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ORIGINAL RESEARCH

Molecular cloning and analysis of the *Catsper1* gene promoter

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ABSTRACT: CatSper channels are essential for hyperactivity of sperm flagellum, progesterone-mediated chemotaxis and oocyte fertilization. *Catsper* genes are exclusively expressed in the testis during spermatogenesis, but the function and regulation of the corresponding promoter regions are unknown. Here, we report the cloning and characterization of the promoter regions in the human and murine *Catsper1* genes. These promoter regions were identified and isolated from genomic DNA, and transcriptional activities were tested *in vitro* after transfection into human embryonic kidney 293, mouse Sertoli cells 1 and GC-1spg cell lines as well as by injecting plasmids directly into mouse testes. Although the human and murine *Catsper1* promoters lacked a TATA box, a well-conserved CRE site was identified. Both sequences may be considered as TATAless promoters because their transcriptional activity was not affected after deletion of TATA box-like sites. Several transcription initiation sites were revealed by RNA ligase-mediated rapid amplification of the cDNA 5′-ends. We also found that the immediate upstream region and the first exon in the human *CATSPER1* gene negatively regulate transcriptional activity. In the murine *Catsper1* promoter, binding sites for transcription factors SRY, SOX9 and CREB were protected by the presence of nuclear testis proteins in DNAse degradation assays. Likewise, the mouse *Catsper1* promoter exhibited transcriptional activity in both orientations and displayed significant expression levels in mouse testis *in vivo*, whereas the suppression of transcription signals in the promoter resulted in low expression levels. This study, thus, represents the first identification of the transcriptional control regions in the genes encoding the human and murine CatSper channels.

Key words: CatSper channel / gene regulation / transcription / promoter / testis

Introduction

The cation sperm channel (CatSper) has been characterized as a Ca^{2+}-permeable transmembrane protein associated with hyperactivation, chemotaxis and the acrosomal reaction. The human and murine *Catsper1* genes are expressed specifically in sperm, and both proteins are located in the principal piece of the flagellum (Ren *et al.*, 2001; Li *et al.*, 2006). *Catsper1* was the first of four identified genes encoding for proteins similar to the pore-forming α_1 subunit of voltage-gated Ca^{2+} channels (Ren *et al.*, 2001). Unlike other CatSper proteins, CatSper1 contains a histidine-rich region that allows channel activation with slight changes in intracellular pH (Kirichok *et al.*, 2006; Lishko and Kirichok, 2010). CatSper channels also include auxiliary subunits called CatSper-β, CatSper-γ and CatSper-δ (Liu *et al.*, 2007; Wang *et al.*, 2009; Chung *et al.*, 2011).

CatSper channels have been associated also with non-genomic actions of progesterone in human sperm, although progesterone seems not to affect murine CatSper channels (Lishko *et al.*, 2011; Strunker *et al.*, 2011). Likewise, CatSper channels seem to mediate Ca^{2+} influx to the flagellum that induces increased Ca^{2+} levels in the sperm head by releasing Ca^{2+} from the internal stores and contributes to the Ca^{2+}-dependent acrosomal reaction (Xia and Ren, 2009; Olson *et al.*, 2010). Interestingly, disruption of the murine *Catsper1* gene yields homozygous male offspring that fail to engender pregnancies as

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a result of sperm abnormal motility. Hyperactivated motility is necessary for exiting the reservoir in the oviductal epithelium and penetrating the oocyte 'zona pellucida' (Carlson *et al*., 2003; Ho *et al*., 2009).

Catsper1 transcripts have been detected in the testis of mice, human, and pigs, as well as in ejaculated human sperm. Murine *Catsper* genes are expressed from post-natal day 18 to the adult testis stage, when the maximum levels of expression are found (Schultz *et al*., 2003; Nikpoor *et al*., 2004; Li *et al*., 2007). The expression profiles of the four Catsper transcripts during post-natal development are distinct, which suggests independent transcriptional regulation (Li *et al*., 2007). Interestingly, the regulation of *Catsper1* expression directly correlates with the onset of spermatogenesis and sexual maturation (Nikpoor *et al*., 2004; Li *et al*., 2006; Song *et al*., 2011).

CatSper1 channel expression seems to be also essential for normal human male fertility. Human *CATSPER1* gene alterations have been associated with autosomal-recessive male infertility, and sequence analysis has revealed insertion mutations that produce frameshifts resulting in the presence of a premature stop codon within the first exon (Avenarius *et al*., 2009). Likewise, in some cases of male infertility, a reduction in *CATSPER1* transcript levels and diminished sperm motility have been observed (Nikpoor *et al*., 2004). In spite of this, studies regarding transcriptional regulation of the *Catsper1* gene have not been previously undertaken. In this report, we describe the first analysis and characterization of the mouse and human *Catsper1* gene promoters.

Materials and Methods

Reporter constructs

DNA was isolated from a BAC clone (DH10B pBAC RPCIB753F22692QC) obtained from the Roswell Park Institute Library. A ~1700-bp region of the first exon and a region of ~2000 bp upstream from the human *CATSPER1* gene were obtained using a double enzymatic digestion (XhoI and HindIII) and ligated into SalI and HindIII sites within the *Renilla* luciferase reporter vector pRL-null (Promega, Madison, WI, USA). Other constructs were derived from the pCAT1 plasmid to yield the ~740-bp promoter without any regions of the first exon. The pCATΔSE construct was obtained by digestion of the pCAT1 vector with EcoRV and StuI, re-ligating the vector keeping a fragment of 2027 bp (nt +14 to −2013) of the *CATSPER1* promoter and eliminating a large portion of the first exon. The pCATΔ3′, pCAT739 and pCATΔbasal deletions were obtained by PCR using the Quikchange site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the two-step protocol (Wang and Malcolm, 2002).

The murine *Catsper1* promoter was amplified by PCR using primers CatS798 (5′CTCAGGCCTGTTCGTGAG3′), CatS1-1215 (5′GAGGGG TAACTTGGAGGAT3′) and CatS1rev (5′TCCCTCCTTGAAGATTG ATCCATG3′) to extend 798-bp and 1215-bp products (−775 to +23 and 1192 to +23 from the start codon) from mouse genomic DNA. The 798-bp region was cloned into the pCR@2.1-TOPO vector and subcloned in sense orientation with XhoI and HindIII (pCatS798) or antisense orientation with KpnI and XhoI (pCatS798AS). The 1215-bp product was cloned into pGEM-T-Easy and subcloned in sense (pCatS1215) and antisense directions (pCatS1215AS) using two flanking EcoRI sites into the *Photinus* luciferase reporter vector pGL3 basic. The full-length *Catsper1* 798-bp promoter was obtained from digestion of the pCRTOPO-798CatS vector with EcoRI; this vector was ligated in either direction into the pIRES_hrGFP_1a vector that encodes green fluorescent protein (GFP) as the reporter. The *CatSper1* promoter was placed in substitution of the cytomegalovirus (CMV) promoter in the pIRES_hrGFP_1a vector to generate the p798CatS-GFP vector. An NsiI-BamHI fragment obtained from the pPCRTOPO-798CatS vector deleted a fragment (nt −66 to +23) at the 3′ end. This fragment was cloned into the NsiI-BamHI sites of the pIRES_hrGFP_1a vector without the CMV promoter to obtain the pΔbasalCatS-GFP construct.

The integrity and orientation of the cloned sequences were confirmed by automatic DNA sequencing using an ABI PRISM 310 sequence analyzer (Perkin-Elmer Applied Biosystems) with the forward (5′GCTCACA TGGCTCGAC3′) and reverse primers (5′CCAGTGCCTCACGACC3′) from the pRL-null, RVprimer3-binding site of the pGL3basic vector and reverse primer (5′CCTTGTAGTCCTCGAGTC3′) from pIRES.

Promoter analysis

The promoter nucleotide sequence was cloned, and its identity was confirmed using the BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi) that identified similarities with the 5′-flanking region of the human *CATSPER1* gene (ID:117144). The cloned nucleotide sequence and the *Catsper1* promoter regions obtained from the human, rat and mouse sequences were aligned using the ClustalX program (http://www-igbmc.u-strasbg.fr/BioInfo/) (Thompson *et al*., 1997). Promoter prediction was performed using ElDorado/Gene2Promoter (http://www.genomatix.de). The prediction of putative transcription binding sites within the *Catsper1* promoters was performed using TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html), Mat-inspector (http://www.genomatix.de) and Mapper programs (http://bio.chip.org/mapper).

RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

Total RNA from human testis was purchased from Clontech (Palo Alto, CA, USA). RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) reactions were performed using a First-Choice kit (Ambion, TX, USA) according to the manufacturer's instructions. Briefly, 2 μg of total RNA was treated with calf intestine alkaline phosphatase at 37°C for 1 h and removed using one volume of phenol:chloroform. Precipitation was performed with 150 μl of isopropanol on ice for 10 min. The RNA pellet was rinsed with cold 70% ethanol, resuspended in nuclease-free water and treated with tobacco acid pyrophosphatase at 37°C for 1 h, and next the RNA was ligated to the 5′RACE adapter (5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUG AUGAAA-3′). For the reverse transcription reaction, 1 μl of the RNA ligation reaction and 1 μl of random decamers were used, and the reaction mix was incubated at 42°C for 1 h.

The first PCR step for the outer 5′RLM-RACE was performed using 1 μl of RT reaction, 10 pM of the 5′RACE outer primer (5′-GCTGATG GCGATGAATGAACACTG-3′) and 10 pM of the *CAT1* outer primer (5′-GTGGAGAGCTCTGCTGTGGC-3′). The reaction conditions were as follows: 94°C for 3 min, 35 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) and an extension step of 7 min at 72°C. The nested PCR for the inner 5′RLM-RACE was performed using 1 μl of the initial PCR amplification reaction, 10 pM of the 5′RACE inner primer (5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′) and 10 pM of the *CAT1* inner primer (5′-CCT CATTCTGAGCCTTTTC-3′). The cycling conditions for the reaction were similar to those applied for the first PCR. The products from the nested PCR were separated by agarose gel electrophoresis, cloned into the pJET 2.1/Blunt vector (Fermentas, Burlington, Canada) and detected via PCR screening of the bacterial colonies. Lastly, the transcription start site (TSS) of the human *CATSPER1* gene was verified via automatic sequencing.

Cell culture, transfection and luciferase assays

The human embryonic kidney cell line (HEK293), mouse Sertoli cells (MSC1) and spermatogonial germ cells (GC-1spg) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% inactivated fetal bovine serum (GIBCO/BRL Life Technologies, Grand Island, NY, USA) and 1% HEPES. Cell lines were cultured in the presence of 50 μg of penicillin, 50 μg of streptomycin and 100 μg of neomycin per millilitre of media (GIBCO) and maintained in a humidified incubator at 37°C and 5% CO_2.

Twenty-four hours before transfection, 2.5 × 10^5 cells/well of each cell line were plated in 24-well plates. The plasmid DNAs were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. To normalize for transfection efficiency, cells were co-transfected with the *Photinus* luciferase expression plasmid, pGL3-CMV for pCAT constructs or with *Renilla* luciferase expression plasmid pRL-CMV for pCatS constructs (Promega). Next, 500 ng of the reporter construct and 20 ng of the control vector were applied to each well. The medium was replaced 24 h post-transfection, and the cells were lysed and subjected to luciferase activity assays using the Dual-Luciferase Reporter Assay System (Promega) 48 h later. The pGL3-CMV or pRL-CMV vectors were used as positive controls according to the construct used. Three independent transfections were performed in triplicate.

Nuclear protein extraction from cell lines and testis

Confluent MSC1, HEK293 and GC-1spg cells were harvested and testes dissected from CD1 male adult mice. Nuclear extracts from these preparations were isolated with a ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) according to the supplier's instructions for cultured cells or tissue samples. Nuclear protein concentration was determined via Bradford assay.

DNAse protection assays

A 798-bp amplicon of the *CatSper1* promoter was obtained by PCR from the pCatS798 vector and labelled with 10 U/μl of T4 polynucleotide kinase in the presence of 10 μC of $^{\alpha 32}$P-dATP; the nucleotides were then removed by centrifugation in a Centri-Sep column (Princeton Separations Inc., Princeton, NJ, USA). Next, the amplicon was used for electrophoretic mobility shift (EMSA) and DNAse protection (footprinting) assays. Binding of different concentrations of testis nuclear proteins to the *Catsper1* promoter probe was performed in binding buffer (10 mM HEPES pH 7.9, 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 1 μg poly dA-dT and 10% glycerol) with 20 ng, 0.4 and 2 μg of nuclear proteins in a final volume of 50 μl for 20 min on ice. DNA protein complex formation was confirmed using non-denaturalizing gels that were dried and then exposed to film for 2 h and revealed.

For footprinting assays, 3 μg of the 798-bp *CatSper1* promoter PCR product and 10 μg of nuclear proteins from murine testis or cell lines were incubated at room temperature in binding buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1.0 mM PMSF, 0.05% Nonidet P-40] in a total volume of 50 ml. After 20 min, 50 ml of a 2X cofactor solution (50 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$) and 0.01 U of DNAse were added. Cleavage was terminated by the addition of 200 μl of stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8 and 40 μg tRNA). The samples were extracted with phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in 4 μl of loading buffer (0.1 xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA and 95% formamide). The DNase I products were separated in a denaturing 6% polyacrylamide gel (7 M Urea, 1x TBE, 40 ul tetramethylethylenediamine and 10% ammonium persulphate) and detected using the DNA silver-staining system (Promega). Maxam–Gilbert sequencing reactions of pCatS798 with CatS1rev primer were run to locate sequence positions of protected regions.

In vivo transfection

CD1 male 8-week-old mice were maintained under constant temperature (28°C) and a 12-h light/12-h dark cycle with free access to pelleted food and water *ad libitum* in the animal facility at the Instituto Nacional de Ciencias Médicas y Nutrición 'Salvador Zubirán' (INCMNSZ). The Animal Resources Laboratory, the animal care procedures and the programs at the INCMNSZ were fully accredited by the Office of Laboratory Animal Welfare of the US Public Health Services under the animal welfare assurance # A5600-1.

Three independent experiments were performed with four male mice. Animals were anaesthetized with ketamine-xylazine (22 and 1.1 mg/kg, respectively) via i.m. injection. Once the mice were anaesthetized, the right testicle was washed with 60% ethanol, and the fur was removed using a stereoscope microscope. The testis and epididymis were exposed through the skin to inject the plasmids. We used the p798CatS-GFP, pΔbasalCatS-GFP and positive control pIRES_hrGFP_1a plasmids with the CMV promoter that drives the expression of GFP *in vivo*. The DNA solution contained 1 mg/ml of plasmid, 100 mM of a caspase inhibitor and 0.1 mg/ml of trypan blue. Each dose consisted of 20 μl of DNA solution and was injected into the seminiferous tubules (intratubular injection) with an insulin syringe and a 32G ultrathin needle. The injection was applied until the trypan blue was visible in 80% of the seminiferous tubules.

For each animal, the right testis was treated as the experimental organ, and the left testis remained untreated and was considered as a control. After DNA injection, the mice were treated with antibiotics (Ampicillin, 0.1 mg/kg;i.m.) and pain medication (Ketoprofen, 3 mg/kg;i.m.); one dose was administered daily over 3 days. These treatments failed to cause noticeable damage to the testicles, and histopathological abnormalities were not observed. The animals were maintained for 1 week before they were euthanized, and their testicles were then prepared for histological analysis using epifluorescence microscopy (Olympus BX51).

Results

Analysis of the human and murine *Catsper1* promoters

Two promoters for the human *CATSPER1* gene were detected. The first one of 739-bp overlapped the first exon (Fig. 1) and the other of 601 bp was located ~17.5 kb upstream of the first exon. A bioinformatic analysis revealed that the *Catsper1* genes of several species, including *Bos taurus*, *Macaca mulatta* and *Pan troglodytes* also have two promoters (Fig. 2A). In *P. troglodytes*, the gene comprises 18 predicted exons with the 5′ end extending ~17 kb in which the first exon is located next to the distal promoter, whereas in humans, the first exon of the *CATSPER1* gene overlaps with the proximal promoter, and the distal promoter may be considered as remnant of a gene that has been reduced in length. In contrast, only one proximal promoter could be predicted for the *Mus musculus* and *Canis familiaris Catsper1* gene.

The 739-bp human promoter proximal sequence contains the start codon and the first 33 codons encoding the amino terminal of the CatSper 1 protein and SRY, CRE and CAAT-box sites (Fig. 1A).

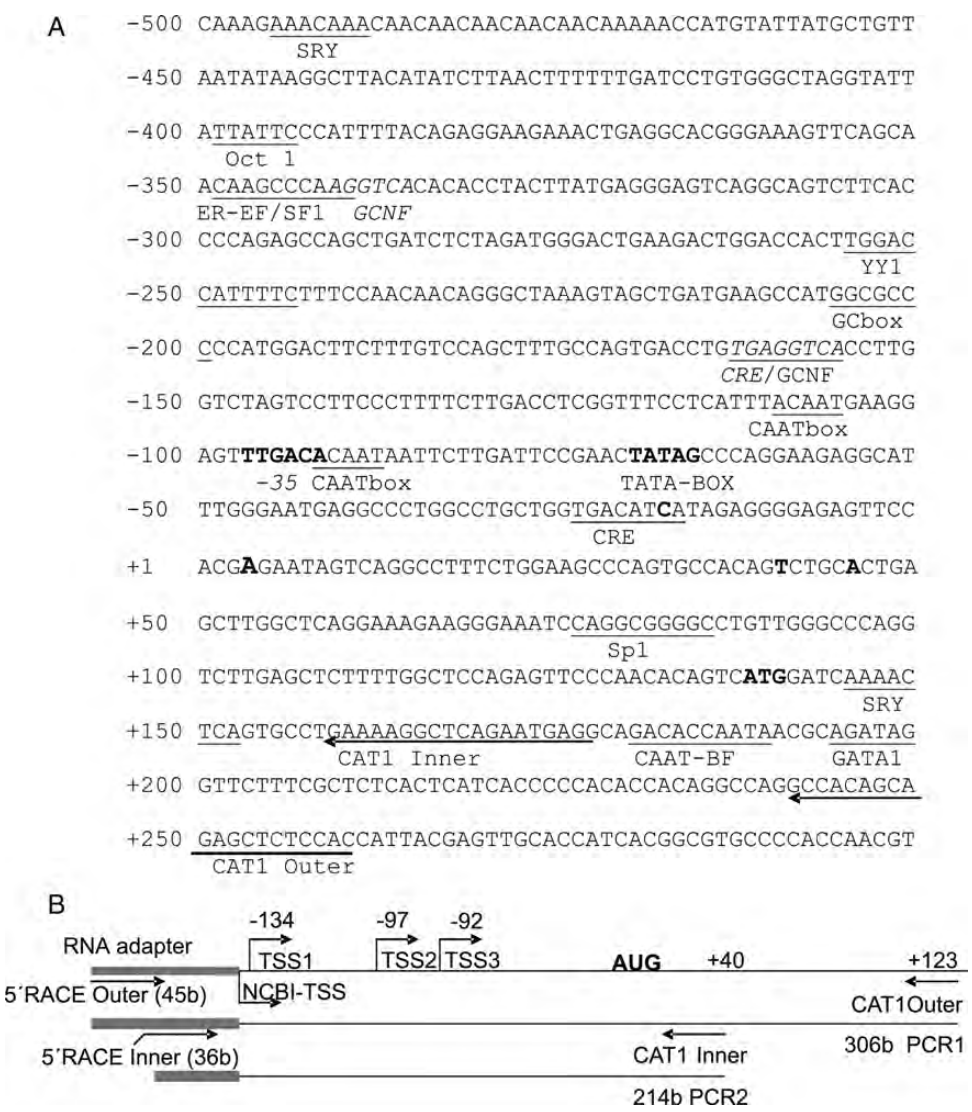

Figure 1 Nucleotide sequence of the human *CATSPER 1* promoter and localization of the TSS. (**A**) A sequence of 740 bp (430 bp in the flanking 5′-upstream region and 300 bp in the first exon) of the human *CATSPER 1* gene is depicted (ID:117144). Transcription factor binding sites for SRY, Oct1, YY1, SF1 GCNF, ER, CRE, Sp1, CAAT-BF and GATA1 are underlined. The putative signals for the basal transcription machinery are represented by bold letters. (**B**) PCR strategy for 5′RACE showing the *Catsper 1*-specific primers and 5′RACE primers for the 5′ ligated RNA adapter to testis RNAm (black arrows). Expected PCR products are marked as PCR1 and PCR2. The TSSs examined in this study are marked as single bold letters, the start codon is indicated by three bold letters and template sequences for oligonucleotides used for 5′RACE-PCR are located above the arrows.

When aligned with mouse and rat sequences, all promoters showed a homology of ~67% and a differential distribution of the binding sites for transcriptional factors with the exception of one CRE site and one CAAT box (Fig. 2B). Interestingly, although a TATA box close to the TSS was observed in the rat promoter, this sequence was not present in the human and the murine promoters. This alignment revealed also a well-conserved CRE site next to the TSS and the presence of a CAAT box upstream of the TATA box in the rat promoter.

Mapping of the TSS

We next sought to determine whether the TSS in the human gene was located within the promoter region (nt −500 to +236) using 5′ RLM-RACE. The first extension with the outer primers should yield a PCR1 product of 306 bp. For the nested PCR with inner primers, a 214-bp PCR2 product was predicted (Fig. 1B). Fifteen candidates with cloned inserts were analysed; 3 candidates displayed a long insert of ~350 bp, 4 candidates displayed a ~300-bp insert and 9 candidates displayed ~250-bp amplicons. All these clones were verified by automatic sequencing. The insert lengths and their corresponding 5′ ends were A174, T135 and A130 bp. Hence, the TSSs in the *CATSPER1* promoter sequences were located at positions −135, −98 and −93 from the start codon. Although we observed a difference of three nucleotides between the NCBI (NM-053054.3)-assigned TSS (adenine at position −138) and our experimental TSS

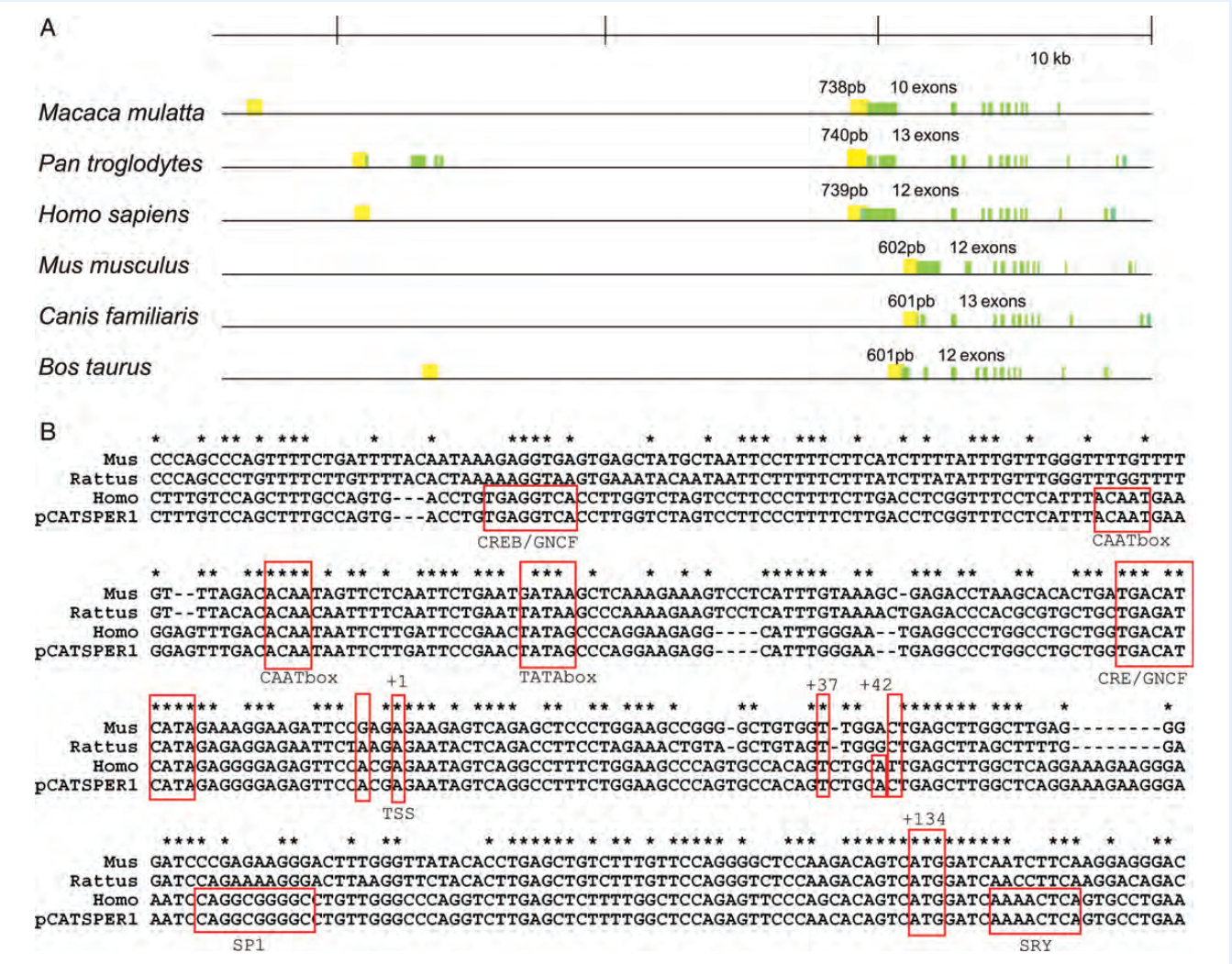

Figure 2 *In silico* analysis of the *Catsper1* promoters. (**A**) Promoter prediction over 30 kb of the genomic DNA upstream of the *Catsper1* gene. Two types of promoter (yellow bars), a distal promoter and a proximal promoter were found adjacent to the *Catsper1* genes; the genomic exons are represented with green bars. (**B**) Alignment of the sequences of ~280 bp for the *Catsper 1* gene promoter region in several species as indicated. The binding sites for various transcriptional factors and the start codon (−134) are indicated within red boxes. The experimentally determined TSSs located at positions +1, +37 and +42 are also indicated in red rectangles. A SNP was identified at nucleotide +430 that varied between the cloned promoter and the GenBank *CATSPER 1* sequence (ID:117144).

(adenine at position −135), we found that this base was well conserved among all the promoters analysed, except in *M. musculus*, where it was substituted by a G (Fig. 2B). These experimental TSSs were present in the *CATSPER1* promoter region of all our constructs.

Analysis of transcriptional activity in transfected cell lines

To identify the regions that regulate the transcriptional activity of the human *CATSPER1* promoter, a region with the first exon and +2153 bp upstream from the TSS was cloned into a promoterless luciferase pRL-null vector (Fig. 3). The pCAT1 construct and the corresponding derivatives were transfected into the spermatogenic mouse cell line GC-1spg, and *Renilla* luciferase reporter activity was monitored. As can be seen in Fig. 4A, a 3-fold induction was observed with the pCAT739 construct (a proximal promoter of 739 bp) that spanned from nt −629 to +102 with respect to the long construct pCAT1 that displayed low transcriptional activity. Interestingly, the loss of the first exon (pCATΔ3′ construct) resulted in only ~2-fold increase in activity, suggesting that the first exon has downstream negative regulatory elements for transcription. A similar effect was observed when another exonic deletion (pCATΔSE construct; nt −115 to +1153) was used (Fig. 4A).

We next compared transcriptional activities among different cell lines. To this end, we reported the *CATSPER1* promoter transcriptional activities as a percentage relative to the transcriptional activity of the CMV promoter. In the MSC1 cells, the transcriptional activity was lower than in GC-1spg cells and was similar with different constructs, suggesting a differential transcriptional control in this cell line (Fig. 4B). Likewise, the transcriptional activity was also low when the constructs

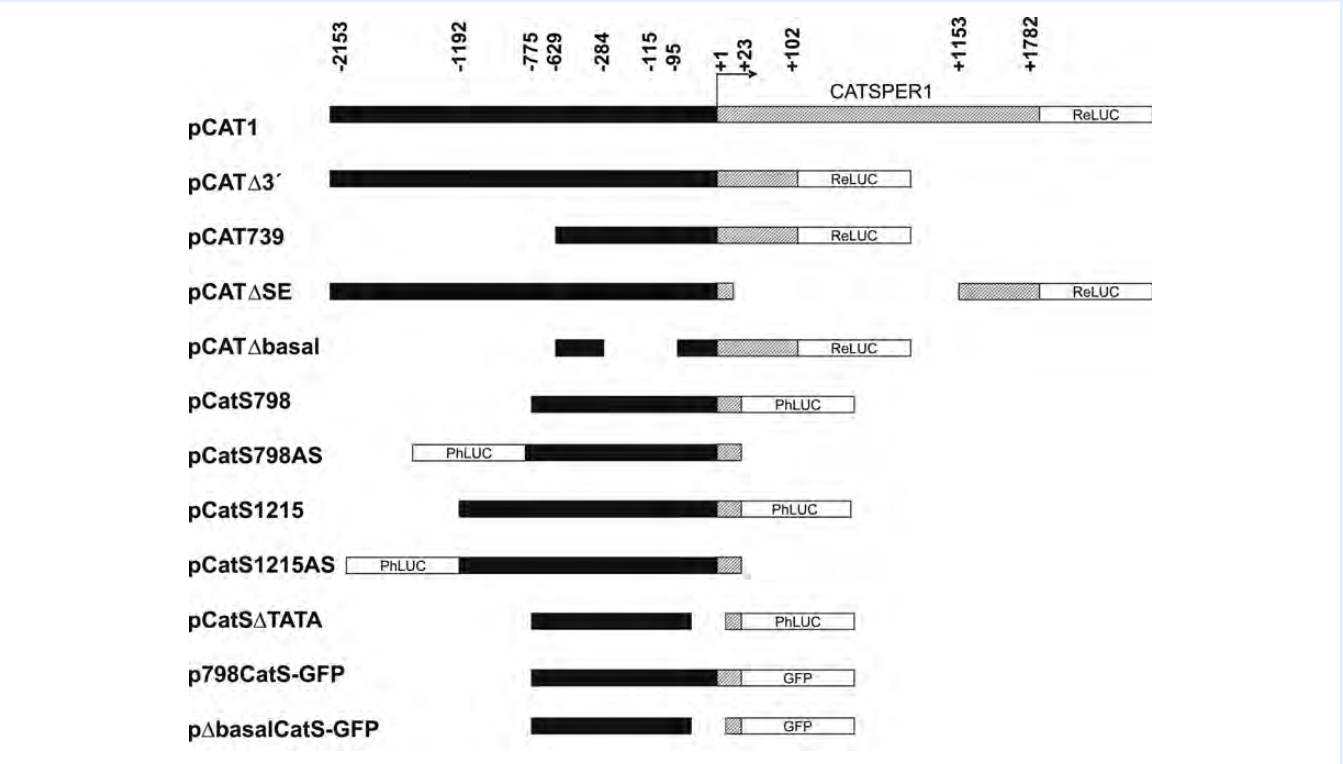

Figure 3 *Catsper1* gene promoter constructs. The pCAT1 plasmid was constructed by ligation of the pRL-null vector with the human *CATSPER1* promoter into the XhoI-HindIII sites preceding the *Renilla* luciferase gene (ReLuc), whereas the derivative constructs were obtained by directed deletion-mutagenesis PCR. The pCATΔ3′ plasmid was constructed by deletion of the first exon at the 3′-end. The pCAT739 construct was generated by eliminating the 5′-end sequence, yielding a 740-bp promoter. The pCATΔSE vector contained a central deletion between the StuI and EcoRV sites of the promoter in which the 5′-end and the putative signals for basal transcription were conserved. The pCATΔbasal vector lacked the putative binding sites for the basal transcription machinery. The plasmids pCatS798 and pCatS1215 contained the murine promoter (798 and 1215 bp, respectively) preceding the *Photinus* luciferase gene (phLuc). The same *Catsper1* promoter regions were cloned in antisense orientation into phLuc to create the pCatS798AS and pCatS1215AS constructs. The p798CatS-GFP plasmid contained the murine *Catsper1* promoter (798 bp), and the pΔbasalCatS-GFP plasmid contained the same region with a deletion between nucleotides −66 and +23 to the TSS; both preceded the *GFP* gene.

were expressed in HEK293 cells (Fig. 4C). Lastly, the activity of the proximal promoter (pCAT739 construct) displayed a high activity GC-1spg cells in comparison with Sertoli cells and the HEK293 cells (Fig. 4D).

CAAT and TATA boxes may not contribute to basal transcription

The *in silico* analysis of the *CATSPER1* promoter revealed one TATA-like box sequence near the TSS, two CAAT boxes and a GC box upstream of the TSS (Fig. 1A). To test whether the CAAT boxes and the TATA-like box contribute to the recruitment of the basal transcriptional machinery, a construct was designed, where these regions were deleted (pCATΔbasal), and its reporter activity was monitored in HEK293 and MSC1 cells. As shown in Fig. 5A and C, similar levels of activity between the human pCAT739 and pCATΔbasal constructs were observed in both cell lines. Similar results were observed after the elimination of the TATA-like boxes in the *Catsper1* murine promoter (Fig. 5B and D). Together, these data rule out a contribution of the CAAT and TATA-like sequences to basal transcription of the *CATSPER1* gene. Interestingly, *in silico* analysis showed that other downstream sites such as Sp1, SRY and Gata1 (Fig. 1A) can be found in the *CATSPER1* promoter, as well as a conserved CRE-binding site located next to the TSS that could function as a substitute for the CAAT and TATA sequences in transcription.

The murine *Catsper1* promoter has bidirectional transcriptional activity

To assess whether a similar size promoter region of the murine *CatSper1* could display transcriptional activity, a ~800-bp region (nt −775 to +23 from the start codon) was cloned in sense orientation adjacent to a *Photinus* luciferase reporter in the promoterless pGL3-basic vector (pCatS798 construct). The same region was cloned in antisense orientation as a negative control. Likewise, the promoter region between nt −1192 to +23 containing an ERα binding site (pCatS1215 construct) was also cloned in both orientations (Fig. 3). These constructs were transfected into the HEK293 and MSC1 cell lines to evaluate their basal transcription activity, and unexpectedly, we observed transcriptional activity in the sequences cloned in the sense and antisense orientations (Fig. 5B and D). The pCatS798 and pCatS1215 sense constructs showed similar transcriptional activity when compared with the promoterless

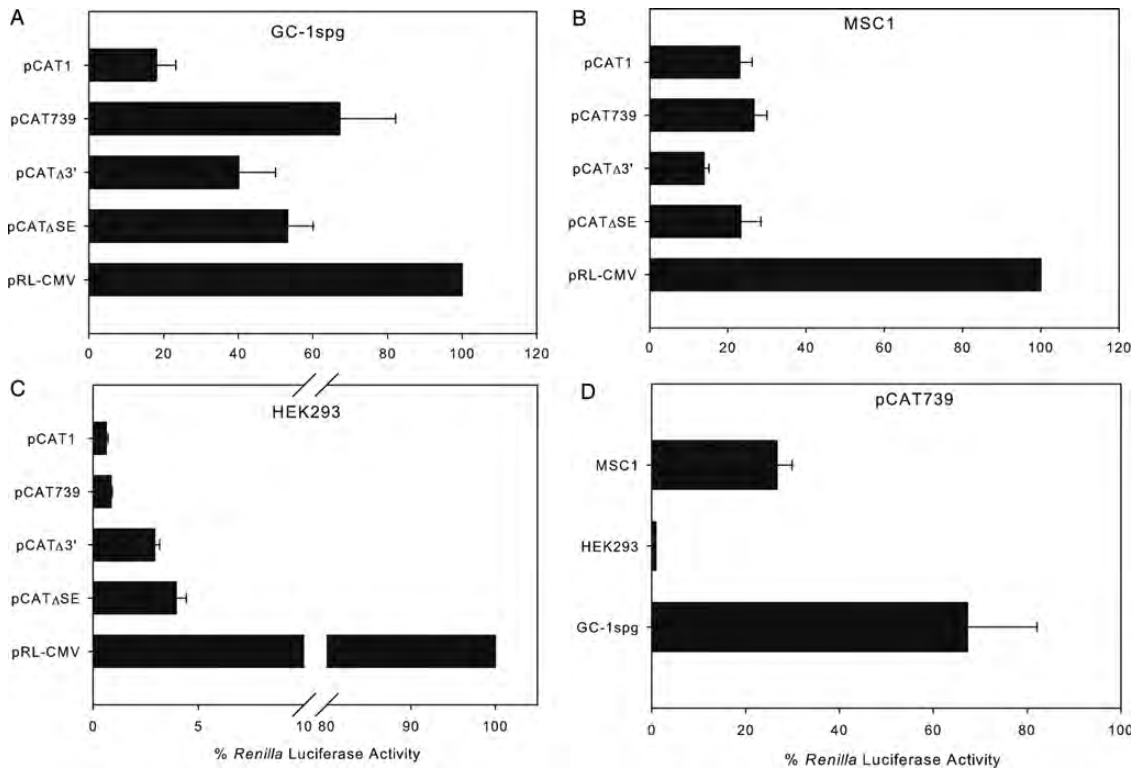

Figure 4 Basal transcription activity of the human *CATSPER1* promoter. The pCAT1 vector and the corresponding derivatives, pCATΔ3′, pCAT 739 and pCATΔSE were transfected into various cell lines: (**A**) Mouse germ cells spermatogonial (GC-1spg), (**B**) MSC1 and (**C**) HEK293 cells. *Renilla* luciferase activity was expressed as a percentage of the pRL-CMV activity observed with the CMV promoter as a positive control. (**D**) Comparison of the transcriptional activity among the tested cell lines with the pCAT739 vector harbouring the proximal *Catsper1* promoter.

pGL3-basic vector, whereas the antisense constructs displayed significant differences in activity between them. The pCatS1215AS showed the highest basal transcriptional activity.

Testis nuclear proteins bind to the *Catsper1* promoter at transcriptional factor sites

The binding of testis nuclear factors to the *CatSper1* promoter was tested via EMSA and footprinting. Nuclear protein extracts were obtained from mouse testis, MSC1, HEK293 and GC-1spg cells. Binding assays with nuclear proteins isolated from mouse testis were performed to determine whether 2 μg of nuclear protein was sufficient to retain 100% of the γ^{32}P-labelled probe. The results indicated that binding with 20 and 400 ng of nuclear proteins can be displaced by an excess of 100 ng of an unlabelled promoter fragment in competition assays (Fig. 6A and B).

Next, a concentration of DNAse1 of 0.01 U was used in a protection assay to obtain a controlled digestion fragment from the *Catsper1* promoter. Protection from DNAse1 degradation was observed in several regions of the *CatSper1* promoter after incubation with nuclear proteins from murine testis, MSC1, HEK293 and GC-1spg cells (Fig. 6C). The binding sequences for the transcription factors Sry, CAATbox (or Sox9 overlapped), TATA-like box and CRE/GNCF next to the TSS were located in the protected regions according to the coordinates of the *Catsper1* promoter given in Fig. 2B.

The murine *Catsper1* promoter directs the expression of GFP in mouse testis

We next tested the activity of the *Catsper1* promoter region in the testicular environment. To this end, a 798-pb *Catsper1* promoter sequence followed by the GFP was constructed as a reporter protein for *in vivo* expression. The pCatS798-GFP plasmid harbours the complete sequence −775 to +23 of the *Catsper1* promoter that was inserted in substitution of the CMV promoter in the pIRES_hrGFP_1a vector to direct GFP expression. A second pΔbasalCatS-GFP construction devoid of the −66 to +23 region of the *Catsper1* promoter (Fig. 3), which contains the identified signals for basal transcription and the annotated TSS for *Catsper1* expression (accession # NM_139301.2), and the pIRES_hrGFP_1a vector with a CMV promoter for the transcription of GFP were used as controls.

The constructs were inoculated (20 μg of each plasmid) in CD1 adult mice via puncture of the right testicle. Histological sections were obtained 7 days after inoculation. The use of the truncated construct (pΔbasalCatS-GFP) resulted in low GFP expression, indicating low transcriptional activity (Fig. 7A). In contrast, the use of the construct containing the intact promoter (p798CatS-GFP) resulted in strong fluorescence signals in germ cells within the seminiferous tubules (Fig. 7B) indicative of strong expression of GFP driven by the *Catsper1* promoter that exceeded the expression levels observed with the control pIRES_hrGFP_1a vector (Fig. 7C), in which GFP transcription is driven by the CMV promoter. These data also suggest that

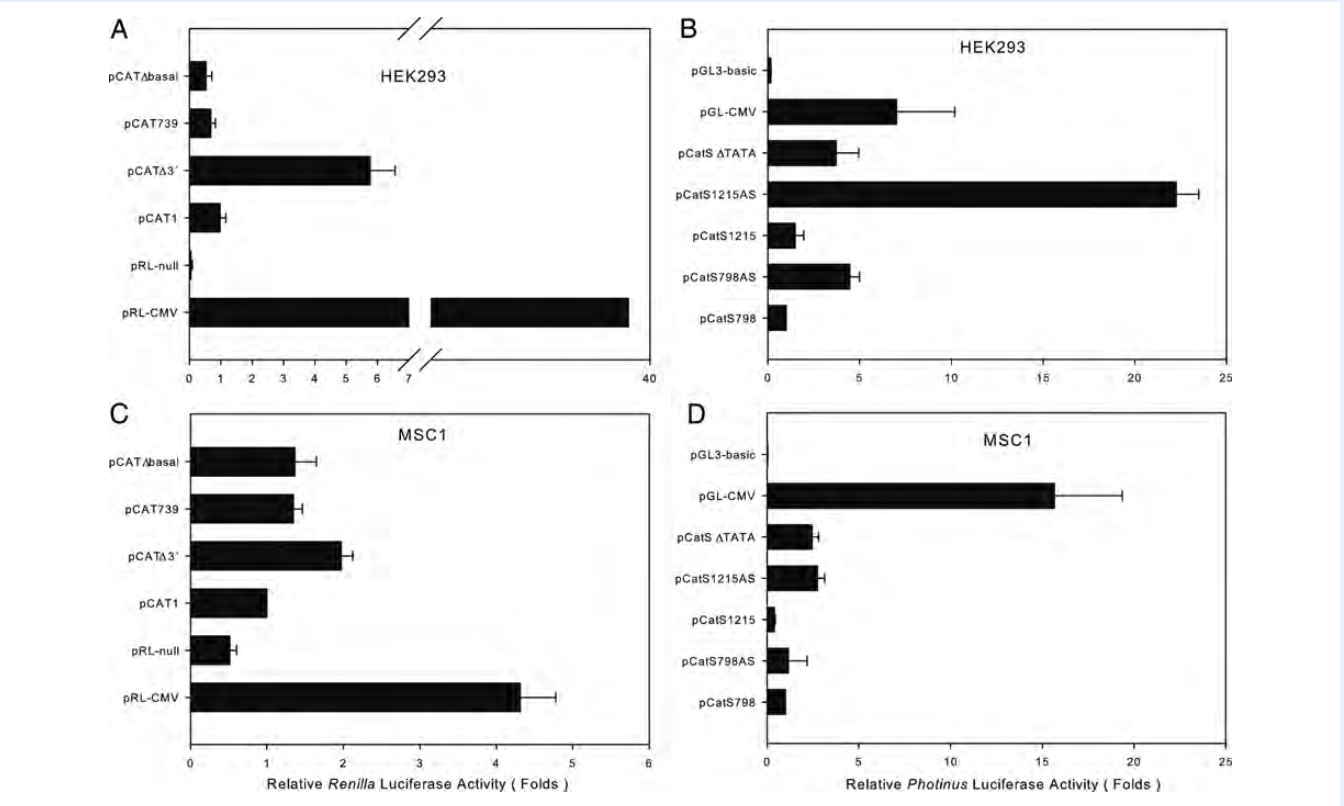

Figure 5 Transcriptional activity of the *Catsper1* promoters in HEK293 and MSC1 cells. Comparison of the transcriptional activity of the pCATΔ basal construct (lacking the putative CAAT and TATA-like boxes and the TSS) with the activities of the pCAT739, pCATΔ3′ and pCAT constructs in HEK293 (**A**) and MSC1 (**C**) transfected cells. *Renilla* luciferase activity is expressed as the fold of activity observed for the pCAT1 vector (the whole promoter region). Bidirectional transcription activity of the murine *Catsper1* promoter in HEK293 (**B**) and MSC1 cells. (**D**) Basal transcription of the murine *Catsper1* promoter cloned in both sense (pCatS1215 and pCatS798 constructs) and antisense orientations (pCatS1215AS and pCatS798AS constructs). Deletion of the TATA-like box and the TSS of *Catsper1* promoter in the pCatSΔTATA construct was assayed for basal transcriptional activity. *Photinus* luciferase activity is expressed as the fold of activity observed in the proximal murine *Catsper1* promoter (pCatS798 construct).

the cloned *Catsper1* promoter region is functional in the testicular environment, as the gene expression occurs in germ cells towards the centre of the seminiferous tubules.

Discussion

Studies using knockout mice and mutations of the *Catsper1* gene have demonstrated the importance of this channel in male germ fertility. Here, we show experimental evidence for the characterization of transcriptional control regions in the genes encoding the human and murine CatSper channels.

Once the human promoter was isolated, we started its characterization by identifying the TSS. The human *Catsper1* mRNA sequence reported in the NCBI (GenBank: NM_053054) includes an untranslated region of 138 nucleotides at the 5′ end, in which the annotated TSS is an adenine. Here, we experimentally identified a TSS only three nucleotides downstream of the annotated TSS at a well-conserved adenine within an Inr sequence (cgAgaat) that had a better identity with the consensus Inr sequence YYANWYY than the previous annotated TSS. Additionally, in the present work, other downstream TSSs were also identified near to the 5′end of the cDNA clone (BC036522.2). Consistent with this, multiple TSSs are frequently found in TATAless promoters. In addition, it is well known that TSSs changes in testis-specific promoters may occur in response to distinct inductor signals (Hagiwara *et al*., 1996; Zheng and Martin-Deleon, 1999; Hata and Ohtsuka, 2000; Gaviraghi *et al*., 2008).

We found that the human *CATSPER1* gene transcription is directed by a proximal promoter that included a 243-bp portion of the first exon. Therefore, the promoter sequence cloned here includes the first exon that includes several transcription factor binding sites. In this study, we also describe that the first exon exerts a negative *cis* effect on transcription. In contrast, the 5′ upstream cloned region (2153-bp) did not display inhibitory effects on the transcription activity of the *CATSPER1* promoter, most likely because this region contains multiple binding sites for transcriptional factors such as SRY, OCT1, SF1, GCNF, CRE, CAATbox, Sp1, GATA1 and YY1 that generally exert a positive effect on transcription. However, confirmation of this hypothesis requires further study. Likewise, a Sp1 binding site is located downstream of the TSS in the *CATSPER1* promoter (Fig. 1A). The Sp family of transcription factors binds to GC-box motifs in promoters and may play a key role in the transcriptional

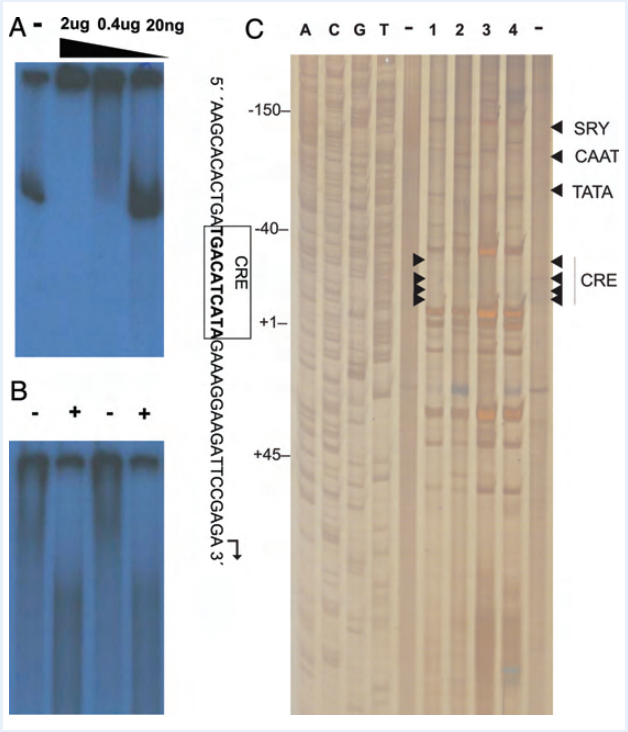

Figure 6 The murine *Catsper1* promoter recruits transcriptional factors. (**A**) The labelled *Catsper1* promoter amplicon (798 bp) was examined for binding to nuclear proteins from mouse testis. Two micrograms, 400 and 20 ng, of nuclear proteins were bound to the promoter and resolved on a 6% non-denaturating polyacrylamide gel. (**B**) The same labelled probe was bound to either 20 or 400 ng of nuclear proteins in the presence of 100 ng of a specific DNA competitor (+). (**C**) DNAse footprinting analysis of the *Catsper1* promoter with testis and cell line nuclear proteins. The *Catsper1* promoter amplicon 3 μg was incubated with 10 μg of nuclear proteins from mouse testis (lane 1), MSC1 (lane 2), HEK293 (lane 3), GC-1spg cells (lane 4) or naked DNA (−). The protected regions are indicated by arrowheads or denoted as vertical text indicating the binding sequence CRE. Horizontal text indicates the TATA-like box, CAATbox/SOX9 and SRY, between nucleotides +45 and −150 of the proximal *Catsper1* promoter. The lanes designates A, C, G, T corresponding to a Maxam–Gilbert sequencing obtained with the pCatS798 and CatS1 reverse primer.

activation of promoters that lack either TATA or CAAT consensus sequences (Suske, 1999).

Our findings in cell lines suggest that the spermatogonial cells (GC-1spg) are suitable for studying the transcription of the proximal human *CATSPER1* promoter. However, although *Catsper1* expression levels and localization in the human germ cells suggest that the promoter must be specific for the germ-cell lineage, its transfection in MSC1 and HEK293 cells revealed some transcriptional activity, as previously characterized (Feng *et al.*, 2000; Borchert *et al.*, 2003; Gaviraghi *et al.*, 2008). This is also consistent with previous studies showing that other germ cell-specific genes display transcriptional activity in HEK293 cells and with the observation that the sperm nucleus glutathione peroxidase (*snGPx*), expressed in the late spermatids, is also detected in testis, kidney and HEK293 cells (Borchert *et al.*, 2003). It is also worth mentioning that the *CATSPER1* promoter was recognized by the transcription machinery in the three cell lines tested, possibly because transcriptional factors that negatively regulate its expression in somatic cells are not present in these cell lines.

Concerning the murine promoter, it was interesting to observe transcriptional activity in the antisense orientation, suggesting that the *Catsper1* promoter may act as a bidirectional promoter. Indeed, the transcriptional activity of the antisense promoter was stronger than the sense *Catsper1* promoter, suggesting that both promoters compete for the transcriptional machinery in a way that is disadvantageous for the sense *Catsper1* promoter. Remarkably, the murine *Catsper1* gene (located on chromosome 19) displays a long non-annotated upstream region spanning 10 488 bp up to the *GM7074* gene (RNA polymerase II-associated protein 2 pseudogene) and 27 002 bp to the galactose-3-O-sulphotransferase 3 (*Gal3st3*) gene. These neighbour genes are transcribed in the same direction as the *Catsper1* gene. In contrast, the *CST6* gene (cystatin E/M) located downstream of the 3′ end of the *Catsper1* gene is transcribed in the opposite direction. Furthermore, the human *Gal3st3*, *CST6* and *CATSPER1* genes kept the same arrangement in chromosome 11. The bidirectional transcriptional activity of the *Catsper1* promoter may produce either divergent gene expression or non-coding RNA expression.

Other testis-specific genes are also expressed by bidirectional promoters. For example, the *PURG* gene located opposite to the *WRN* gene encodes the PURG-B protein (Liu and Johnson, 2002); the male-enhancer antigen-1 *(Mea1)* expressed in germ cells is transcribed from a testis-expressed bidirectional promoter that also drives the *Peas* gene (Ohinata *et al.*, 2003); Haspin (*Gsg2*), a protein kinase expressed in haploid male germ cells is the product of a gene with a short bidirectional promoter of 193 bp shared by the *Aed* gene (Tokuhiro *et al.*, 2007); and last, a repressor of the TATA box, the *Dfa* gene (divergent from *Ate1*), results from the activation of the bidirectional *Ate1* promoter that transcribes a highly expressed isoform in the testis (Hu *et al.*, 2006; Brower *et al.*, 2010).

The putative signals for recruiting the transcriptional machinery within the *Catsper1* promoter, such as the CAAT and TATA-like boxes identified by *in silico* analysis, were also examined in this study. First, the deletion of these sequences in the promoter resulted in similar levels of activity in both the pCATΔbasal and pCAT739 vectors when compared with the promoter-less pRL-null vector. Although no consensus TATA box sequences were identified in the *Catsper1* gene promoter, it is possible that other factors may recruit the transcriptional machinery upstream of the TSS as seen in several other TATAless promoters. Many testis-specific promoters, described as TATAless such as those associated with the *Bax inhibitor-1*, *PURG-A* and *Oxct2b* genes, present multiple TSSs and use the CRE or Sp1 elements for transcriptional initiation (Jean *et al.*, 1999; Liu and Johnson, 2002; Somboonthum *et al.*, 2005).

Other upstream signals might control also the transcriptional initiation of the *Catsper1* promoters. Our bioinformatic analysis revealed binding sites for spermatogenesis-specific transcriptional factors that may activate or repress the promoter such as SRY, CREB and

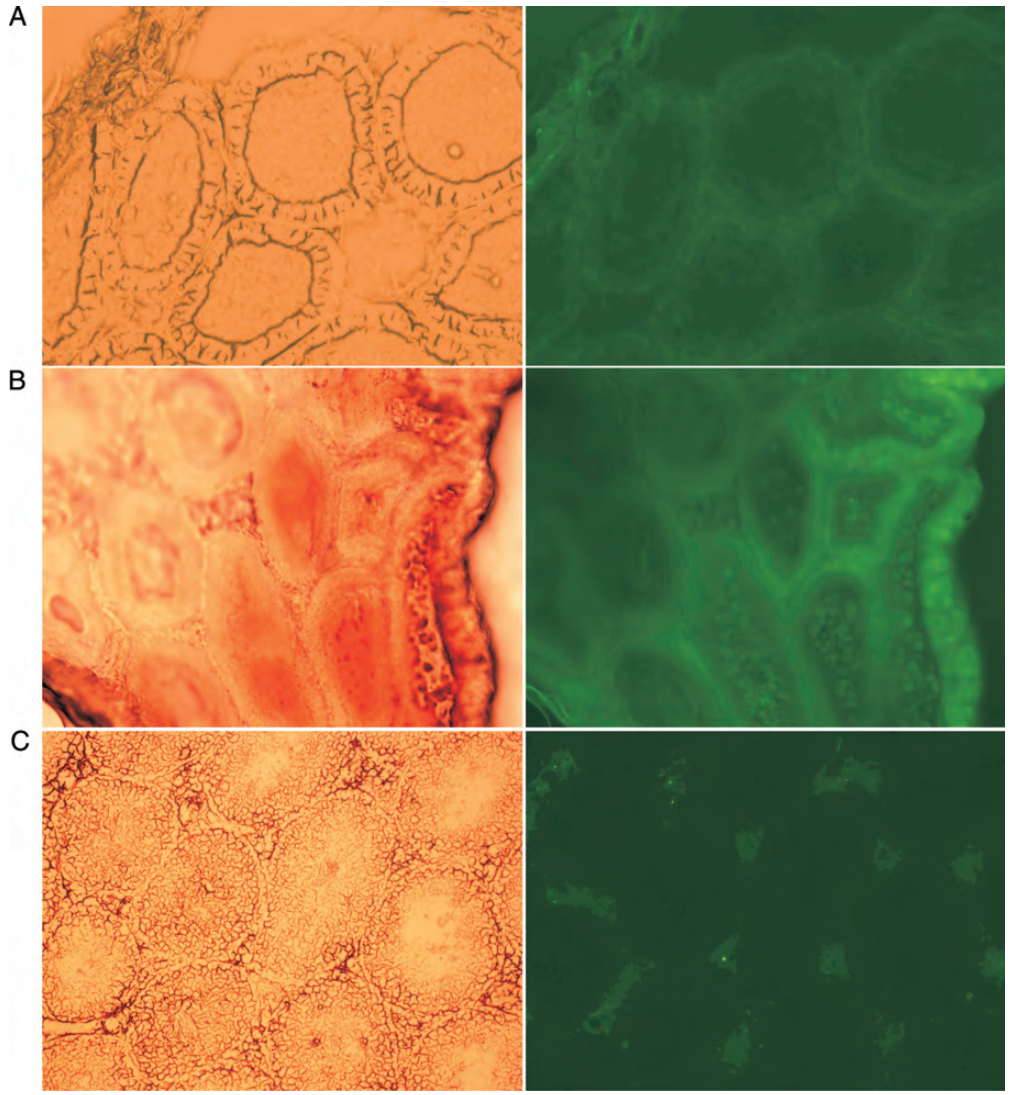

Figure 7 Transcriptional activity of the murine *Catsper1* promoter in testis. *Catsper1* promoter constructs followed by the reporter protein *gfp* gene were injected (20 μg) into the testicles of adult CD1 mice. (**A**) Injection of the first construct (pΔbasalCatS-GFP) devoid of the nt −66 to +23 region that contains the putative signals for the recognition of transcriptional machinery; (**B**) injection of the second construct (p798CatS-GFP) containing the complete sequence of the proximal murine *Catsper1* promoter (nt −775 to +23); and (**C**) injection of the control pIRES_hrGFP_1a vector with the CMV promoter for constitutive transcription of the *gfp* gene. Bright field- and epifluorescence images of the same sections (right and left panels, respectively) are shown together for comparison.

CREM, as well as YY1 and GCNF (Fig. 1A). The protection conferred by the testis nuclear proteins on the SRY, CAAT-box and CRE binding sites of the *Catsper1* promoter suggests that these transcriptional factors may assist the recruitment of the transcriptional machinery or act as transcriptional transactivators of the promoter in germ cells (Martianov *et al.*, 2010). Likewise, we identified one CRE binding site that overlaps with a GCNF site for the repressor germ cell nuclear factor that is sufficient to direct cell type-specific expression *in vivo* (Rajkovic *et al.*, 2010). The promoter exhibited strong transcriptional activity in the seminiferous tubules *in vivo*, suggesting that the *Catsper1* promoter is a specific promoter recognized by the transcriptional machinery in germ cells.

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Authors' roles

N.O. and J.H.-S. designed the study and the interpreted data. D.C. and E.A.-C. performed the bioinformatic analysis. E.A.-C., M.M.-R. and A.H.-R. coordinated the experimental work. E.T.-C. contributed to the *in vivo* experimentation. N.O. and E.T.-C. both analysed and interpreted the data and drafted the manuscript. J.H.-S. and R.F.

critically revised the manuscript for substantial intellectual content. All authors have read and approved the submitted manuscript.

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Conflict of interest

None declared.

References

Avenarius MR, Hildebrand MS, Zhang Y, Meyer NC, Smith LL, Kahrizi K, Najmabadi H, Smith RJ. Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 2009;**84**:505–510.

Borchert A, Savaskan NE, Kuhn H. Regulation of expression of the phospholipid hydroperoxide/sperm nucleus glutathione peroxidase gene. Tissue-specific expression pattern and identification of functional cis- and trans-regulatory elements. *J Biol Chem* 2003;**278**:2571–2580.

Brower CS, Veiga L, Jones RH, Varshavsky A. Mouse Dfa is a repressor of TATA-box promoters and interacts with the Abt1 activator of basal transcription. *J Biol Chem* 2010;**285**:17218–17234.

Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. *Proc Natl Acad Sci USA* 2003;**100**:14864–14868.

Chung JJ, Navarro B, Krapivinsky G, Krapivinsky L, Clapham DE. A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. *Nat Commun* 2011;**2**:153.

Feng ZM, Wu AZ, Zhang Z, Chen CL. GATA-1 and GATA-4 transactivate inhibin/activin beta-B-subunit gene transcription in testicular cells. *Mol Endocrinol* 2000;**14**:1820–1835.

Gaviraghi M, Caricasole A, Costanzo C, Diamanti D, Dandrea M, Donadelli M, Scarpa A, Palmieri M. Identification of a candidate alternative promoter region of the human Bcl2L11 (Bim) gene. *BMC Mol Biol* 2008;**9**:56.

Hagiwara T, Tanaka K, Takai S, Maeno-Hikichi Y, Mukainaka Y, Wada K. Genomic organization, promoter analysis, and chromosomal localization of the gene for the mouse glial high-affinity glutamate transporter Slc1a3. *Genomics* 1996;**33**:508–515.

Hata M, Ohtsuka K. Cloning and expression of murine sp40 gene: differences in initiation sites between heat-induced and constitutive transcripts. *DNA Seq* 2000;**11**:213–223.

Ho K, Wolff CA, Suarez SS. CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. *Reprod Fertil Dev* 2009; **21**:345–350.

Hu RG, Brower CS, Wang H, Davydov IV, Sheng J, Zhou J, Kwon YT, Varshavsky A. Arginyltransferase, its specificity, putative substrates, bidirectional promoter, and splicing-derived isoforms. *J Biol Chem* 2006;**281**:32559–32573.

Jean JC, Oakes SM, Joyce-Brady M. The Bax inhibitor-1 gene is differentially regulated in adult testis and developing lung by two alternative TATA-less promoters. *Genomics* 1999;**57**:201–208.

Kirichok Y, Navarro B, Clapham DE. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature* 2006;**439**:737–740.

Li HG, Liao AH, Ding XF, Zhou H, Xiong CL. The expression and significance of CATSPER1 in human testis and ejaculated spermatozoa. *Asian J Androl* 2006;**8**:301–306.

Li HG, Ding XF, Liao AH, Kong XB, Xiong CL. Expression of CatSper family transcripts in the mouse testis during post-natal development and human ejaculated spermatozoa: relationship to sperm motility. *Mol Hum Reprod* 2007;**13**:299–306.

Lishko PV, Kirichok Y. The role of Hv1 and CatSper channels in sperm activation. *J Physiol* 2010;**588**:4667–4672.

Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca2+ channel of human sperm. *Nature* 2011;**471**:387–391.

Liu H, Johnson EM. Distinct proteins encoded by alternative transcripts of the PURG gene, located contrapodal to WRN on chromosome 8, determined by differential termination/polyadenylation. *Nucleic Acids Res* 2002;**30**:2417–2426.

Liu J, Xia J, Cho KH, Clapham DE, Ren D. CatSperbeta, a novel transmembrane protein in the CatSper channel complex. *J Biol Chem* 2007;**282**:18945–18952.

Martianov I, Choukrallah MA, Krebs A, Ye T, Legras S, Rijkers E, Van Ijcken W, Jost B, Sassone-Corsi P, Davidson I. Cell-specific occupancy of an extended repertoire of CREM and CREB binding loci in male germ cells. *BMC Genomics* 2010;**11**:530.

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.

Ohinata Y, Sutou S, Mitsui Y. Peas-Mea1-Ppp2r5d overlapping gene complex: a transposon mediated-gene formation in mammals. *DNA Res* 2003;**10**:79–84.

Olson SD, Suarez SS, Fauci LJ. A model of CatSper channel mediated calcium dynamics in mammalian spermatozoa. *Bull Math Biol* 2010; **72**:1925–1946.

Rajkovic M, Iwen KA, Hofmann PJ, Harneit A, Weitzel JM. Functional cooperation between CREM and GCNF directs gene expression in haploid male germ cells. *Nucleic Acids Res* 2010;**38**:2268–2278.

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.

Schultz N, Hamra FK, Garbers DL. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. *Proc Natl Acad Sci USA* 2003;**100**:12201–12206.

Somboonthum P, Ohta H, Yamada S, Onishi M, Ike A, Nishimune Y, Nozaki M. cAMP-responsive element in TATA-less core promoter is essential for haploid-specific gene expression in mouse testis. *Nucleic Acids Res* 2005;**33**:3401–3411.

Song C, Gao B, Wu H, Xie Y, Wang X, Li B, Chen G, Mao J. Molecular cloning, spatial and temporal expression analysis of CatSper genes in the Chinese Meishan pigs. *Reprod Biol Endocrinol* 2011;**9**:132.

Strunker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature* 2011;**471**:382–386.

Suske G. The Sp-family of transcription factors. *Gene* 1999;**238**:291–300.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;**25**:4876–4882.

Tokuhiro K, Miyagawa Y, Yamada S, Hirose M, Ohta H, Nishimune Y, Tanaka H. The 193-base pair Gsg2 (haspin) promoter region regulates germ cell-specific expression bidirectionally and synchronously. *Biol Reprod* 2007;**76**:407–414.

Wang H, Liu J, Cho KH, Ren D. A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein. *Biol Reprod* 2009;**81**:539–544.

Wang W, Malcolm BA. Two-stage polymerase chain reaction protocol allowing introduction of multiple mutations, deletions, and insertions, using QuikChange site-directed mutagenesis. *Methods Mol Biol* 2002;**182**:37–43.

Xia J, Ren D. Egg coat proteins activate calcium entry into mouse sperm via CATSPER channels. *Biol Reprod* 2009;**80**:1092–1098.

Zheng Y, Martin-Deleon PA. Characterization of the genomic structure of the murine Spam1 gene and its promoter: evidence for transcriptional regulation by a cAMP-responsive element. *Mol Reprod Dev* 1999; **54**:8–16.