata* 2 on mRNA obtained from rat testis detects the presence of two distinct transcripts of 2.2 and 4.0 kb: the first one corresponds to cDNA sequence reported here since repeated 5′ RACE-PCR performed using oligos within the first 200 bp failed to identify a larger transcript, while for the second one the difference in size may be due to an alternative 3′ untranslated region (UTR) as demonstrated for human *pd*1 by Nagase *et al.* (2). The 3′ UTR

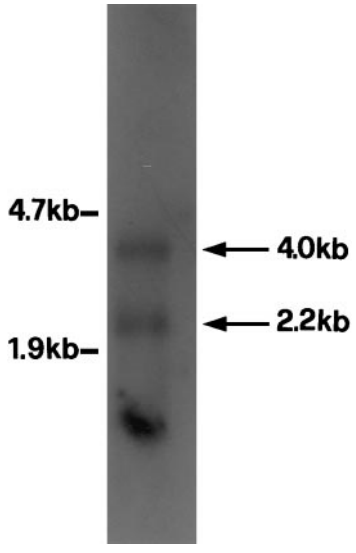

FIG. 3. Expression of *spata* 2 mRNA in adult rat testis by Northern blot analysis. Two μg of mRNA were electrophoresed and blotted to nylon membrane and hybridized with a radiolabeled cDNA fragment of *spata* 2 (from base 1217 to base 1655). Two distinct transcripts of 2.2 and 4.0 kb for *spata* 2 mRNA are indicated by arrows. On the left are shown the molecular size of the 28S and 18S ribosomal RNA.

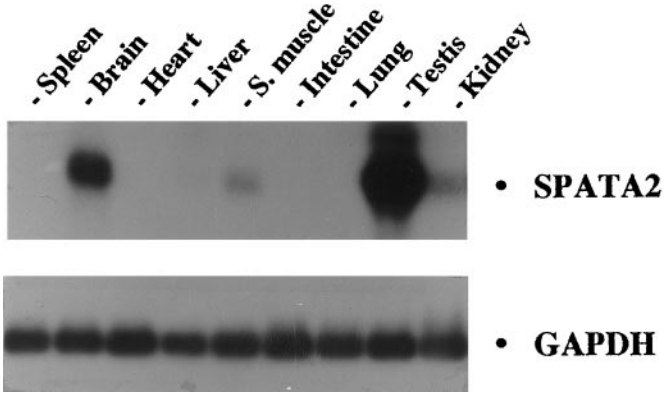

FIG. 4. RT-PCR analysis of *spata* 2 in several adult rat tissues. 30 ng of total RNA from each tissue were reverse-transcribed into cDNA and subsequently amplified for *spata* 2 and GAPDH by PCR. Aliquots (10 μl) of the PCR reaction were electrophoresed, blotted to nylon membrane, and detected after hybridization with the relative radiolabeled probes. The highest expression of *spata* 2 (top) was detected in testis and brain while kidney and skeletal muscle showed expression several fold lower. GAPDH expression (bottom) was utilized as an internal control for the equal amount of RNA used.

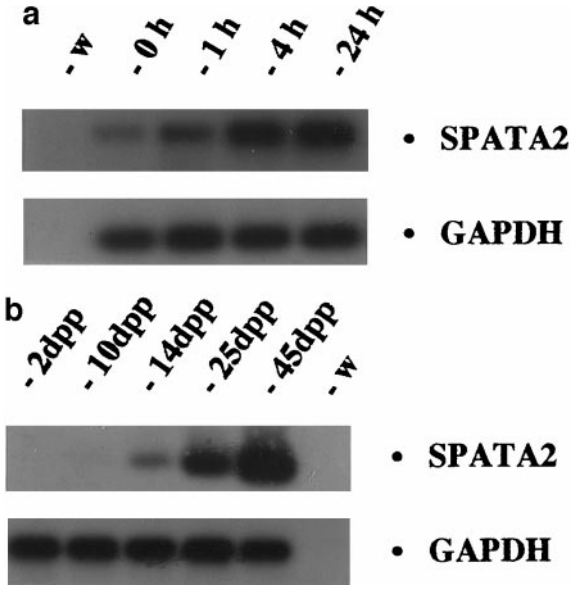

FIG. 5. Time course and developmental changes of *spata* 2 mRNA level in the rat testis detected by RT-PCR analysis. (a) Total RNA was isolated from Sertoli cells in culture either before (0 h) or 1, 4, or 24 h after FSH addition. RT-PCR products of *spata* 2 (26 cycles) and GAPDH (20 cycles) from a representative experiment are shown: a marked increase for *spata* 2 was seen after 4 h and some more after 24 h. (b) Total RNA was isolated from rat testis at 2, 10, 14, 25, and 45 days post partum (dpp). The PD1 transcripts were first detected between 10 and 14 days then they increased steadily with an advancement of age. A sample (w) was run on PCR by using water as negative control as well as a "no RT" control for each RNA sample (data not shown).

lacks the canonical polyadenylation signal, AATAAA, but it contains the most common variant, ATTAAA.

The presence of a signal peptide was analysed by means of PSORT II program and, even if no significant subcellular localization signals or transmembrane segment were found, a possible nuclear localization was suggested. To obtain additional information on the localization of SPATA2 in living cells, we constructed a SPATA2–GFP fusion protein which was detected primarily in the nucleus of HLtat transfected cells. Further analyses are ongoing (e.g., yeast two hybrid assay) to better define if SPATA2 might interact with any nuclear proteins.

The expression pattern of *spata* 2 in nine rat tissues was examined by RT-PCR and showed that the relative transcript is not widely distributed, the tissue with highest expression is testis, a lower expression was observed in brain, whereas very faint signals were detected in skeletal muscle and kidney. A similar pattern was observed in human (1, 2). This is not unusual since other genes with specific and essential roles in spermatogenesis have been reported to be expressed in testis and indeed in other tissue as well (15).

RT-PCR analysis on mRNA from Sertoli cells in culture demonstrated the presence of *spata* 2 transcripts. These results are in agreement with previous immunohistochemical analyses on human testis that showed PD1 to be present in the Sertoli cells while no reactivity was observed in any spermatogenic cells (1).

Since FSH is the most important hormone regulating Sertoli cell function we evaluated the effect of FSH on *spata* 2 mRNA level in isolated rat Sertoli cells: in two separate experiments this hormone induced, in a time-dependent manner, a significant increase in *spata* 2 mRNA level demonstrating that the expression of this protein is FSH-responsive. These results exclude a constitutive role for SPATA2 and suggest an involvement of this protein in the FSH-dependent function of Sertoli cells.

One of the physiological steps of the FSH-dependent Sertoli cell function is the induction of puberty. Previously studies demonstrated that the *spata* 2 mRNA level became detectable at 14 dpp during testicular development (16). In the present report, we analyzed the expression of *spata* 2 mRNA in rat testis during puberty. We used a more sensitive method which allowed us to detect *spata* 2 transcripts between 10 and 14 days (in the middle of the infantile period) when type A and B spermatogonia and leptotene spermatocytes are present. The *spata* 2 signal became then clearly visible at 25 days (during the juvenile period) when young spermatids start to be present and increased at 45 days when the rat reaches the sexual maturity and mature spermatozoa appear in the seminiferous tubules (17–19). The mechanism determining the increase in the *spata* 2 mRNA level during puberty is not clear. One hypothesis is that increasing the serum FSH level during this period may have a role in this phenomenon. Alternatively, as for inhibin, the neighbouring germ cells may modulate the FSH-induced *spata* 2 mRNA expression by Sertoli cells (20, 21). In summary the results of this study demonstrate that SPATA2, a novel rat protein: (i) is expressed at the testicular level, (ii) originates from Sertoli cells, (iii) appears during the infantile-juvenile period and increases steadily with advancement of age, (iv) increases the relative mRNA level after FSH stimulation *in vitro*.

ACKNOWLEDGMENTS

We wish to thank Dr. B. K. Felber and Dr. G. N. Pavlakis for the kindly providing of HLtat cells. We also thank Dr. P. Downey and Dr. P. Scannapieco for their advice in the course of the investigation and for critically reading the manuscript.

REFERENCES

1. Graziotto, R., Foresta, C., Scannapieco, P., Zeilante, P., Russo, A., Negro, A., Salmaso, R., and Onisto, M. (1999) cDNA cloning and characterization of PD1: A novel human testicular protein with different expressions in various testiculopathies. *Exp. Cell Res.* **248** (2), 620–626.
2. Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Miyajima, N.,

Tanaka, A., Hirokazu, K., Nomura, N., and Ohara, O. (1998) Prediction of the coding sequences of unidentified human genes. XI. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. *DNA Research* **5,** 277–286.

3. Taenasky, H., Gill, D., Murthy, S., Shao, X., Demetrick, D. J., and van der Hoorn, F. A. (1998) A novel testis-specific gene, SPAG4, whose product interacts specifically with outer dense fiber protein ODF27, maps to human chromosome 20q11.2. *Cytogenet. Cell Genet.* **81,** 65–67.
4. Maruyama, O., Nishimori, H., Katagiri, T., Miki, Y., Ueno, A., and Nakamura, Y. (1998) Cloning of TCFL5 encoding a novel human basic helix-loop-helix motif protein that is specifically expressed in primary spermatocytes at the pachytene stage. *Cytogenet. Cell Genet.* **82,** 41–45.
5. Scarpino, S., Morena, A. R., Petersen, C., Froysa, B., Soder, O., and Boitani, C. (1998) A rapid method of Sertoli cell isolation by DSA lectin, allowing mitotic analysis. *Mol. Cell. Endocrinol.* **146,** 121–127.
6. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Rapid production of full-length cDNA from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
7. Kyte, J., and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.
8. Nakai, K., and Horton, P. (1999) PSORT: A program for detecting the sorting signals of protein and predicting their subcellular localization. *Trends Biochem. Sc.* **24,** 34–35.
9. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.
10. Pearson, W. R., and Lipman, D. J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85,** 2444–2448.
11. Bairoch, A., Bucher, P., and Hofmann, K. (1995) The PROSITE database: Its status in 1995. *Nucleic Acids Res.* **24,** 189–196.
12. Schwartz, S., Felber, B. K., Benko, D. M., Fenyo, E. M., and Pavlakis, G. N. (1990) Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type I. *J. Virol.* **64,** 2519–2529.
13. Ferre, F. (1992) Quantitative or semi-quantitative PCR: Reality versus myth. *PCR Methods and Applications* **2,** 1–9.
14. Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.
15. Cooke, H. J., Hargreave, T., and Elliott, D. J. (1998) Understanding the genes involved in spermatogenesis: A progress report. *Fertil. Sterility* **69,** 989–995.
16. Onisto, M., Graziotto, R., Scannapieco, P., Marin, P., Merico, M., Slongo, M. L., and Foresta, C., (2000) A novel gene (PD1) with a potential role on rat spermatogenesis. *J. Endocrinol. Invest.* **23,** 605–608.
17. Clermont, Y., and Perey, B. (1957) Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am. J. Anat.* **100,** 241–267.
18. Ojeda, S. R., and Urbanski, H. F. (1988) Puberty in the rat. *in* The Physiology of Reproduction. (Knobil, E. and Neill, J., Eds.), pp. 1699–1728, Raven Press, New York.
19. de Kretser, D. M., and Kerr, J. B. (1988) The cytology of testis. *in* The Physiology of Reproduction. (Knobil, E., and Neill, J. Eds.) pp. 837–932, Raven Press, New York.
20. Pineau, C., Sharpe, R. M., Saunders, P. T., Gerard, N., and Jegou, B. (1990) Regulation of Sertoli cell inhibin production and of inhibin alpha-subunit mRNA levels by specific germ cell types. *Mol. Cell Endocrinol.* **72,** 13–22.
21. Anawalt, B. D., Bebb, R. A., Matsumoto, A. M., Groome, N. P., Illingworth, P. J., McNeilly, A. S., and Bremner, W. J. (1996) Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *J. Clin. Endocrinol. Metab.* **81,** 3341–3345.