Short Communication

2P1, a novel male mouse cDNA specifically expressed during meiosis

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ABSTRACT We screened a mouse germinal cell expression library with a probe derived from Sob1, a human testis-specific cDNA, and identified 2P1, a new mouse cDNA. A database search revealed that 2P1 was 91% identical to ORF1 of E3-3, a rat gene probably involved in the regulation of alternative splicing. Sequencing showed that 2P1 has a destabilization motif in its 3'-untranslated region. Northern blotting showed strong gene expression in the testis and weak expression in the epididymis, with no signal detected in other tissues. RT-PCR analysis confirmed testis and epididymis expression. In situ hybridization revealed that 2P1 mRNA was absent in spermatogonia but expressed in spermatocytes. This last result was confirmed by RT-PCR of FACS isolated primary spermatocytes (pachytene stage). Using RT-PCR, purified spermatids were also shown to express 2P1.

KEY WORDS: spermatogenesis, pachytene spermatocyte, mRNA splicing, post-transcriptional regulation, ARE.

Spermatogenesis is a complex process involving a sequence of stages. This process must have two essential properties if it is to fulfill its function, the translation of proteins with new functions, and very flexible regulation making it possible to switch genes on and off in a precise temporal fashion. There are currently many gaps in our knowledge concerning spermatogenesis. For example, the mechanisms enabling stem cells to undergo meiosis are currently unknown. The identification of genes specifically expressed during the course of male germ cell differentiation may shed light on this process.

A set of genes expressed in postmeiotic cells only has been identified in the mouse. It includes genes encoding specific sperm components (protamines: PRM-1 and PRM-2), transition proteins (TP1 and TP2) (Meistrich, 1989; Shih and Kleene, 1992) and SP-10 (Kurth et al., 1993), as well as genes encoding regulatory proteins such as MSY2, a member of the Y box protein family (Gu et al., 1998). Alternative splicing of the initial transcripts may also generate variants with new functions expressed uniquely in the testis (Williams et al., 1995). One such variant, DNA ligase III, loses its DNA repair function when produced in primary spermatocytes and is thought to be involved in the completion of meiotic recombination (Mackey et al., 1997). Several splicing-dependent changes in function have been identified during the course of spermatogenesis. CREM (cyclic AMP-responsive element modulator) protein is produced as an antagonist in premeiotic cells but switches to CREMt, the testis-specific activator form, from the pachytene spermatocyte stage onwards (Foulkes et al., 1992). Conversely, SOX17 protein, which seems to function as a transcriptional activator in premeiotic germ cells, switches to t-SOX17 in postmeiotic germ cells and loses its apparent DNA-binding activity (Kanai et al., 1996).

The original aims of this study was to identify the mouse counterpart of the human Sob1 gene, which encodes a sperm protein potentially involved in sperm-oocyte binding (Lefevre et al., 1999). We screened a λgt11 mouse germinal cell library with a probe derived from the human Sob1 cDNA sequence and isolated mouse Sob1 and 2P1 cDNAs. The 2P1 cDNA displays sequence similarity, over some of its length, to a variant specifically produced from the rat E3-3 gene by alternative splicing (Hartmann and Stamm, 1997). We also describe the germ cell type pattern of expression of the 2P1 cDNA in the seminiferous tubules. All these observations are in favor of accurately regulated activity during the spermatogenic cycle for 2P1.

Abbreviations used in this paper: ACE, angiotensin converting enzyme; FACS, fluorescence-activated cell sorter; RT-PCR, reverse transcriptase polymerase chain reaction.

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Sequence Note: The Genbank accession number of the 2P1 clone reported in this paper is: AF235017.

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Results

cDNA Sequencing
A 1079-nucleotide (nt) cDNA was isolated and named 2P1. The open reading frame (ORF) region consisted of 555 nt (Fig. 1). The 5'-untranslated region (UTR) was 422 nt long. An ATTGG motif that could account for a CCAAT box in its complementary form was observed at position – 118, as well as two GC-rich regions at positions -107 and -70 respectively. No TATA box was detected. The 3'-end contained no polyadenylation signal; it had an ATTTA destabilization motif at position + 637. The corresponding protein has a predicted size of 20.72 kDa with an isoelectric point of 8.31.

2P1 Expression
The distribution of 2P1 mRNA was determined by Northern blotting of total RNA from various tissues of mice. The 2P1 transcript was detected as a single band of 1.3 Kb (Fig. 2). It was present in large amounts in the testis, giving a strong signal whereas only a faint band was detected in the epididymis. No signal was detected for any other tissue.

RT-PCR was carried out from the testis and epididymis used as the template. Amplification generated a strong 627 bp band (Fig. 3A). This band corresponded to the expected size according to the primers chosen. Prm2 was used as a positive control; it was expressed in both tissues.

In situ hybridization was then used to identify cell types containing 2P1 transcripts in sections of mature mouse testis. With an antisense 2P1 probe, we detected a significant signal in primary spermatocytes (pachytene stage step), while spermatagonia were negative (Fig. 4). Hybridization with an ACE antisense probe used as control was detected in haploid cells only, and the 2P1 sense probe acted as a negative control. We found that 2P1 was expressed from stage I in pachytene spermatocytes, with expression persisting until stage XII of the spermatogenic cycle.

Fig. 1. The nucleotide sequence of the testis-specific 2P1 cDNA and the deduced amino acid sequence of its product. Genbank accession number AF235017. The nucleotide sequence contains a 422 base pair upstream untranslated segment. The box-like CAAT and GC in the 5'UTR and the destabilization signal in the 3'UTR are underlined.
Furthermore, we found that four recently identified mouse ESTs share 95 to 98% sequence identity with 2P1. One of these ESTs, isolated from spermatocyes (GenBank accession number BG101073), corresponds to nt 28 to 623 of 2P1. Moreover, the RIKEN cDNA Expression Array Database has recently reported the identification of two major cDNAs corresponding to nt 415 to 712 (90% identity) and nt 881 to 1073 (97% identity) of the 2P1 cDNA, respectively (Miki et al., 2001).

The presence of a CCAAT box-like in its complementary form ATTTGG was detected in the long 5'UTR of 2P1. We found no evidence of specific regulation except the two GC-rich regions. In return we identified a destabilization motif in the 3' region that may account for precise temporal regulation. Such A/U rich element sequences (ARE) have recently been proved to be responsible for the shortening of mRNA half-life (Powell et al., 2000; Gay and Babajko, 2000).

Northern blot and RT-PCR studies showed strong 2P1 expression in the testis. In situ hybridization studies of testis were undertaken to identify the cells that synthesize 2P1 mRNA. No signal was observed in somatic cells while, in the germinal lineage, transcripts were detected in pachytyne spermatocytes only. This result was confirmed by RT-PCR of FACScan sorted 4C spermatocytes. The positive result of purified spermatids displayed by RT-PCR suggests that some 2P1 mRNA, that could not be detected by in situ hybridization, remain at this stage. The signals observed in epididymis using Northern blots and RT-PCR probably account for a residual expression in spermatooa. Using flow cytometry, we are currently studying the expression of 2P1 in each germinal cell type.

In summary, 2P1 gene is expressed during spermatogenesis in a stage dependant manner. Furthermore, it displays in its 3'-UTR a destabilization motif that may lead to its transient expression recently described in several cell types (Paillard et al., 2002; Schaaf and Cidlowski, 2002; Suda et al., 2002). This posttranscriptional regulation pathway has been suggested to account, in association with translational regulation, for gene expression in response to hormonal environment or during embryonic development (Gay and Babajko, 2000; Ueno and Sagata, 2002).

At this stage, two major questions arise: when is the protein encoded by the 2P1 transcript synthesized and what is its physiological role? Hartmann et al. (1997) isolated E3-3 cDNA with the yeast two-hybrid system using SRp30c, a protein involved in pre-mRNA splicing, as bait. Their results suggest that E3-3 belongs to the spliceosome protein family. Furthermore, they showed the nuclear location of the protein encoded by ORF1.
in transfected A293 cells, that is consistent with a role in gene regulation. Given its high level of similarity to E3-3, the presence of a destabilization motif in its 3'UTR region and its specific expression in pachytene spermatocytes, 2P1 may be one transiently expressed gene involved in male meiotic process.

**Experimental Procedures**

**Plasmids and Probes**

We screened the Uni-ZAP XR male mouse germ cell expression library (Stratagene, La Jolla, CA, USA) with the Qb8 cDNA probe corresponding to nucleotides 1495 to 2780 of the human cDNA encoding the spermatocyte binding protein SOB1 (Lefevre et al., 1999). Qb8 was obtained from human testis mRNA by 3'RACE using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) with an upstream primer specific for SobI (5'-GATATTGGCAACCTCAGCCTTC-3'), the anchor primer provided by the manufacturer and Taq Gold polymerase (Perkin Elmer, Foster City, CA, USA). The resulting products were inserted into the PCR 2-1 TOPO Vector (Invitrogen). Before screening the library, we showed by Southern blotting that Qb8 hybridized to mouse testis DNA (data not shown).

The probe used for Northern blotting was obtained from 10 µg of mouse testis total RNA treated with 10 U of RNase-free DNase. The first-strand cDNA was synthesized with MMLV reverse transcriptase (Life Technologies, Cergy Pontoise, France) and the 627 bp fragment was amplified by PCR with Taq Gold polymerase (Perkin Elmer) and 2P1 specific primers (upstream 5'-GGCTACTGCTCTAGGATTTCGC-3' and downstream 5'-CTGGGTGAGCATGACAAGTGTG-3'). The amplified cDNA was then inserted into the PCR 2-1 TOPO Vector (Invitrogen).

**Fig. 4. (Left) In situ hybridization of adult testicular tissue sections.** Hybridization with the [35S]-UTP labeled antisense 2P1 RNA probe (A,B, A',B'). Hybridization controls were performed with the 2P1 sense RNA probe as a negative control (C,D) or the angiotensin converting enzyme (ACE) antisense RNA probe as a positive control (E,F). Arrows in A' and B' point to spermatogonies. Bright field (A, A', C and E) and dark field (B, B', D and F) images of the same sections are shown. Original magnification X 16 in all cases, except A' and B' (X63).

**Fig. 5. (Right) In situ hybridization at various stages of the mouse seminiferous tubule cycle.** Hybridization with the [35S]-UTP labelled antisense 2P1 RNA probe. Stages I-II (A), III - V (B), VII (C), VIII and IX (D), XI (E) and XII (F). Original magnification X 63 in all cases.
For *in situ* hybridization, the first-strand cDNA was produced by reverse transcription from mouse testis total RNA, then amplified by PCR with Advantage Taq polymerase (Clontech) and upstream and downstream primers containing XbaI and EcoRI restriction sites (5'-GCTCTAGAATGCTCTAGCTATGGATCTTGC-3' and 5'-GGAATTCCTGATGTGTCATTCTGAGGCTTGACC-3' respectively). The amplified DNA was digested with XbaI (Boehringer Mannheim, Germany) and EcoRI (Gibco BRL, Paisley, Scotland), and inserted into the Bluescript KS vector (Stratagene). Single-stranded sense and antisense 2P1 probes were synthesized with 50 µCi [32P]-dCTP ([α-32P]-UTP (Amersham Pharmacia Biotech Ltd, UK) as described by Sibony *et al.* (1995).

All cDNAs were sequenced on both DNA strands.

cDNA Library Screening and Molecular Cloning

The mouse testis germ cell cDNA library expressed in the Uni-ZAP XR vector containing the BlueScript KS phagemid was used to infect *Escherichia coli* XL1-blue MRF' (Stratagene) and 50,000 plaque-forming units (pfu) were plated on 150-mm N2 8% top agar plates. The plates were incubated at 37°C for 8 h, and bacteriophage particles were then replicated onto 0.45 µm nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). Membranes were screened with the 2P6R probe radiolabeled with [32P]-dCTP by random priming with the Rediprime II Kit (Amersham). They were washed once with 1 X SSC, 0.1% SDS at 65°C, finally with 0.5 SSC 0.1% SDS at room temperature. Positive clones were selected and Phagemid BlueScript was excised *in vivo* from the λ ZAP vector, using the ExAssist helper phage (106 pfu) in *Escherichia coli* non suppressing Su SORL strain (Stratagene). Recombinant phagemids were then purified by alkaline lysis (Sambrook *et al.*, 1989) and digested with EcoRI (Gibco BRL). Digestion products were size-fractionated by electrophoresis in a 1% agarose gel. Four positive recombinant phagemid clones were sequenced with the T3 and T7 primers, which bound to opposite sites of the polylinker in the λ Uni-ZAP XR vector. The 2P1 cDNA was sequenced on both strands and the resulting nucleotide sequence is available from GenBank under accession number AF235017.

Primary Spermatocyte and Spermatid Purification

Pachytene spermatocytes (4C) and spermatids were isolated from adult mouse testes, and purified using a dual-laser FACStar Plus (Becton Dickinson, San Jose, CA, USA) according to Kottun and McMurray (2001). Cells were discriminated by size and DNA content via Hoechst 33342 staining. Then they were evaluated for morphology and gene expression (I. Allemand, personal communication).

Northern Blotting

Total RNA was extracted from adult male mouse organs (testis, epididymis, seminal vesicle, vas deferens, kidney, bladder, lung, liver, muscle, heart and brain) by guanidine thiocyanate/phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The RNA was quantified and then treated with 10 U of RNase-free DNase (Boehringer) for 15 min at 37°C. Equal amounts of total RNA (25 µg/lane) were denatured, subjected to electrophoresis in a 1.5% formaldehyde-agarose gel for 4 h at 70 volts and transferred to nitrocellulose membranes (Hybond N, Amersham) by capillary action overnight in 20 X SSC. The RNA was cross-linked to the membrane by exposure to UV light. Membranes were incubated for 4 h at 65°C in prehybridization buffer (50% (w/v) formamide, 0.75 M NaCl, 0.05 M NaH₂PO₄, 0.5 mM EDTA, 5 X Denhardt’s solution, 0.1 mg/ml salmon sperm) to which we added 0.5% SDS. The 2P1 probe was labeled with 50 µCi ([32P]-dCTP, using a random priming kit (Amersham) and added to the prehybridization buffer with 0.1% SDS. The membrane was incubated in this mixture for 16 h. Membranes were then washed once with 3 X SSC 0.1% SDS at room temperature, twice with 2 X SSC, 0.1% SDS at 65°C for 15 min and once with 1 X SSC, 0.1% SDS at 65°C for 30 min. Hybridized membranes were placed against X-ray film (Hyperfilm MP, Amersham) at – 80°C with intensifying screens for 48h.

RT-PCR

Total RNA was isolated from 10 µg mouse testes or epididymis tissue (Chomczynski and Sacchi, 1987). Samples were treated with MMLV reverse transcriptase (Life Technologies) at 42°C for 1 h. The cDNA was diluted to 20 µl and 2 µl were used per tube. Upstream (5'-GGCTACTGCTCTAGGATCTC-3') and downstream (5'-CTGGGTGAGCATGACAAGTGTG-3') 2P1 primers were used. Amplification of the mouse *PmⅡ-2* gene was used as a positive control, (upstream 5'-CGCTACCAATGAGAGGCCCAGT-3' and downstream 5'-TTAGTGAGTTGCTTCTCATATTCC-3'). PCR was then carried out with enzyme Taq Gold (Perkin Elmer) as follows: initial denaturation for 10 min at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 60°C and 1 min at 72°C, then a final extension at 72°C for 10 min. Total RNA of sorted germinal cells was purified using Rneasy Mini kit according to manufacturer’s instruction (Qiagen GmbH, Hilden, Germany). Reverse transcription was the same as above. The cDNA was diluted to 150 µl and 10 µl were used per control. Amplification of the mouse *actin* gene was used as a positive control (upstream 5'-ACCCACACTGTTGCCCATCTAC-3' and downstream 5'-CTCATGTGTTGCTGAGGACCAG-3'). PCR was carried out with HotStart Taq Polymerase (Qiagen) and conditions were the following: initial denaturation for 15 min at 95°C, then 30 cycles of 30 s at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension of 10 min at 72°C. For all amplifications, negative controls without the cDNA matrix were included (data not shown).

In Situ Hybridization

Prehybridization, hybridization and posthybridization of paraformaldehyde-fixed, paraffin-embedded testis sections (4-6 µm) were carried out as previously described (Sibony *et al.*, 1995). The procedure included a treatment with proteinase K, overnight hybridization at 50°C with 0.7 x 10⁶ cpm of labeled probe per section, washing under stringent conditions and RNase A treatment. The dried sections were dipped in Kodak NTB-2 photographic emulsion (IBS INTEGRA Biosciences, Cergy-Pontoise, France) and stored at 4°C for 15 and 21 days in the dark. After photographic processing, sections were counterstained in 0.2 % toluidine blue, and mounted in EUKITT mounting medium (Elvetec Services, Vénissieux, France). Sections were viewed and photographed under dark-field illumination.

The location of the grains was determined by reference to the same section viewed under bright-field illumination. For quantification, sections were examined with a computer-assisted image analysis system, Visilog 4.15 (Noesis, Les Ulis, France), consisting of an IMC 500 camera and an IMC 500 digitizer. The stages of the seminiferous epithelium cycle were identified according to the morphological criteria for mouse spermatogenesis established by Oakberg (1956) and Russell *et al.* (1990). The intensity of the hybridization signal for the various stages of the spermatogenic cycle was quantified and expressed as the percentage of the pixels within a marked area with silver grains.

Statistical Analysis

Analyses were performed with SYSTAT (Wilkinson, 1990). Means were compared by one-way ANOVA, and Tukey-Kramer multiple comparison tests. Error estimates are given as standard error of the mean (SEM). Results are expressed as means ± SEM. Data were considered statistically significant if *P* < 0.05.

Sequence Data Analysis

Sequence data were compiled and DNA sequences were aligned with the BLASTN and ALIGN programs.

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