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Research Paper

Identification and characterization of a spermatogenesis-related gene *Ube1* in rat testis

DU Ying[1,2], LIU Mei-Ling[2], JIA Meng-Chun[2,*]

[1]Peking Union Medical College Postgraduate School, Beijing 100730, China; [2]Department of Reproductive Endocrinology, National Research Institute for Family Planning, Beijing 100081, China

Abstract: A gene that could be potentially involved in spermatogenesis was identified and characterized by using suppression subtractive hybridization (SSH) and rapid amplification of cDNA ends (RACE) with total RNA from type A spermatogonia and pachytene spermatocytes of rat. This gene consists of 3 433 base pairs (bp) with a complete open reading frame (ORF) of 3 171 bp and encodes a putative protein containing 1 057 amino acids. The nucleotide sequence displays a 93% identity to mouse ubiquitin-activating enzyme E1, Chr Y 1 (*Ube1y1*) and an 82% identity to human ubiquitin-activating enzyme E1 (*UBE1*). The putative protein of this gene contains an ubiquitin-activating enzyme signature 1 and an ubiquitin-activating enzyme active site, which are also existed in mouse ubiquitin-activating enzyme E1, human ubiquitin-activating enzyme E1 *et al*. So we named this gene as *Rattus norvegicus* ubiquitin-activating enzyme E1 (*Ube1*). The sequence of *Ube1* was submitted to GenBank and the accession number is EF690356. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that *Ube1* was specifically expressed in testis, while its expression was not detected in heart, brain, spleen, lung, liver, muscle, kidney and ovary. Comparison of the expression of *Ube1* in different developmental stages of testis and Sertoli cells (real-time PCR) indicated that *Ube1* was expressed more highly in spermatogonia than in spermatocytes, spermatids and Sertoli cells. In conclusion, *Ube1* is a gene encoding rat ubiquitin-activating enzyme E1 and specifically expressed in testis, which might play a key role in ubiquitin system and influence spermatogenesis.

Key words: ubiquitin-activating enzyme; spermatogenesis; suppression subtractive hybridization; rapid amplification of cDNA ends; RT-PCR; real-time PCR

大鼠睾丸特异表达基因 *Ube1* 的分离鉴定及生物学特征

杜 颖[1,2]，刘美玲[2]，贾孟春[2,*]

[1] 北京协和医学院研究生院，北京 100730； [2] 国家人口计生委科学技术研究所生殖内分泌室，北京 100081

摘 要：本研究采用抑制性消减杂交(suppression subtractive hybridization, SSH)和 cDNA 快速扩增(rapid amplification of cDNA ends, RACE)技术从大鼠 A 型精原细胞和粗线期精母细胞中成功克隆出大鼠泛素激活酶(ubiquitin-activating enzyme)基因 *Ube1* (GenBank 登录号 EF690356)。该基因序列全长 3 433 bp，其中开放阅读框有 3 171 bp，编码一个含 1 057 个氨基酸的蛋白质。Blast 比对显示，*Ube1* 与小鼠泛素激活酶基因 *Ube1y1* 的同源性为 93%，与人泛素激活酶基因 *UBE1* 的同源性为 82%。*Ube1* 基因编码的蛋白质含泛素激活酶信号位点和泛素激活酶活化位点，这些位点也存在于人类和小鼠的泛素激活酶 1 中。RT-PCR 分析显示，*Ube1* 在睾丸中大量表达，而在心、肝、脾、肺、肾、肌肉、脑、卵巢中没有表达。荧光定量 PCR 分析不同生精细胞中 *Ube1* 的表达，显示 *Ube1* 在 A 型精原细胞中大量表达，在粗线期精母细胞、圆形精子细胞和支持细胞中微弱表达。以上结果提示，*Ube1* 是大鼠睾丸特异表达基因，可能通过参与泛素 / 蛋白酶体途径来影响精子发生。

关键词：泛素激活酶；精子发生；抑制性消减杂交；cDNA 快速扩增；逆转录 - 聚合酶链反应；荧光定量 PCR
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*Corresponding author. Tel: +86-10-62179164; Fax: +86-10-62173457; E-mail: jmchun@163bj.com

Spermatogenesis is a complex cyclical process leading to the formation of mature spermatozoa. Germ cells undergo a series of unique stage-specific events including the mitotic proliferation of spermatogonial stem cells, meiotic division of spermatocytes and spermiogenesis of haploid spermatids[1]. These events take place in the seminiferous tubules of the testis, in which spermatogonia and Sertoli cells sit on the basement membrane, with spermatocytes interior to spermatogonia, and spermatids and mature spermatozoa facing the lumen. The genes expressed in spermatogenesis encode proteins necessary to the development of germ cells in specific stages. And many genes that regulate spermatogenesis have been studied via various methods[2-8]. However, many aspects of the mechanism of gene expression regulation in spermatogenesis remain elusive. Thus identification of genes specifically involved in spermatogenesis and analysis of the phenotypes and other characteristics could provide insight into this developmental process.

The ubiquitin-activating enzyme has been found in yeast, plants and mammals (including mouse, human, rabbit), but has not been reported in rat. In this study, our research group has found *Ube1*, which encodes the *Rattus norvegicus* ubiquitin-activating enzyme E1 and is highly expressed in testis. The characteristics and tissue distribution of *Ube1*, its expression in different developmental stages of testis and its possible correlation with spermatogenesis were discussed.

1 MATERIALS AND METHODS

1.1 *Isolation of type A spermatogonia, pathytene spermatocytes, round spermatids and Sertoli cells*

Male Sprague-Dawley rats, 9 days old, were purchased from Weitonglihua Company (Beijing, China) for isolation of type A spermatogonia and Sertoli cells. Testes were excised and decapsulated. The seminiferous epithelial cells were dispersed and separated. Briefly, the decapsulated testes were suspended in Dulbacco's minimum Eagle's medium/F12 (DMEM/F12) containing collagenase (1.5 mg/mL) and DNase (1 μg/mL), incubated at 34 ºC for 15 min in a shaking water bath operated at 100 cycles/min. After twice washes in DMEM/F12 medium, seminiferous cord fragments mostly devoid of interstitial cells were incubated with DMEM/F12 containing collagenase (1.5 mg/mL), hyaluronidase (1.5 mg/mL), trypsin (0.5 mg/mL) and DNase (1 μg/mL) for 20-30 min under the similar conditions. The disperse cells were washed twice with medium and filtered through 80- and 40-μm nylon mesh (Teco Inc., Briarcliff Manor, NY), successively. The seminiferous epithelial cells were separated by sedimentation velocity at unit gravity at 4 ºC, using a 2%-4% BSA gradient in DMEM/F12 medium. The cells were allowed to settle for a standard period of 2.5 h. Thirty-five aliquots of 15 mL volume each were examined at 90-s intervals. The cells in each aliquot were examined under a phase contrast microscope, and fractions containing cells of similar size and morphology were pooled and spun down by low-speed centrifugation and resuspended in DMEM/F12 medium. The isolated type A spermatogonia were characterized by light and electron microscopy and identified by immunocytochemical and Northern blot analysis of c-kit receptor[10]. The pachytene spermatocytes and round spermatids were isolated from adult rat testes by the same method described above.

All chemicals were purchased from Sigma Chemical Co. (St. Lout, MO).

1.2 *RNA extraction*

The total mRNAs of spermatogonia, spermatocytes, spermatids and Sertoli cells were extracted respectively according to the TRIzol RNA isolation protocol (Gibco-BRL, Grand Island, NY) with DNase I to eliminate DNA contamination. The reaction containing 20 μg total RNA, 10 U RNase-free DNase I (Gibco-BRL) and 10 U RNasin (Promega) was carried out at 37 ºC for 30 min and extracted with phenol and chloroform. The quality and concentration of RNA samples were verified by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

1.3 *Suppression subtractive hybridization (SSH) and generation of subtracted cDNA libraries*

SSH was performed based on the original procedures of Diatchenko *et al*[9]. Following the instructions of the PCR-Select cDNA Subtraction Kit (BD Biosciences) and the SMART PCR cDNA Synthesis Kit (BD Biosciences), SMART cDNA from pathytene spermatocytes was used as the ''driver'' sample and the corresponding cDNA from type A spermatogonia as the ''tester'' sample. To estimate the efficiency of subtraction, PCR was performed on the subtracted and unsubtracted PCR product with β-actin 5' and 3' primers. Aliquots of the samples were taken after 18, 23, 28 and 33 cycles of PCR amplification and the products were analyzed on a 2% agarose gel. If subtraction was efficient, transcripts of housekeeping genes should be significantly reduced.

The final PCR products were cloned into pGM-T easy vector (TIANGEN) to generate subtracted cDNA libraries. After overnight growth on agar plates, 576 individual

bacterial colonies each for forward- and reverse-subtracted libraries (1 152 total colonies) were individually transferred with sterile toothpicks to 96-well microplates containing 100 μL LB medium with ampicillin (100 μg/mL) per well. After overnight growth, all isolated colonies were stored in 60% glycerol at -80 ºC until differential screening.

1.4 *Dotting blot*

Clones containing subtracted cDNAs were screened for false-positives with a PCR-Select Differential Screening Kit (BD Biosciences). Nylon membranes were blotted with candidate cDNAs amplified from SSH. Each duplicate set of membranes was screened with ^{32}P-labeled probes from forward- and reverse-subtracted cDNAs. Membranes were prehybridized, hybridized and washed according to the manufacturer's instructions.

1.5 *Screening of genes differentially expressed in type A spermatogonia and pathytene spermatocytes*

Clones confirmed by dotting blot analysis to be differentially expressed were sequenced by using an ABI 377 autosequencer at AuGCT Biotechnology (Beijing, China). The sequences were then blasted in GenBank (http://www.ncbi.nlm.nih.gov) by using the software Blast to determine the homology among various species and locations in chromosomes. The nucleotide and deduced amino acid sequences were also analyzed by using NCBI-ORF Finder (http://www.ncbi.nlm.nih.gov), DNAMAN, Prosite Database (http://www.expasy.ch/prosite/) and SMART software (http://smart.embl-heidelberg.de/).

1.6 *Rapid amplification of cDNA ends (RACE)*

Full-length sequence of *Ube1* was obtained by using 3'-Full RACE Core Set Ver.2.0 (TaKaRa) and 5'-Full RACE Kit (TaKaRa).

1.6.1 *3'-RACE*

The first strand cDNA was synthesized using 1 μg total RNA and an oligo dT-3 site adaptor primer provided by the RACE kit in a total volume of 10 μL. The cDNA was then amplified using the adaptor primer (included in the RACE kit) and the gene specific primers (Table 1). Both outer and inner PCR was performed as following procedure: 3 min at 94 ºC, 30 cycles of 30 s at 94 ºC, 30 s at 56 ºC, 1 min at 72 ºC, and then 10 min at 72 ºC. The inner PCR products were resolved on an agarose gel and the appropriate band was excised, purified, cloned into pGM-T vector (TIANGEN) and sequenced.

1.6.2 *5'-RACE*

The gene specific outer and inner primers (Table 1) were designed based on the sequence of the internal conservative fragment. About 2 μg total RNA was reacted with alkaline phosphatase (calf intestine), tobacco acid pyrophosphatase, 5'-RACE adaptor, T4 RNA ligase and reversely transcribed using random 9mers and reverse transcriptase *M-MLV* (RNase H$^-$). Both outer and inner PCR was performed at 94 ºC for 30 s, 56 ºC for 30 s, 72 ºC for 1 min with 30 cycles. The amplified products were resolved on an agarose gel and the appropriate band was excised, purified and cloned into pGM-T vector followed by sequencing.

1.7 *RT-PCR*

Total RNAs from adult rat multi-tissues (heart, brain, spleen, lung, liver, muscle, kidney, ovary and testis) were prepared as described above. The cDNA templates for RT-PCR analysis were synthesized from the total RNA according to the instruction of the first strand cDNA synthesis kit (TaKaRa). The cDNAs were amplified with the

Table 1 Primers used for RT-PCR, RACE-PCR and real-time PCR

Gene	Primers	Sequences
Ube1	3'-RACE outer primer	5'-ACTCAAGATTCGCTACCTACTC-3'
	3'-RACE inner primer	5'-TTCATTGTGGCAGCATCCAACCTC-3'
	5'-RACE 1st outer primer	5'-AAAAGAATCCAGTTGTTGGTGAC-3'
	5'-RACE 1st inner primer	5'-CTGGAGTAGGTAGCGAAGTCTTGAGT-3'
	5'-RACE 2nd outer primer	5'-CTGCTGGATGTTGTGAGAATAC-3'
	5'-RACE 2nd inner primer	5'- CCTTCA AACTCATCCCGAGCCCATTCTA-3'
	RT-PCR	5'-TCATGGAGCGGACACTG-3'
		5'-CCTTCTCGAAATCAATGGG-3'
	real-time PCR	5'-GCTCTATTCCTTCTTTATG-3'
		5'-AATGTCGTCTCCACTCTCA -3'
β-actin		5'-CCCTGTATGCCTCTGGTCGTA-3'
		5'-CCATCTCTTGCTCGAAGTCT-3'

specific primers in Table 1 and PCR was performed by denaturation for 5 min at 94 ºC, 30 cycles of 95 ºC for 40 s, 56 ºC for 40 s and 72 ºC for 40 s, and then 72 ºC for 10 min. The β-actin mRNA was also amplified as positive control. The PCR products were analyzed by electrophoresis.

1.8 *Real-time PCR*

Total RNAs from three different spermatogenic cells and Sertoli cells were prepared as described above. The cDNA templates for real-time PCR analysis were synthesized as described above. Real-time PCR was conducted using SYBR Green PCR Master Mix Reagent (SYBR Premix Ex Taq kit, TaKaRa) by an ABI 7700 Sequence Detection System (PE Applied-Biosystems). The reaction was performed in a volume of 25 μL containing primers, SYBR Premix Ex Taq and cDNA templates. PCR was carried out with 40 cycles at 95 ºC for 15 s, 52 ºC for 30 s, and 72 ºC for 30 s. The β-actin was used as internal control under the same conditions. To obtain relatively accurate results, each template of different spermatogenic cells and Sertoli cells was processed in three tubes in the same PCR mixture. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in *Ube1* gene transcription. The *Ube1* Ct values were firstly normalized by subtracting the Ct value obtained from the β-actin control ($\Delta Ct = Ct_{Ube1} - Ct_{control}$). The relative concentration was determined by $2^{-\Delta\Delta Ct}$. The primer pairs used in real-time PCR analysis were listed in Table 1. Differences among groups were determined by SPSS 10.0 software.

2 RESULTS

2.1 Isolation of different germ cells and Sertoli cells

It is difficult to isolate highly purified spermatogonia, because they are only about 1% among adult testis cells and 10% among immature testis cells. Jia MC, the corresponding author of this paper, successfully established the method to separate the highly enriched spermatogonia from the testis of 9-day-old rats in Dym's laboratory[10]. The purity of approximately 90% of spermatogonia and Sertoli cells from testis of 9-day-old rats, spermatocytes and spermatids from testis of adult rats were separated respectively.

2.2 SSH and dotting blot

To screen the stage-specific expression genes during the processes of spermatogenesis, SSH combined with dotting blot hybridization was performed and 36 differentially expressed cDNA fragments were obtained. Among them, 3 cDNA fragments were identified as positive different expression EST (expression sequence tag). They were cloned and sequenced using an automated DNA sequencer (ABI 373A).

The efficiency of subtraction was evaluated by PCR amplification of a housekeeping gene, β-actin. Figure 1 showed that the β-actin fragment was detectable after 28 cycles of amplifications in the subtracted sample, while it was clearly detectable in the unsubtracted sample after 18 cycles. Partial results of differential screening for the subtracted libraries were showed in Fig.2.

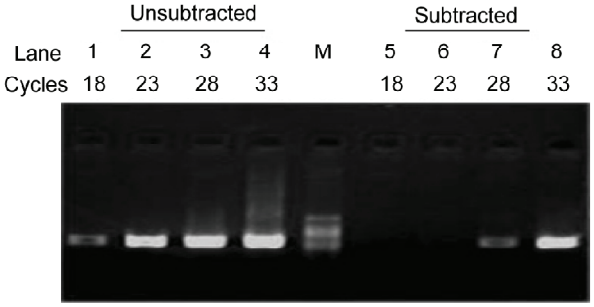

Fig. 1. Reduction of β-actin abundance by PCR-select subtraction. PCR was performed on the unsubtracted (lanes 1-4) or subtracted (lanes 5-8) secondary PCR products with β-actin 5' and 3' primers. Lanes 1, 5: 18 cycles; Lanes 2, 6: 23 cycles; Lanes 3, 7: 28 cycles; Lanes 4, 8: 33 cycles. Lane M: 100 bp DNA marker.

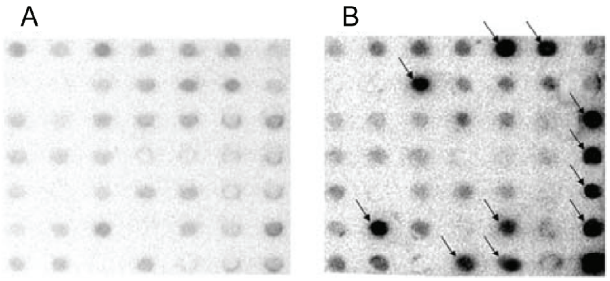

Fig. 2. Partial results of differential screening for the forward subtractive library. *A*: Membrane A was hybridized with cDNA probes made from reverse subtracted products. *B*: Membrane B was hybridized with cDNA probes made from forward subtracted products. Membrane B was identical to the corresponding A. The arrows designate the positive clones screened from the forward subtractive library.

2.3 Cloning and analysis of *Ube1* cDNA clone

One new EST screened by SSH, which was predominantly expressed in spermatogonia, was used as probe. To obtain the full length of the gene, RACE method was used

and finally cDNA ends of 2 607 bp and 426 bp were amplified by 5'-RACE and 3'-RACE respectively. According to the analysis by using ORF Finder of NCBI, the full-length cDNA of *Ube1* gene is 3 433 bp, consisting of a 169-bp 5' untranslated region, a 90-bp 3' untranslated region and a 3 171-bp open reading frame (ORF), encoding a 1 057-amino acid protein. The 3' untranslated region (3'UTR) possesses typical low G+C content and a putative polyadenylation signal ATAAA is detected 11 bp upstream from the poly(A) tail. The full nucleotide and deduced amino acid sequences of *Ube1* were displayed in Fig.3. The *Ube1* nucleotide sequence displays a 93% identity to mouse ubiquitin-activating enzyme E1, Chr Y 1 (*Ube1y1*) and an 82% identity to human ubiquitin-activating enzyme E1 (*UBE1*). The cDNA sequence of this gene is deposited in GenBank. The accession number is EF690356.

2.4 Feature of *Ube1* amino acid sequence

Ube1 encodes a 1 057-amino acid putative protein with predicted molecular weight of 11.78 kDa and isoelectric point 5.32. The putative protein displays a 91% identity to mouse ubiquitin-activating enzyme E1, Chr Y 1 (Ube1y1) and an 85% identity to human ubiquitin-activating enzyme E1 (UBE1). Analysis of the amino acid sequence by using DNAMAN software revealed that the protein had no typical transmembrane domain, suggesting that Ube1 protein may be a soluble protein. The protein contained an ubiquitin-activating enzyme signature 1 (PS00536) and an ubiquitin-activating enzyme active site (PS00865). The ubiquitin-activating enzyme signature 1 was located at 410-418 aa and the ubiquitin-activating enzyme active site was located at 629-637 aa (Fig.3). So we named this gene as a *Rattus norvegicus* ubiquitin-activating enzyme E1 (*Ube1*).

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1       GAAAAATCTTGTTCCTCGTGTGAGGACGCGGACGCGCCTTTTGTGGAAGGAGTTTCTGAAA
61      AAACTGGTCAGGGACCTGAAGGTGGAGGCTGCACCATCCTGGGACCACGTCTTCGAGGAA
121     GAGAAAGTACGAAGAGAGCGACAGGGAGCGCAAACAGGCACCAGCATTGATGTCCAGCTC
                                                      M  S  S  S
181     GGTCCTGTCCAAGAAACGCAGAGTGTCTGGACCTGACTCTGAGGTGGATTCTAGTTGGCC
         V  L  S  K  K  R  R  V  S  G  P  D  S  E  V  D  S  S  W  P
241     TTCTACCCATTCTGTGATGTTTGGCGCACCCCCAGGGCCAAACAGCGGAATGTCAAAAAA
         S  T  H  S  V  M  F  G  A  P  P  G  P  N  S  G  M  S  K  N
301     CAAGGAAATGGATATAGATGAAAGCCTTTACTCCCGCCAGCTGTATGTACTAGGTCATGA
         K  E  M  D  I  D  E  S  L  Y  S  R  Q  L  Y  V  L  G  H  E
361     GGCAATGAAACATCTCCAGACGTCCAGTGTACTGATATCAGGCCTGCAGGGTCTGGGTGT
         A  M  K  H  L  Q  T  S  S  V  L  I  S  G  L  Q  G  L  G  V
421     GGAAATTGCCAAGAATATCATCCTTGGTGGGGTCAAGGCTGTCACTCTCCATGACCAGGG
         E  I  A  K  N  I  I  L  G  G  V  K  A  V  T  L  H  D  Q  G
481     CACTGCCCAGTGGGCTGATCTGTCTTCCCAGTTCTACCTGCATGAGGAAGATATTGGCAA
         T  A  Q  W  A  D  L  S  S  Q  F  Y  L  H  E  E  D  I  G  K
541     AAATCGAGCTGAGGTATCCCAACCACGCCTTGCTGAACTCAACAGTTATGTTCCTGTGCA
         N  R  A  E  V  S  Q  P  R  L  A  E  L  N  S  Y  V  P  V  H
601     CACTTACACTGGACCTCTAGTTGACGACTTCCTTAGTGGTTTTCAGGTGGTGGTTCTTAC
         T  Y  T  G  P  L  V  D  D  F  L  S  G  F  Q  V  V  V  L  T
661     CAACACTCCTTTGGAATATCAGCTGCAGGTGGGTGAATTCTGCCATAGCCATGGAATCAA
         N  T  P  L  E  Y  Q  L  Q  V  G  E  F  C  H  S  H  G  I  K
721     GCTGGTAGTAGCAGACACTCGGGGCCTAGTTGCACAACTCTTCTGTGACTTTGGAGAGGA
         L  V  V  A  D  T  R  G  L  V  G  Q  L  F  C  D  F  G  E  E
781     AATGATTCTCACTGATGCAAATGGAGAACAGCCACTCAGTGCTATGGTTTCAATGATCAC
         M  I  L  T  D  A  N  G  E  Q  P  L  S  A  M  V  S  M  I  T
841     TAAGGAGAATCCAGGGATTGTCACCTGCTTGGAGGAAACCCGGCATGGATTTGAAAGTGG
         K  E  N  P  G  I  V  T  C  L  E  E  T  R  H  G  F  E  S  G
901     TGACTTTGTCTCTTTCACAGAAGTTCAAGGCATGAGTGAACTGAATGGCATTGGTCCTAT
         D  F  V  S  F  T  E  V  Q  G  M  S  E  L  N  G  I  G  P  M
961     GGAGATCAAAGTTCTGGGTCCCTATTCCTTTAGTATCTGTGATACCTCCAGCTTCTCTGA
         E  I  K  V  L  C  P  Y  S  F  S  I  C  D  T  S  S  F  S  E
1021    GTACACCCGTGGAGGCATTGTCAGTCAAGTGAAAGTATCTCAGAAGATCAGTTTTAAATC
         Y  T  R  G  G  I  V  S  Q  V  K  V  S  Q  K  I  S  F  K  S
1081    CCTAGTTGCCTCGCTGGCAGAGCCAGAGTTTGTGATAACAGATTTTGCTAAGTGCTGTCG
         L  V  A  S  L  A  E  P  E  F  V  I  T  D  F  A  K  C  C  R
1141    CCCTGCTCAGCTCCACATTGGCTTCCAGGCCCTGCATCAGTTCTGTACTCAGCACAGCAG
         P  A  Q  L  H  I  G  F  Q  A  L  H  Q  F  C  T  Q  H  S  R
1201    GCCTCCTCGGCCCCATAATGAGGAGGATGCTGCAGAAATGGTGACCTTAGCACAGGCTGT
         P  P  R  P  H  N  E  E  D  A  A  E  M  V  T  L  A  Q  A  V
1261    GAATGCTCAATCTTTGCCAGCAGTCCAGCAGGATTGCCTAGATATAGACCTCATCCGGAA
         N  A  Q  S  L  P  A  V  Q  Q  D  C  L  D  I  D  L  I  R  K
1321    GTTGGCCTATGTAGCAGCTGGGGATCTGGCACCCATGAGTGCTTTCATTGGTGGTTTGGC
         L  A  Y  V  A  A  G  D  L  A  P  M  S  A  F  I  G  G  L  A
1381    TGCCCAGGAGGTCATGAAGGCTTGTTCTGGAAAGTTTATGCCTATTAGGCAGTGGCTGTA
         A  Q  E  V  M [K  A  C  S  G  K  F  M  P] I  R  Q  W  L  Y
1441    CTTTGATGCCCTCGAATGTCTCCCGGAGCACAGAGTGGCCTTCATGGAAGATAAGTGCCT
         F  D  A  L  E  C  L  P  E  H  R  V  A  F  M  E  D  K  C  L
1501    GCCGCGCCAGAACCGTTACGATGGGCAAGTGGCGGTATTTGGATCAGACCTACAAGAGAA
         P  R  Q  N  R  Y  D  G  Q  V  A  V  F  G  S  D  L  Q  E  K
1561    GCTTGGCAAGCAGAAGTACTTCCTGGTAGGTGCAGGTGCCATTGGTTGTGAGCTGCTCAA
         L  G  K  Q  K  Y  F  L  V  G  A  G  A  I  G  C  E  L  L  K
1621    GAACTTTGCCATGATTGGCCTTGGCTGTGGAGAGGGTGGAGAAATCACAGTTACAGACAT
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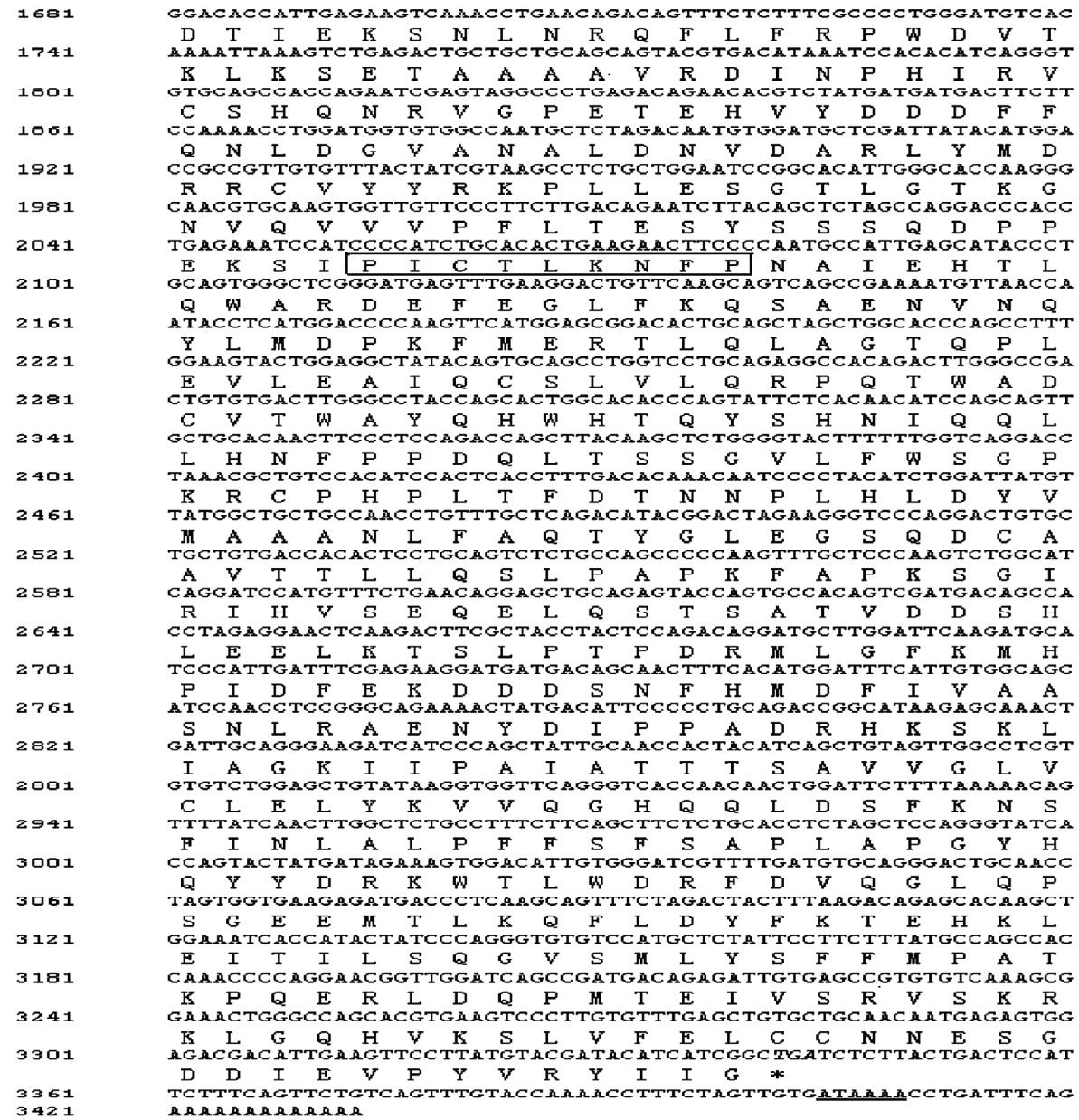

Fig. 3. The nucleotide sequence and deduced amino acid sequence of ubiquitin-activating enzyme 1 (Ube1). The initiation and stop codons are in italic. The trailing signal (ATAAA) is underlined. Ubiquitin-activating enzyme signature 1 (PS00536) and ubiquitin-activating enzyme active site (PS00865) are boxed.

2.5 Expression of *Ube1* in nine different tissues

RT-PCR analysis of RNA from nine different tissues (heart, brain, spleen, lung, liver, muscle, kidney, ovary and testis) showed that *Ube1* was only expressed in testis (Fig.4).

2.6 Expression of *Ube1* in different spermatogenic cells and Sertoli cells

To detect the expression of *Ube1* mRNA in different spermatogenic cells and Sertoli cells, SYBR Green-based quantitative PCR was conducted to analyze the expression of *Ube1* mRNA in type A spermatogonia, pathytene spermatocytes, round spermatids and Sertoli cells respectively. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative quantity of *Ube1* gene transcription. The $2^{-\Delta\Delta Ct}$ values of *Ube1* were 0.03230, 0.00706, 0.00786, 0.00129 in spermatogonia, spermatocytes, round spermatids and Sertoli cells, respectively. These values were analyzed by SPSS 10.0 software and were significantly different from each other (P<0.01, ANOVA). These results suggested that the expression of *Ube1* was strong in spermatogonia and very weak in spermatocytes, round spermatids and Sertoli cells (Fig.5).

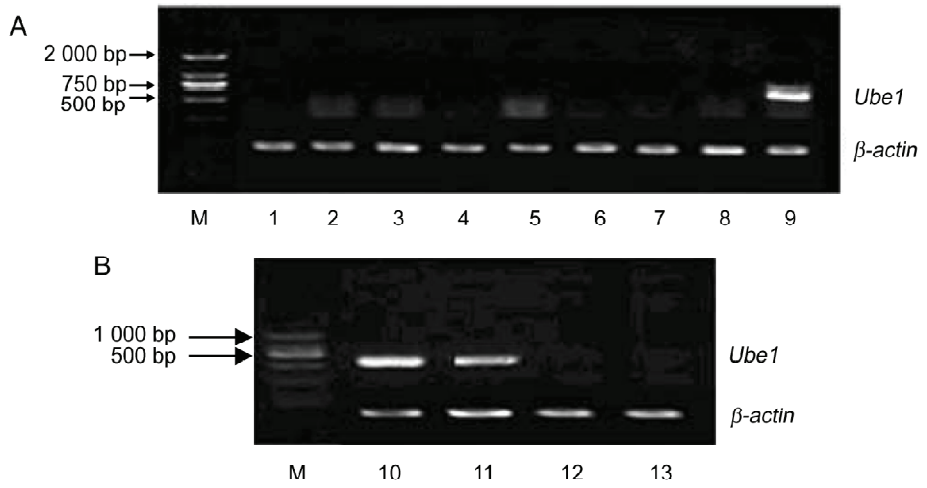

Fig. 4. Expression of *Ube1* in nine different tissues and different spermatogenic cells and Sertoli cells by RT-PCR. *A*: Expression of *Ube1* in nine different tissues with *Ube1* sequence-specific primers. Expression of *Ube1* was only found in the testis and the PCR product was 450 bp. *B*: *Ube1* cDNAs were amplified in spermatogonia, spermatocytes, round spermatids and Sertoli cells. *Ube1* was more highly expressed in spermatogonia than that in spermatocytes. There was no *Ube1* expression in spermatids and Sertoli cells. β-actin in corresponding tissues and cells were displayed in the bottom panel of *A* and *B* as control. Lane 1, heart; Lane 2, liver; Lane 3, spleen; Lane 4, lung; Lane 5, kidney; Lane 6, muscle; Lane 7, brain; Lane 8, ovary; Lane 9, testis; Lane 10, spermatogonia; Lane 11, spermatocytes; Lane 12, round spermatids; Lane 13, Sertoli cells; Lane M, Marker.

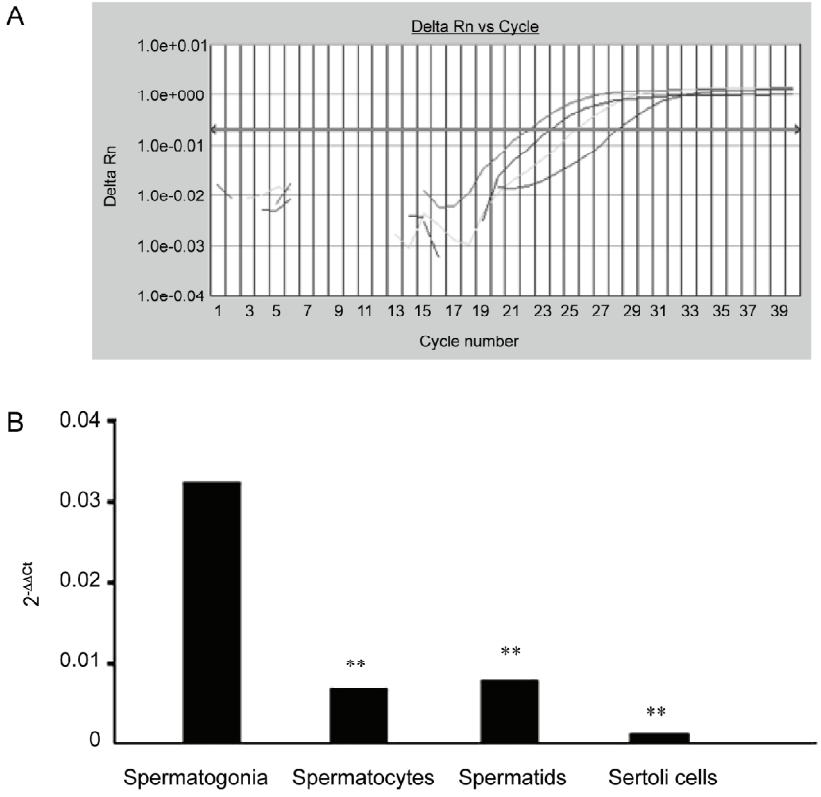

Fig. 5. A representative amplification plot of real-time PCR (*A*) and quantitive comparison of the expression of *Ube1* in different spermatogenic cells and Sertoli cells (*B*). The $2^{-\Delta\Delta Ct}$ values were 0.03230, 0.00706, 0.00786, 0.00129 in spermatogonia, spermatocytes, round spermatids and Sertoli cells, respectively. These values were analyzed by SPSS 10.0 software and were significantly different from each other ($^{**}P<0.01$ *vs* spermatogonia, ANOVA).

3 DISCUSSION

SSH is a widely used method for separating DNA molecules that distinguish two closely related DNA samples[11]. The development of the SSH procedure has led to the identification of many genes that are differentially regulated in various cell and tissue types. Here we screened the genes which were differentially expressed in isolated testicular type A spermatogonia and pathytene spermatocytes by SSH. As a result, we found a new gene in rat, *Ube1*, which was predominately expressed in spermatogonia and expressed weakly in spermatocytes.

Ube1 gene is 3 433 bp in length and encodes a 1 057-amino acid putative protein. The *Ube1* nucleotide sequence displays a 93% identity to mouse ubiquitin-activating enzyme E1, ChrY1 (*Ube1y1*) and an 82% identity to human ubiquitin-activating enzyme E1 (*UBE1*). Comparison of amino acid sequence of Ube1 protein with that of mouse Ube1y1, human UBE1 and wheat ubiquitin-activating enzyme E1 indicates that all of them contain the characteristic ubiquitin-activating enzyme signature 1 (PS00536) and ubiquitin-activating enzyme active site (PS00865). Hence, we consider Ube1 protein as a rat ubiquitin-activating enzyme E1 protein that might play key roles in ubiquitin system.

The salient features of *Ube1* transcription were: (1) While *Ube1* was expressed specifically in testis, its expression was not detected in other tissues of rat; (2) *Ube1* was expressed predominantly in spermatogonia and weakly in spermatocytes and round spermatids; (3)*Ube1* was expressed weakly in Sertoli cells; (4) In the present context, the important feature of *Ube1* expression was that it was highly expressed in spermatogonia and that subsequently there was suppression of expression in spermatocytes and round spermatids. The stage-specific and tissue-specific expression characteristics of *Ube1* indicated that it was probably involved in rat spermatogenesis.

Spermatogenesis is a complex system leading to the formation of mature spermatozoa which can be divided into three distinct stages: the mitotic proliferation of spermatogonial stem cells, meiotic division of spermatocytes and spermiogenesis of haploid spermatids[1]. The transition from the somatic cell-like phenotype of spermatogonia to the unique and motile phenotype of fully differentiated spermatids during mammalian spermatogenesis requires regulated proteolysis and organelle degradation. The ubiquitin-proteasome pathway (UPP) fulfills necessary requirements for the substrate specificity and developmental programming of proteolysis within the differentiating male germ cells. Consequently, a set of genes that encode for testis-specific or alternatively spliced, unique ubiquitin-activating and -conjugating enzymes, along with polyubiquitin genes and those encoding for proteasomal subunits are expressed during spermatogenesis. Some of these genes, if disrupted, lead to altered or arrested spermatogenesis[12-15].

The UPP requires the sequential action of three enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-protein ligase E3. E1 catalyzes the formation of a thiol ester bond between the C-terminal glycine of ubiquitin and a cysteine residue of E1. Then activated ubiquitin moiety is transferred to E2. E2 ligates ubiquitin directly to substrate proteins with or without the assistance of E3[16]. Varied roles of the UPP in cellular physiology and pathology include the endoplasmic reticulum-associated quality control during protein sorting into the secretory pathway (ERAD)[17], antigen presentation[18], cell cycle control[19] and apoptosis.

As the first enzyme in the pathway, ubiquitin-activating enzyme E1 plays an essential role in yeast since the deletion of the yeast ubiquitin-activating enzyme E1 enzyme called UBA1 is lethal[20]. Ube1 protein may regulate diverse events of spermatogenesis through UPP pathway. Ubiquitin-mediated proteolysis activated by the ubiquitin-activating enzyme E1 is involved in different steps and processes during speratogenesis. Ubiquitin-activating enzyme E1 is highly conserved in yeast, plants and mammals (including mouse, human, rabbit)[20-23]. Our research has found the ubiquitin-activating enzyme E1 in rat.

It is known that progression through the cell cycle depends on the specific proteolysis of cyclin. Different cyclins, specific for the G-, S-, or M-phase of the cell cycle, accumulate and activate cyclin-dependent kinases (Cdks) at appropriate time during the cell cycle, and then they are degraded, causing kinase inactivation. Thus they conform the transition of each phase. All cyclins are degraded through the ubiquitin-proteasome pathway[19]. The ubiquitin-activating enzyme E1 plays a key role in cell-cycle progression[24]. So the role of ubiquitin system on cell-cycle control would influence the mitosis of spermatogonia in testes. Our study showed that the *Ube1* gene was just highly expressed in spermatogonia, which might start the ubiquitin pathway and regulate the proliferation of spermatogonia.

Ube1 that we have identified is a rat ubiquitin-activating enzyme E1 gene specifically expressed in testis, so it may play key roles in spermatogenesis and male fertility. We are going to use RNA interference to knock down *Ube1* gene expression in the rat *in vivo* to investigate the effect

of *Ube1* in spermatogenesis. We predict that the further investigation of *Ube1* would promote insight into mammalian spermatogenesis.

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