BIOLOGY OF REPRODUCTION **73,** 1235–1242 (2005)
Published online before print 17 August 2005.
DOI 10.1095/biolreprod.105.045468

Catsper3 and *Catsper4* Encode Two Cation Channel-Like Proteins Exclusively Expressed in the Testis

Jing-Ling Jin, Alan M. O'Doherty, Shouhua Wang, Huili Zheng, Kenton M. Sanders, and Wei Yan[1]

Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, Nevada 89557

ABSTRACT

CATSPER1 and CATSPER2 are two cation channel-like proteins exclusively expressed in the testis and essential for normal sperm motility and male fertility. Using in silico subtraction and database mining, we identified expressed sequence tags encoding two previously uncharacterized cation channel-like proteins structurally homologous to CATSPER1 and CATSPER2. Similar to CATSPER1 and CATSPER2, these two proteins contain a single-ion transport domain comprised of six transmembrane spanning regions, in which the fourth transmembrane region resembles a voltage sensor and a pore-forming region lies between transmembrane regions 5 and 6. The pore contains the consensus sequence T × D × W, which is indicative of a potential calcium-selective channel. The mRNAs for *Catsper3* and *Catsper4* were detected exclusively in the testis using multitissue Northern blot and RT-PCR analyses. The onsets of both genes coincide with the first appearance of spermatids during testicular development. In situ hybridization analyses revealed that *Catsper3* and *Catsper4* mRNAs displayed identical localization patterns and were confined to spermatids of steps 1–8. Immunofluorescence and immunohistochemistry analyses demonstrated that these two proteins were expressed within the acrosome of late spermatids and spermatozoa. Our data suggest that CATSPER3 and CATSPER4 are two cation-channel proteins and have roles in acrosome reaction and male fertility.

acrosome reaction, calcium, sperm, sperm motility and transport, spermatogenesis

INTRODUCTION

Many ion channels, especially calcium channels, are present in sperm. These channels include high voltage-gated calcium channels (Ca_v 1.2, 2.1/2/3) [1]; cyclic nucleotide-gated channels [2]; the transient receptor potential channel [3, 4]; the voltage-operated Ca^{2+}-channels α1A, α1C, and α1E [5, 6]; the T-type voltage-operated Ca^{2+}-channels α1G and α1H [7–10]; the N-type, R-type [11], and L-type voltage operated Ca^{2+} channels [12]; and pkD2 cation channel [13]. However, the molecular identity and/or physiological roles of these channels remain largely undefined.

CATSPER1 and CATSPER2 are putative six-transmembrane, voltage-gated ion channels located in the sperm flagellum [14–18]. Gene targeting studies show that both are required for cAMP-induced Ca^{2+} current essential for normal sperm motility and male fertility [14]. Similar structure and expression patterns of these two proteins, as well as identical phenotypes in the *Catsper1* and *Catsper2* knockout mice, strongly suggest that they may work together as a heterodimer. However, heterologous expression of either or both fails to induce detectable ionic current and to modify intracellular Ca^{2+} concentration [14–16]. It has been suggested that CATSPER1 and CATSPER2 require additional subunits and/or interacting partners to function, because the pore-forming units of voltage-gated cation channels form a tetramer [14–16, 19]. Recently, Lobley et al. reported the in silico identification of *Catsper3* and *Catsper4* cDNAs [19]. However, little is known about their potential function because of the lack of experimental data.

In a search for male germ cell-specific genes using in silico subtraction and database mining [20–24], we identified ESTs encoding CATSPER3 and CATSPER4 from several testis libraries. Here, we report the expressions and localizations of *Catsper3* and *Catsper4* mRNAs and proteins. Our data suggest that CATSPER3 and CATSPER4 may not form a tetramer with CATSPER1 and CATSPER2 because of their different expression and localization patterns, and CATSPER3 and CATSPER4 may be involved in acrosome reaction.

MATERIALS AND METHODS

Animals

C57BL/6J F_1 mice were maintained under constant temperature (22°C) in a standard animal facility with free access to pelleted food and water at the University of Nevada School of Medicine. All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Nevada, Reno.

In Silico Subtraction and Database Mining

Expressed sequence tags (ESTs; n = 49,064) from three libraries (Lib.6786 [round spermatids], Lib.2547 [adult testis], and Lib.2511 [adult testis]) were downloaded from the mouse Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Mm) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). In silico subtraction was performed as described previously [22]. Using the BLAST program at the NCBI, mouse *Catsper3* and *Catsper4* cDNA and protein sequences were used as baits to search against the nucleotide and protein databases and to identify orthologs of these two proteins in other species, including the rat, dog, chimpanzee, and human.

Protein Alignment Analyses

Alignments of CATSPER3 and CATSPER4 proteins of different species were performed using the MegAlign program of the DNASTAR software package (DNASTAR, Inc.). The sequence identity analyses were performed using the same program.

Northern Blot Analyses

Total RNA (15 μg) isolated from multiple mouse tissues was fractionated on a 1.2% formaldehyde-agarose gel and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech). A 369-bp PCR fragment

[1]Correspondence: Wei Yan, Department of Physiology and Cell Biology, University of Nevada School of Medicine, 1664 North Virginia Street, MS 352, Reno, NV 89557. FAX: 775 784 6903;
e-mail: weiyan@unr.edu

Received: 12 July 2005
First decision: 29 July 2005
Accepted: 15 August 2005

ISSN: 0006-3363. http://www.biolreprod.org

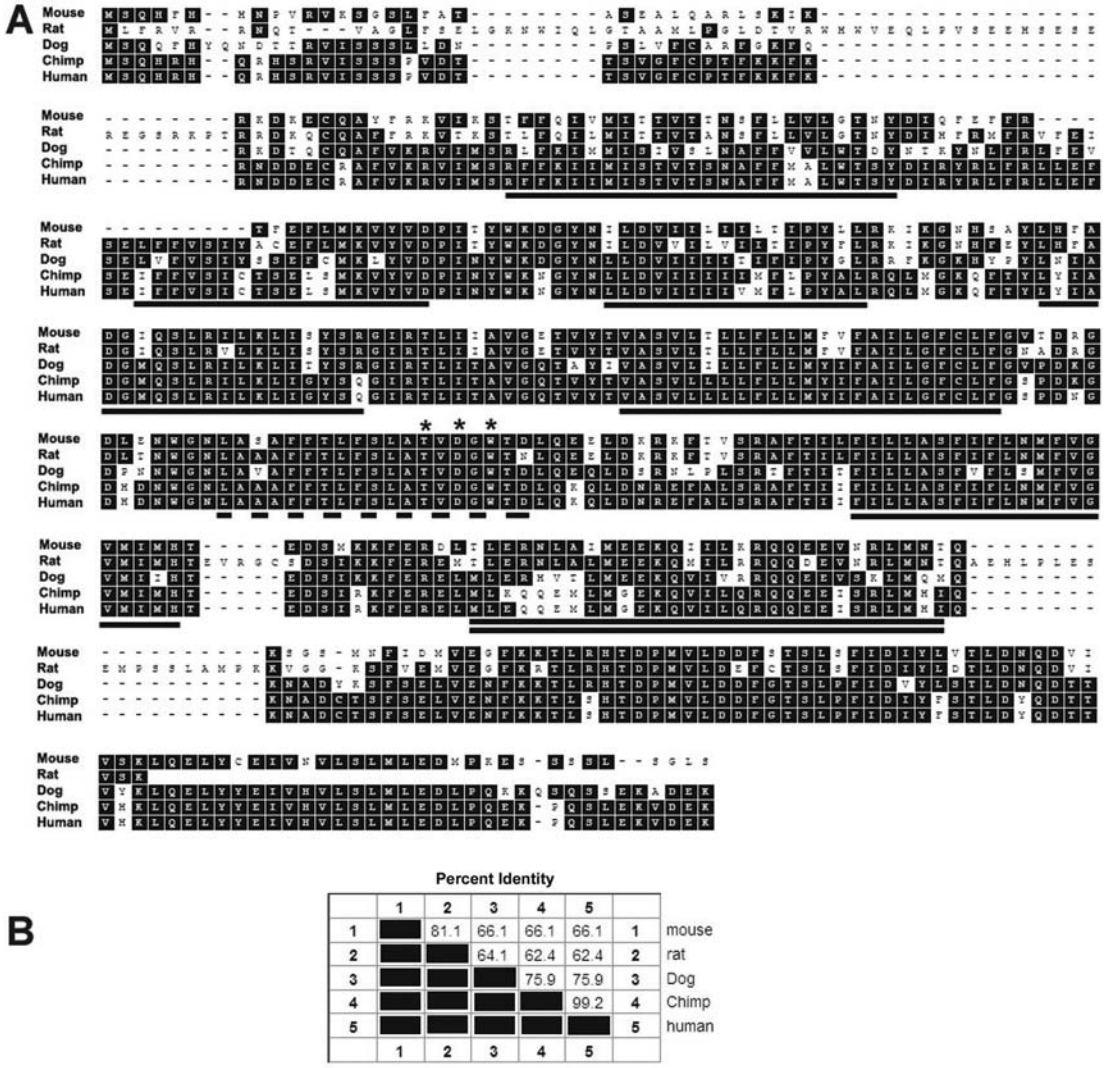

FIG. 1. Alignments of orthologous CATSPER3 proteins in different species. **A**) The mouse, rat, dog, chimpanzee, and human CATSPER3 proteins are aligned, and identical residues are shaded. Predicted transmembrane regions are underlined. The pore-forming region is dash lined, and the coiled-coil domain is double-underlined. **B**) Percentage identity of CATSPER3 orthologs in different species. Numbers show the percentage identity of amino acid sequences.. The human and chimpanzee CATSPER3 proteins are 99.2% identical, whereas the mouse CATSPER3 shares 66.1% amino acid sequence identity with the human CATSPER3.

corresponding to nucleotides 98–466 of the *Catsper3* cDNA (GenBank accession no. BC089518) and a 532-bp PCR fragment corresponding to nucleotides 364–895 of the *Catsper4* cDNA (GenBank accession no. NM_177866) were labeled with [α-^{32}P]dCTP using a RediPrime TM II labeling system (Amersham Pharmacia Biotech). Membrane hybridization, washing, and autoradiography, as well as stripping and reprobing, were performed according to the manufacturer's instructions. Blots were stripped and hybridized with an 18S rRNA cDNA labeled with [α-^{32}P]dCTP to control for equal RNA loading.

Semiquantitative RT-PCR

Reverse transcription reaction was performed in a 50-μl volume containing 2 μg of total RNA, 1× RT Buffer, 100 pmol of random primer, 1 mM deoxynucleoside triphosphate, 10 U of RNase inhibitor (RNasin), and 5 U of avian myeloblastosis virus reverse transcriptase at room temperature overnight. All RT reagents were purchased from Promega Corporation. An aliquot (2 μl) of the cDNAs synthesized as described above was used as template for a semiquantitative PCR analysis. Primers (upstream, 5′-TTCACCACAACCCTGTACGA-3′; downstream, 5′-ATGCAGAAT-GATTCCCCTTG-3′) were used to amplify a 369-bp fragment of *Catsper3* cDNA, and another pair of primers (upstream, 5′-CCTTATCTGC-GAGGTTCTGC-3′; downstream, 5′-TTGTTCCAGGTTTGTGGTCA-3′) were employed to yield a 532-bp fragment of *Catsper4* cDNA. As a loading control, a housekeeping gene, *Hprt,* was amplified using a primer set described previously [22].

In Situ Hybridization

In situ hybridization was performed as described previously [25]. Briefly, paraffin-embedded testes were cut into sections (thickness, 5 μm), and cDNA fragments of *Catsper3* and *Catsper4* were subcloned into a pGEM-T vector (Promega). Next, [α-^{35}S]UTP-labeled sense and antisense riboprobes were generated using a Riboprobe In Vitro Transcription System (Promega). Hybridization signals were detected by autoradiography using NTB-2 emulsion (Eastman Kodak Co.). After development and fixation, the slides were counterstained with hematoxylin and mounted for photography.

Productions of CATSPER3 and CATSPER4 Recombinant Proteins and Polyclonal Antibodies

A pET protein production system (EMD Biosciences, Inc.) was employed to produce mouse CATSPER3 and CATSPER4 proteins. A cDNA fragment (1108 bp) from the coding region of *Catsper3* was amplified by PCR using the primers 5'-GGATCCCACCACAACCCTGTACGA-3′ (with *Bam*HI adaptor, GGATCC) and 5'-CTCGAGGCTCTCCTTGGGCATGTCTTC-3′ (with *Xho*I

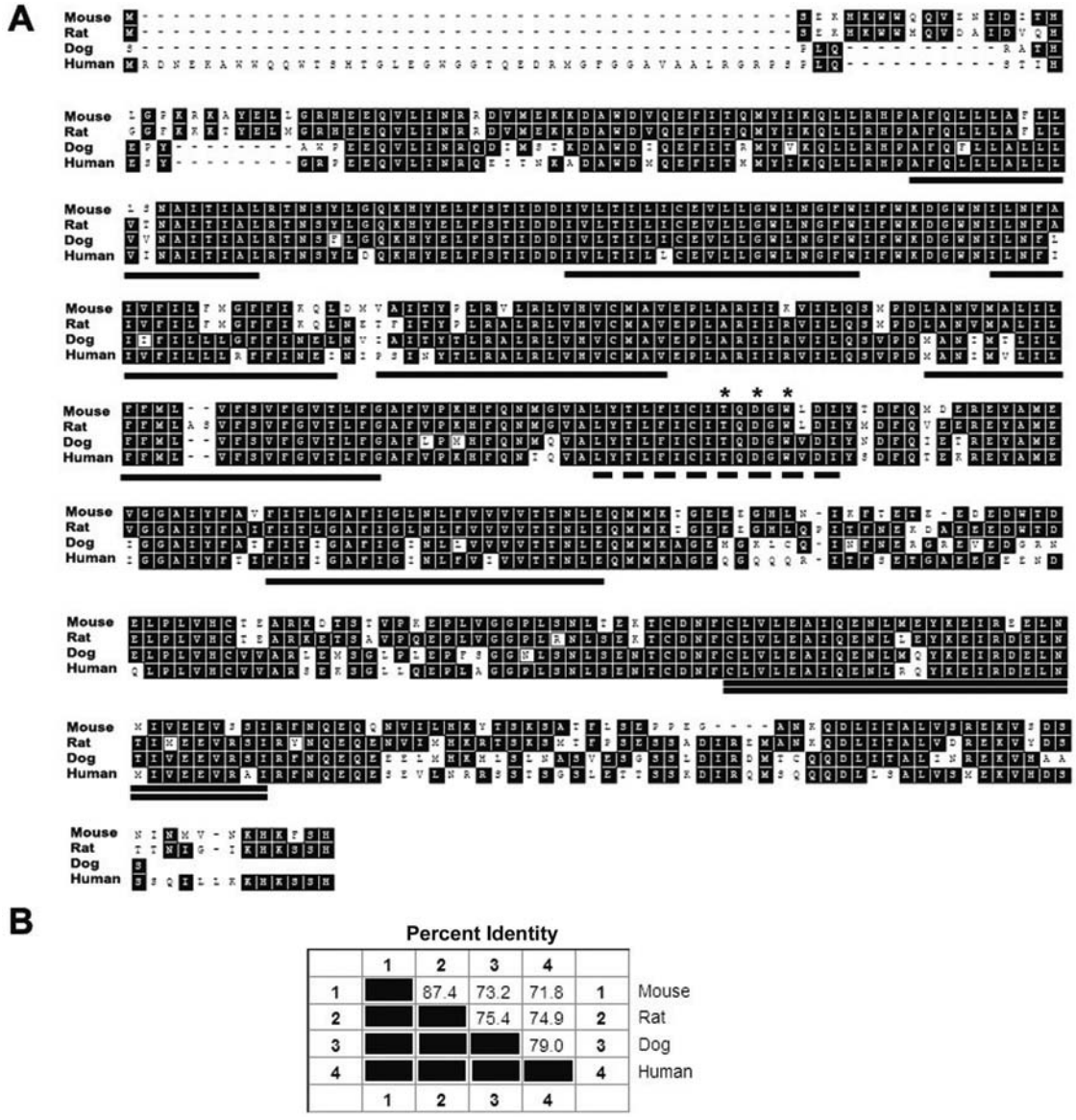

FIG. 2. Alignments of orthologous CATSPER4 proteins in different species. **A**) The mouse, rat, dog, and human CATSPER4 proteins are aligned, and identical residues are shaded. Predicted transmembrane regions are underlined. The pore forming region is dash lined, and the coiled-coil domain is double-underlined. **B**) Percentage identity of CATSPER4 orthologs in different species. Numbers show the percentage identity of amino acid sequences. The mouse and rat CATSPER4 proteins are 87.4% identical, whereas the mouse CATSPER4 shares 71.8% amino acid sequence identity with the human CATSPER4.

adapter, CTCGAG). For *Catsper4,* a cDNA fragment (1261 bp) from the coding region was amplified by PCR using the primers 5'-GGATCCG-CAGGTGGAGAACATCGAC-3′ (with *Bam*HI adaptor, GGATCC) and 5'-AAGCTTATCAGACACCTTTTCCCTGCT-3′ (with *Hin*dIII adapter, AAGCTT).

The PCR products were subcloned into pGEM-T vector (Promega Corp.) and sequenced to confirm the sequence accuracy. The *Bam*HI/*Xho*I fragment of *Catsper3* and the *Bam*HI/*Hin*dIII fragment of *Catsper4* were then subcloned into pET-23b vector (EMD Biosciences), respectively. Protein induction and purification were performed according to the manufacturer's instructions. Fusion proteins of CATSPER3 and CATSPER4 containing the full-length protein, N-terminal T7 flag, and C-terminal histidine tag were used to immunize rabbits to produce polyclonal antibodies (Cocalico Biologicals, Inc.).

Western Blot Analyses

Proteins were isolated from adult mice tissues, including heart, liver, spleen, lung, kidney, brain, testis, and ovary. Western blot analyses were performed as described previously [26]. Rabbit anti-CATSPER3 and anti-CATSPER4 polyclonal antibodies were used at a dilution of 1:1000.

Immunofluorescence and Confocal Microscopy

Testis cryosections were prepared as described previously [27]. Sections were incubated with anti-CATSPER3 and anti-CATSPER4 antibodies (diluted at 1:1000) for 1 h, followed by three washes (15 min each) in TBST (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 0.1% Tween 20). The slides were then incubated with 3 μg/ml of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 45 min. The DNA was counterstained with propidium iodide (1 μg/ml) for 10 min. Preimmune serum was used for controls. The staining patterns were observed and photographed using a confocal laser-scanning microscope (model LSM500; Carl Zeiss Microimaging) and its imaging system.

Immunohistochemistry

Cauda epididymal sperm were spread onto Superfrost Plus slides (Fisher Scientific, Inc.) and air-dried. Microwave antigen retrieval was employed as described previously [25]. An aliquot of 100 μl of primary antibody diluted at 1:1000 was applied to each slide and incubated at 4°C overnight. Incubation with secondary antibody and visualization of positive cells were performed

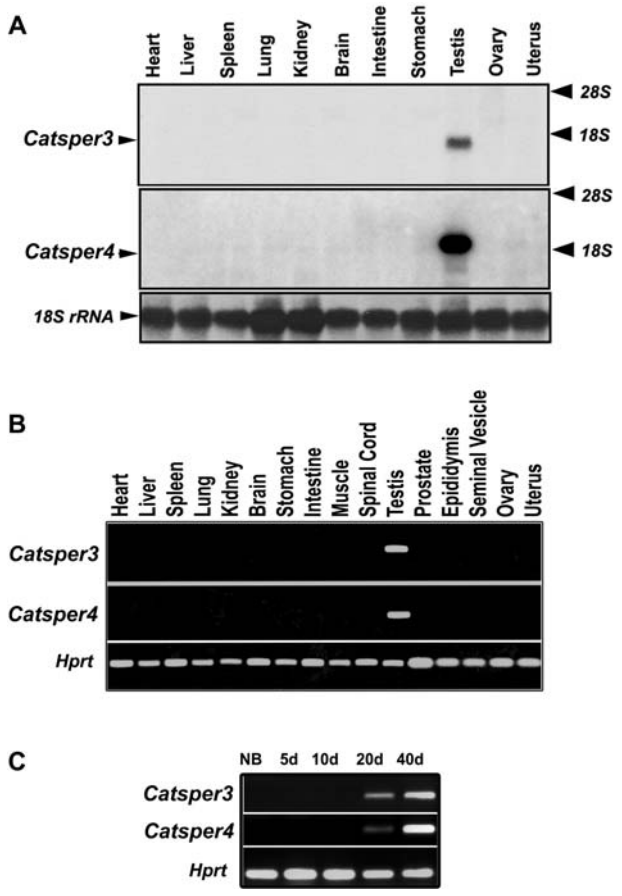

FIG. 3. Expressions of *Catsper3* and *Catsper4* mRNAs in mouse tissues. **A**) Northern blot analyses of *Catsper3* and *Catsper4* mRNA expression in mouse tissues, including heart, liver, spleen, lung, kidney, brain, intestine, stomach, testis, ovary, and uterus. The blots were stripped and rehybridized with an 18S rRNA cDNA probe, which served as a loading control. **B**) RT-PCR analyses of *Catsper3* and *Catsper4* mRNAs in multiple mouse tissues, including heart, liver, spleen, lung, kidney, brain, stomach, intestine, muscle, spinal cord, testis, prostate, epididymis, seminal vesicle, ovary, and uterus. The PCRs for *Catsper3* and *Catsper4* were run for 40 cycles, whereas *Hprt* (as a loading control) was amplified for 20 cycles. **C**) Semiquantitative PCR analyses of mouse *Catsper3* and *Catsper4* mRNAs during testicular development. Mouse *Catsper3* and *Catsper4* cDNAs from newborn (NB; ~0.5 day), 5 (5d), 10 (10d), 20 (20d), and 40-day-old (40d) testes were used as templates. *Hprt* was amplified as a loading control.

using a VectaStain Elite kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. Preimmune serum was used in control slides.

RESULTS

Characteristics of CATSPER3 and CATSPER4

Among the 10 ESTs identified to contain open reading frames (ORFs) encoding a protein structurally homologous to CATSPER1 and CATSPER2, one clone contained an 1149-bp ORF. We designed primers and successfully amplified the entire ORF (data not shown), indicating that this EST contained the full-length cDNA for *Catsper3* (GenBank accession no. BC089518). Similarly, we found another EST (of 17 identified) with a 1329-bp ORF encoding another CATSPER homolog that contained the full-length cDNA for *Catsper4* (GenBank accession no. NM_177866).

Similar to CATSPER1 and CATSPER2, both CATSPER3 and CATSPER4 contain six transmembrane regions, which are close together and joined by short loop regions. A short, conserved hydrophobic stretch representing the pore-forming region is present in a longer loop region between the fifth and sixth transmembrane domains (Figs. 1A and 2A). The specific arrangement of these transmembrane helices is characteristic of the voltage-gated channel ion-transport domain found in the voltage-gated K^+, Ca^{2+}, and Na^+ channels and also reported for CATSPER1 and CATSPER2 [14–16].

The voltage sensor is located within the fourth transmembrane helix and participates in channel activation through positively charged residues positioned every three to four amino acids [28]. Ion specificity is determined by a pore consensus sequence, [T/S] × [D/E] × W, in voltage-gated calcium channels [29]. Sequence analyses of this region revealed the presence of a similar conserved motif, T × D × W, suggesting that CATSPER3 and CATSPER4 may be selective for calcium ions, as discussed previously for CATSPER1 and CATSPER2 [14–16]. Interestingly, a coiled-coil motif recently described and believed to be a protein-protein interaction interface [19] is also present in CATSPER3 and CATSPER4 (Figs. 1A and 2A). Therefore, CATSPER1 through CATSPER4 comprise a CATSPER protein family characteristic of a single-ion transport domain and a C-terminal coiled-coil motif. Despite the structural similarities, the four CATSPER proteins are distinct in sequence relationship; sequence identity ranges between 16% and 27% across the ion-transport domain (data not shown). This low sequence identity contrasts with that observed for the voltage-gated sodium- and calcium-channel families, which display higher sequence identity between their corresponding ion-transport repeat regions [19]. Interestingly, we could not detect CATSPER-like proteins in species lower than the mouse. These data strongly suggest that the CATSPER family proteins neither were derived from an early duplication event nor resulted from convergent evolution of ion-channel genes at different chromosomes toward a common function. Lobley et al. [19] suggest that *Catsper* genes may result from a more recent evolutionary event or rapid evolution. Nevertheless, when we compare orthologous CATSPER3 and CATSPER4 in several mammalian species, we found that these two proteins were highly conserved during evolution. The sequence identity of CATSPER3 orthologs ranges from 61.1% (mouse vs. rat) to 99.2% (chimpanzee vs. human), and the sequence identity of CATSPER4 orthologs varies from 71.8% (mouse vs. human) to 87.4% (mouse vs. rat) (Figs. 1B and 2B).

Expression and Localization of Mouse Catsper3 *and* Catsper4 *mRNAs*

The PCR fragments amplified from mouse *Catsper3* and *Catsper4* cDNAs were used as probes for multitissue Northern blot analyses. The *Catsper3* probe hybridized to an approximately 1.6-kb transcript, and the *Catsper4* probe detected an approximately 2.0-kb transcript. Both transcripts were detected exclusively in the testis (Fig. 3A). To reveal potential low-level expression in other tissues, we employed RT-PCR analysis in which *Catsper3* and *Catsper4* cDNAs were amplified for 40 cycles, but the housekeeping gene *Hprt* (loading controls) was amplified for 20 cycles (in the exponential range). In this way, we can achieve maximal sensitivity in amplifying target templates with very low expression levels. As shown in Figure 3B, no signals were detected from multiple tissues, except for the testis.

We further examined levels of *Catsper3* and *Catsper4* mRNAs during testicular development using a semiquantitative RT-PCR analysis [22]. Neither *Catsper3* nor *Catsper4* mRNA was detected before Postnatal Day (P) 10 (Fig. 3C). They were

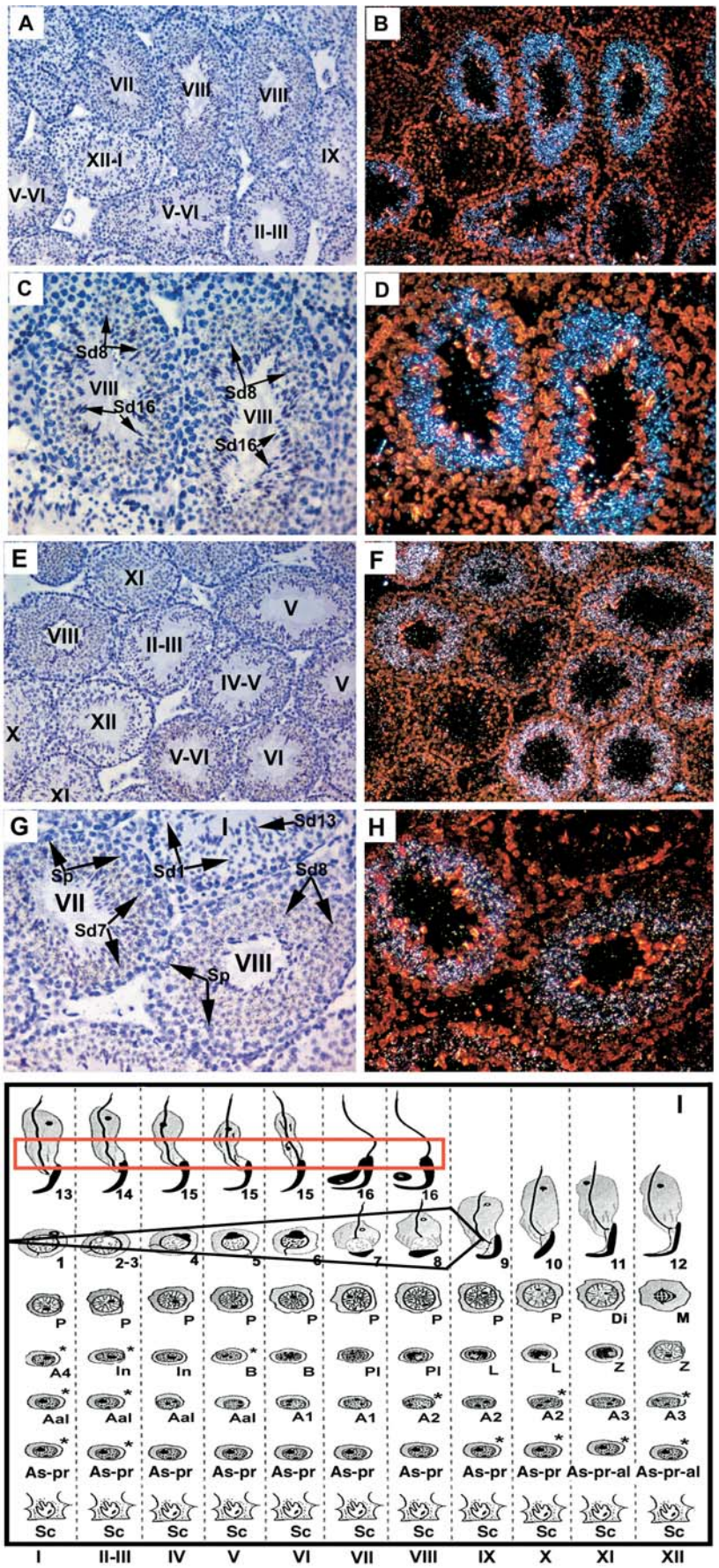

FIG. 4. Localizations of *Catsper3* and *Catsper4* mRNAs in the mouse testis by in situ hybridization. **A**) Localization of *Catsper3* mRNA in the mouse testis. Bright-field (**A** and **C**) and dark-field (**B** and **D**) images are shown. Specific hybridization signals are confined to step-1 to step-8 spermatids (Sd) of the adult testis (**A** and **B**). Roman numerals represent stages of the tubules. High-magnification images (**C and D**) show two-stage-VIII tubules with intensive hybridization signals in step-8 spermatids but not in step-16 spermatids. **B**) Localization of *Catsper4* mRNA in the mouse testis. Bright-field (**E** and **G**) and dark field (**F** and **H**) images are shown. Specific hybridization signals are confined to step-1 to step-8 spermatids (Sd) in the adult testis (**E** and **F**). High-magnification images (**G** and **H**) show a stage-VII tubule and a stage-VIII tubule with intensive hybridization signals in step-7 (Sd7) and step-8 (Sd8) spermatids but not in step-16 spermatids. **C**) Schematic illustration of the expression sites of *Catsper3* and *Catsper4* mRNAs and proteins. Both CATSPER3 and CATSPER4 display identical mRNA and protein expression patterns. The black frame represents mRNA expression sites of *Catsper3* and *Catsper4*, and the red frame illustrates protein expression sites of CATSPER3 and CATSPER4. The width of the frames represents levels of expression. The specific cell associations in the vertical columns represent specific stages (Roman numerals) of the epithelial cycle. Arabic numbers represent the steps of spermatides. Sc, Sertoli cells; A1–4, type A1–4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; Di, diplotene spermatocytes; M, meiotically dividing spermatocytes. Original magnification ×200 (**A**, **B**, **E**, and **F**) and ×400 (**C**, **D**, **G**, and **H**).

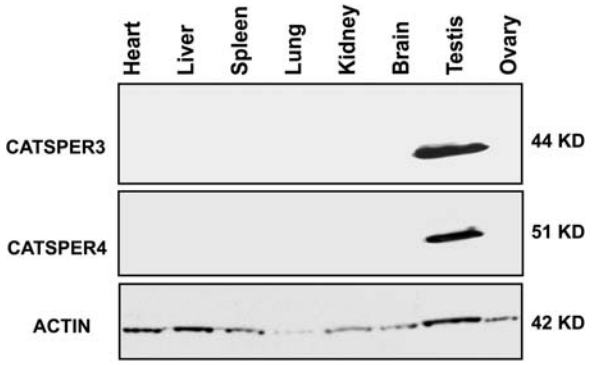

FIG. 5. Western blot analyses of CATSPER3 and CATSPER4 proteins in multiple mouse tissues, including heart, liver, spleen, lung, kidney, brain, testis, and ovary. A 44-kDa band for CATSPER3 and a 51-kDa band for CATSPER4 were only detected in the testis (two upper panels). The membrane was stripped and subsequently blotted with an anti-ACTIN monoclonal antibody to monitor the loading (lower panel).

first detected at P20, and the levels increased at P40 (Fig. 3C). The onset of *Catsper3* and *Catsper4* mRNA expression coincides with the first appearance of haploid cells in the developing testis (P18), suggesting that spermatids are the germ cell type expressing these two transcripts. To confirm further the expression sites of *Catsper3* and *Catsper4* mRNAs, we performed in situ hybridization analyses. We found that hybridization signals were confined to spermatids of steps 1–8, and the hybridization patterns of *Catsper3* and *Catsper4* were identical (Fig. 4). The intensity of hybridization signals displayed a stage-specific pattern, in which both mRNA levels gradually increased from step 1 through step 7, reached a peak in step 8, and diminished thereafter (Fig. 4).

Productions of CATSPER3 and CATSPER4 Proteins and Antibodies

Full-length mouse CATSPER3 and CATSPER4 proteins fused with histidine tags at their C termini were produced using

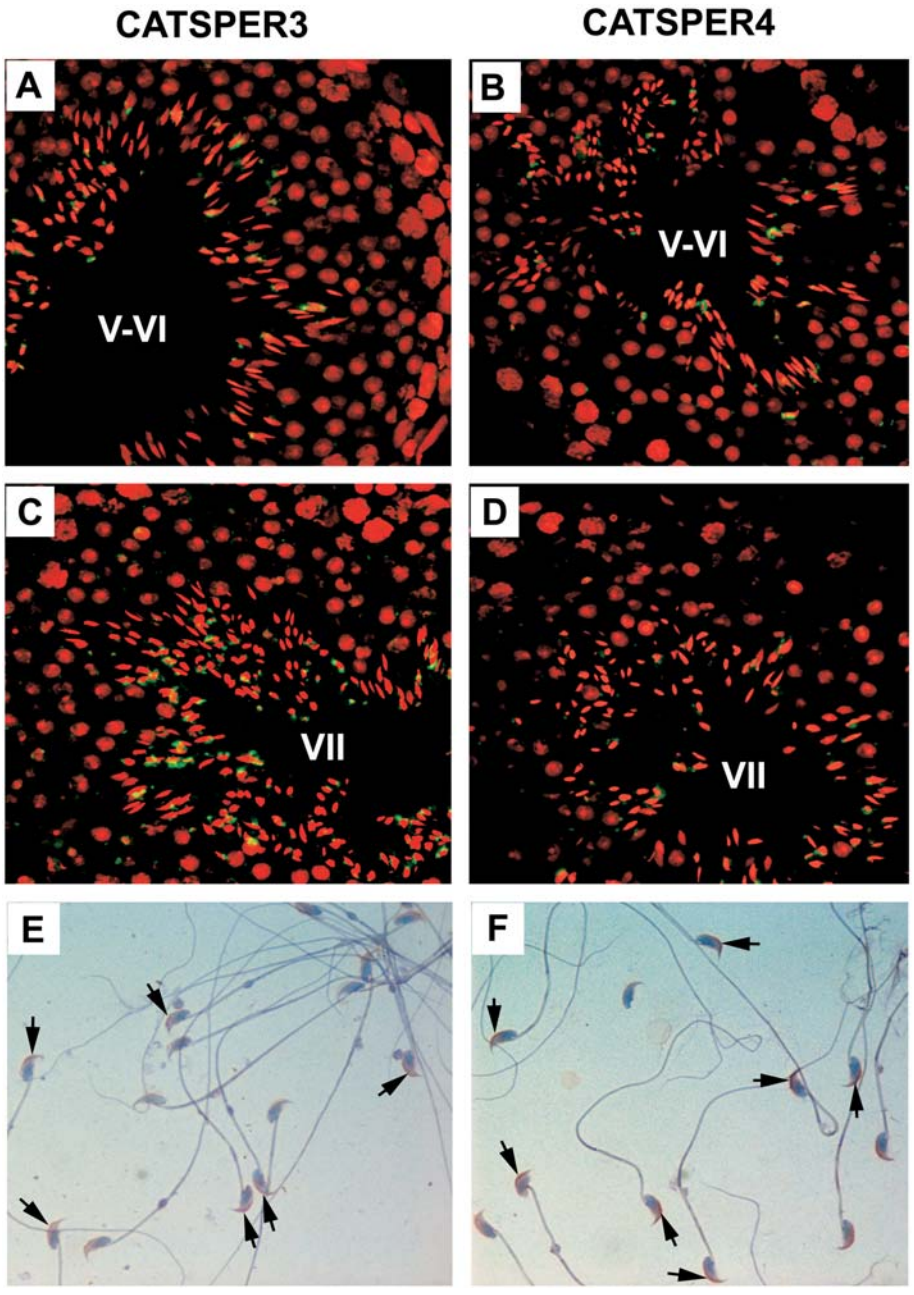

FIG. 6. Localization of CATSPER3 and CATSPER4 proteins. **A–D**) immunofluorescent detection of CATSPER3 and CATSPER4 proteins in the mouse testis. Confocal microscopic images of stage-V to stage-VI tubules (**A** and **B**) and stage-VII tubules (**C** and **D**) demonstrate that CATSPER3 (**A** and **C,** labeled in green) and CATSPER4 (**B** and **D,** labeled in green) localize to the acrosome of step-15 to step-16 spermatids. Propidium iodide (red) was used to stain the nuclei. **E** and **F**) Immunohistochemical detections of CATSPER3 (**E**) and CATSPER4 (**F**) proteins in the mouse sperm. Both CATSPER3 (**E**) and CATSPER4 (**F**) proteins are detected in the acrosome of sperm (shown in brown). Original magnification ×630 (**A**-**D**) and ×1000 (**E** and **F**).

the pET protein production system. The proteins were predominantly expressed in inclusion bodies in *Escherichia coli* (strain BL-21). Using a His affinity purification method [22], we purified the recombinant CATSPER3 and CATSPER4 proteins, which were then used to immunize rabbits to generate polyclonal antibodies. To test further the specificity and confirm the tissue distribution of CATSPER3 and CATSPER4 in the mouse, we performed Western blot analysis using proteins isolated from multiple mouse tissues (Fig. 5). The rabbit anti-CATSPER3 and anti-CATSPER4 antibodies detected as little as 1 ng of recombinant CATSPER3 and CATSPER4 proteins, respectively, and the preimmune sera did not react with the recombinant protein (data not shown). Western blot analyses revealed an approximately 44-kDa band for CATSPER3 and an approximately 51-kDa band for CATSPER4, which are consistent with the predicted sizes of both proteins. These results are consistent with our Northern blot (Fig. 3A) and RT-PCR (Fig. 3B) results, which showed that these two proteins were exclusively expressed in the testis and demonstrated that the polyclonal antibodies raised against CATSPER3 and CATSPER4 were specific.

Expressions and Localizations of CATSPER3 and CATSPER4 Proteins in Mouse Testis and Sperm

Indirect immunofluorescent labeling with the anti-CATSPER3 and anti-CATSPER4 antisera and confocal microscopy were used to assess protein expressions in the testis. Both CATSPER3 and CATSPER4 were detected in the acrosomal region of elongated spermatides (steps 13–16) (Fig. 6, A–D). The immunoreactivity for CATSPER3 and CATSPER4 was detected mainly in the acrosome of sperm isolated from cauda epididymis (Fig. 6, E and F). No immunoreactivity was detected in control slides using preimmune sera as the primary antibodies (data not shown).

DISCUSSION

Our data demonstrated that *Catsper3* and *Catsper4* mRNAs were expressed in round spermatids (steps 1–8), whereas CATSPER3 and CATSPER4 proteins were located in the acrosome of the late spermatids and spermatozoa. The delayed protein expression reflects the fact that transcription ceases when spermatid elongation and chromatin condensation start in step 9. Therefore, mRNAs for proteins needed during late spermiogenesis must be produced before step-9 spermatids. This is a phenomenon common to many spermatid-specific genes [30–35].

Both CATSPER3 and CATSPER4, together with previously well-studied CATSPER1 and CATSPER2, constitute a CATSPER protein family. Its members share similar domain structure but display low amino acid sequence identity. Both CATSPER1 and CATSPER2 localize to the flagellum of sperm, and disruption of either *Catsper1* or *Catsper2* results in infertility because of defects in sperm motility. These data suggest that these two CATSPER proteins may form a single heterodimeric channel. However, attempts to coimmunoprecipitate these two proteins have failed [15], and to our knowledge, neither CATSPER1 nor CATSPER2 has been shown to function as a cation channel when transfected into cells either singly or in conjunction. The presence of CATSPER3 and CATSPER4 prompted suggestions that the four CATSPER proteins form a functional heterotetrameric channel in sperm [16, 19]. However, our data are not supportive to this hypothesis, because CATSPER3 and CATSPER4 are present in the acrosome of sperm but CATSPER1 and CATSPER2 are located in the flagellum of sperm. Distinct localization patterns between CATSPER1/2 and CATSPER3/4 suggest that CATSPER3 and CATSPER4 have different roles in sperm. Based on their acrosomal localization, CATSPER3 and CATSPER4 may be involved in the acrosome reaction, which is a Ca^{2+}-dependent secretory event and an essential early step in the fertilization process [36, 37]. It has been reported that Ca^{2+} and cyclic nucleotides can induce the acrosome reaction [38–40]. Voltage-gated Ca^{2+} channels are believed to mediate the entry of extracellular calcium [41–43]. Therefore, CATSPER3 and CATSPER4 may have a role in regulating intracellular Ca^{2+} levels during the acrosome reaction.

Based on their identical patterns of mRNA and protein expression as well as localization, we postulate that CATSPER3 and CATSPER4 may form a heterodimer and, thus, function as a single channel. The interactions between CATSPER3 and CATSPER4 could be mediated directly through their C-terminal coiled-coil domains or indirectly via other intracellular adaptor proteins binding to the coiled-coil domains of CATSPER3 and CATSPER4. The α-helical coiled-coil motifs have been shown to mediate subunit multimerization of a large number of proteins [44, 45]. Further biochemical and genetic studies are needed to reveal the physiological roles of CATSPER3 and CATSPER4. A widely used calcium-channel blocker, nifedipine, has been shown to display a reversible contraceptive effect [46], which likely is mediated via voltage-gated calcium channels, primarily the L-type voltage-gated channels. Therefore, CATSPER ion channels appear to be attractive targets for nonhormonal contraceptives.

REFERENCES

1. Trevino CL, Felix R, Castellano LE, Gutierrez C, Rodriguez D, Pacheco J, Lopez-Gonzalez I, Gomora JC, Tsutsumi V, Hernandez-Cruz A, Fiordelisio T, Scaling AL, et al. Expression and differential cell distribution of low-threshold Ca^{2+} channels in mammalian male germ cells and sperm. FEBS Lett 2004; 563:87–92.
2. Wiesner B, Weiner J, Middendorff R, Hagen V, Kaupp UB, Weyand I. Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. J Cell Biol 1998; 142:473–484.
3. Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM. Trp2 regulates entry of Ca^{2+} into mouse sperm triggered by egg ZP3. Nat Cell Biol 2001; 3:499–502.
4. Castellano LE, Trevino CL, Rodriguez D, Serrano CJ, Pacheco J, Tsutsumi V, Felix R, Darszon A. Transient receptor potential (TRPC) channels in human sperm: expression, cellular localization and involvement in the regulation of flagellar motility. FEBS Lett 2003; 541:69–74.
5. Westenbroek RE, Babcock DF. Discrete regional distributions suggest diverse functional roles of calcium channel α1 subunits in sperm. Dev Biol 1999; 207:457–469.
6. Park JY, Ahn HJ, Gu JG, Lee KH, Kim JS, Kang HW, Lee JH. Molecular identification of Ca^{2+} channels in human sperm. Exp Mol Med 2003; 35: 285–292.
7. Son WY, Lee JH, Han CT. Acrosome reaction of human spermatozoa is mainly mediated by α1H T-type calcium channels. Mol Hum Reprod 2000; 6:893–897.
8. Stamboulian S, Kim D, Shin HS, Ronjat M, De Waard M, Arnoult C. Biophysical and pharmacological characterization of spermatogenic T-type calcium current in mice lacking the CaV3.1 (α1G) calcium channel: CaV3.2 (α1H) is the main functional calcium channel in wild-type spermatogenic cells. J Cell Physiol 2004; 200:116–124.
9. Arnoult C, Cardullo RA, Lemos JR, Florman HM. Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci U S A 1996; 93:13004–13009.
10. Santi CM, Darszon A, Hernandez-Cruz A. A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. Am J Physiol 1996; 271:C1583–C1593.
11. Wennemuth G, Westenbroek RE, Xu T, Hille B, Babcock DF. CaV2.2 and CaV2.3 (N- and R-type) Ca^{2+} channels in depolarization-evoked entry of Ca^{2+} into mouse sperm. J Biol Chem 2000; 275:21210–21217.
12. Goodwin LO, Leeds NB, Hurley I, Mandel FS, Pergolizzi RG, Benoff S.

Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception. Mol Hum Reprod 1997; 3:255–268.
13. Gao Z, Ruden DM, Lu X. PKD2 cation channel is required for directional sperm movement and male fertility. Curr Biol 2003; 13:2175–2178.
14. Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE. A sperm ion channel required for sperm motility and male fertility. Nature 2001; 413:603–609.
15. Quill TA, Ren D, Clapham DE, Garbers DL. A voltage-gated ion channel expressed specifically in spermatozoa. Proc Natl Acad Sci U S A 2001; 98:12527–12531.
16. Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, Garbers DL. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. Proc Natl Acad Sci U S A 2003; 100:14869–14874.
17. Nikpoor P, Mowla SJ, Movahedin M, Ziaee SA, Tiraihi T. CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. Hum Reprod 2004; 19:124–128.
18. Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. Proc Natl Acad Sci U S A 2003; 100:14864–14868.
19. Lobley A, Pierron V, Reynolds L, Allen L, Michalovich D. Identification of human and mouse CatSper3 and CatSper4 genes: characterization of a common interaction domain and evidence for expression in testis. Reprod Biol Endocrinol 2003; 1:53.
20. Rajkovic A, Yan C, Yan W, Klysik M, Matzuk MM. Obox, a family of homeobox genes preferentially expressed in germ cells. Genomics 2002; 79:711–717.
21. Rajkovic A, Lee JH, Yan C, Matzuk MM. The ret finger protein-like 4 gene, Rfpl4, encodes a putative E3 ubiquitin-protein ligase expressed in adult germ cells. Mech Dev 2002; 112:173–177.
22. Yan W, Rajkovic A, Viveiros MM, Burns KH, Eppig JJ, Matzuk MM. Identification of Gasz, an evolutionarily conserved gene expressed exclusively in germ cells and encoding a protein with four ankyrin repeats, a sterile-α motif, and a basic leucine zipper. Mol Endocrinol 2002; 16:1168–1184.
23. Yan W, Burns KH, Ma L, Matzuk MM. Identification of Zfp393, a germ cell-specific gene encoding a novel zinc finger protein. Mech Dev 2002; 118:233–239.
24. Suzumori N, Yan C, Matzuk MM, Rajkovic A. Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. Mech Dev 2002; 111:137–141.
25. Yan W, Kero J, Suominen J, Toppari J. Differential expression and regulation of the retinoblastoma family of proteins during testicular development and spermatogenesis: roles in the control of germ cell proliferation, differentiation, and apoptosis. Oncogene 2001; 20:1343–1356.
26. Yan W, Suominen J, Samson M, Jegou B, Toppari J. Involvement of Bcl-2 family proteins in germ cell apoptosis during testicular development in the rat and pro-survival effect of stem cell factor on germ cells in vitro. Mol Cell Endocrinol 2000; 165:115–129.
27. Yan W, Ma L, Burns KH, Matzuk MM. Haploinsufficiency of kelch-like protein homolog 10 causes infertility in male mice. Proc Natl Acad Sci U S A 2004; 101:7793–7798.
28. Stuhmer W, Conti F, Suzuki H, Wang XD, Noda M, Yahagi N, Kubo H, Numa S. Structural parts involved in activation and inactivation of the sodium channel. Nature 1989; 339:597–603.
29. Ellinor PT, Yang J, Sather WA, Zhang JF, Tsien RW. Ca^{2+} channel selectivity at a single locus for high-affinity Ca^{2+} interactions. Neuron 1995; 15:1121–1132.
30. Hecht NB. Molecular mechanisms of male germ cell differentiation. Bioessays 1998; 20:555–561.
31. Hecht NB. Postmeiotic gene expression during spermatogenesis. Prog Clin Biol Res 1988; 267:291–313.
32. Hecht NB. Regulation of 'haploid expressed genes' in male germ cells. J Reprod Fertil 1990; 88:679–693.
33. Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol (Berl) 1999; 199:471–487.
34. Steger K. Haploid spermatids exhibit translationally repressed mRNAs. Anat Embryol (Berl) 2001; 203:323–334.
35. Eddy EM. Regulation of gene expression during spermatogenesis. Semin Cell Dev Biol 1998; 9:451–457.
36. Wassarman PM, Jovine L, Litscher ES. A profile of fertilization in mammals. Nat Cell Biol 2001; 3:E59–E64.
37. Wassarman PM. Fertilization in animals. Dev Genet 1999; 25:83–86.
38. Darszon A, Labarca P, Nishigaki T, Espinosa F. Ion channels in sperm physiology. Physiol Rev 1999; 79:481–510.
39. Purohit SB, Laloraya M, Kumar GP. Role of ions and ion channels in capacitation and acrosome reaction of spermatozoa. Asian J Androl 1999; 1:95–107.
40. O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 2000; 11:1571–1584.
41. Gonzalez-Martinez MT, Galindo BE, de la Torre L, Zapata O, Rodriguez E, Florman HM, Darszon A. A sustained increase in intracellular Ca^{2+} is required for the acrosome reaction in sea urchin sperm. Dev Biol 2001; 236:220–229.
42. Florman HM, Corron ME, Kim TD, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol 1992; 152:304–314.
43. Florman HM. Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. Dev Biol 1994; 165:152–164.
44. Kammerer RA, Frank S, Schulthess T, Landwehr R, Lustig A, Engel J. Heterodimerization of a functional GABAB receptor is mediated by parallel coiled-coil α-helices. Biochemistry 1999; 38:13263–13269.
45. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 1998; 395:347–353.
46. Benoff S. Voltage dependent calcium channels in mammalian spermatozoa. Front Biosci 1998; 3:D1220–D1240.