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Promoter analysis of the gene encoding GDNF in murine Sertoli cells ☆

Dante Lamberti, Elena Vicini *

Fondazione Pasteur Cenci Bolognetti, Department of Anatomy, Histology, Forensic Medicine and Orthopedic, Section of Histology Sapienza University of Rome, Italy

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ABSTRACT

GDNF is a Sertoli-cell-derived factor that controls the balance between self-renewal and differentiation of the spermatogonial stem cells. Although research in recent years has concentrated on the impact of GDNF on target germ cells rather little attention has been paid to the molecular control of GDNF expression in Sertoli cells. Here, we aimed to characterize the promoter region of the mouse *gdnf* gene active in Sertoli cells. We identified the transcriptional start sites and analyzed the promoter activity of the 5′-flanking regions. By in-silico analysis of evolutionarily conserved DNA sequences we identified several putative transcription factor-binding regions. Deletion analysis showed the involvement of the three CRE sites for basal and cAMP-induced expression of *gdnf* in murine Sertoli cells. These results provide the basis for future studies to analyze how hormonal or paracrine signals modulate the transcriptional activity of *gdnf* in Sertoli cells.

1. Introduction

The glial cell line-derived neurotrophic factor (GDNF) is involved in many aspects of embryonic development and homeostasis of adult tissues (Sariola and Saarma, 2003). As a neurotrophic factor, GDNF plays important roles in the ontogeny of a great number of neurons, synaptic plasticity, neurite branching and the development of neuron electrophysiological properties. Outside the nervous system, GDNF is a critical regulator of urogenital system development and spermatogenesis. Several studies have highlighted the importance of GDNF for correct spermatogenesis. Disruption of one GDNF allele leads to spermatogonial depletion and the appearance of Sertoli-cell-only tubules in the adult. In contrast, GDNF over-expression causes an accumulation of clusters of undifferentiated spermatogonia (Meng et al., 2000). GDNF is also required for the initiation and maintenance of spermatogonial stem cells (SSCs) in culture (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). The ability of GDNF to support the spermatogonial stem cell compartment in vivo may partially rely on its ability to function as chemoattractant for stem/progenitor cells (Dovere et al., 2013; Kanatsu-Shinohara et al., 2012). Taken together, these results suggest that GDNF is critical for self-renewal maintenance of SSCs, both in vivo and in vitro. In the seminiferous epithelium of the testis, GDNF is produced and secreted by Sertoli cells (He et al., 2007; Tadokoro et al., 2002). Sertoli cells, the somatic cells of the seminiferous epithelium, provide structural support and create an adequate ionic and metabolic environment in which germ cells can differentiate and maturate (Griswold, 1998). Sertoli cells regulate spermatogenesis under a complex hormonal interplay that includes FSH, the glycoproteic hormone produced in the pituitary in response to the hypothalamic gonadotropin releasing hormone (Heckert and Griswold, 1991). Sertoli cells express the FSH receptor that, upon hormone binding, activates an intricate intracellular signaling pathway, leading to the phosphorylation of the CREB protein that binds the cAMP-responsive elements (CRE) and thus inducing transcription (Walker et al., 1995). Notably, GDNF production is responsive to FSH, and it has been shown that the levels of GDNF in different mammalian species vary during the cycle of the seminiferous epithelium (Caires et al., 2012; Grasso et al., 2012; Hasegawa et al., 2013; Johnston et al., 2011; Makela et al., 2011; Sato et al., 2011; Tadokoro et al., 2002). However, little is known about the molecular mechanisms that regulate GDNF expression in Sertoli cells.

Due to the important role of GDNF in the nervous system, transcriptional regulation of the *gdnf* gene has mostly been analyzed in neural models. The gene coding for GDNF was first cloned from the mouse (Matsushita et al., 1997) and soon after from human (Baecker et al., 1999; Grimm et al., 1998; Woodbury et al., 1998). In these earlier studies, it was shown that *gdnf* in both species is organized into one promoter and three exons that are separated by two introns. The localization of the transcriptional start sites, as well as the sequence 5′-flanking to exon 1, show an high degree

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* Corresponding author. Address: Via Antonio Scarpa 14, 00161 Roma, Italy. Tel.: +39 06 49766804; fax: +39 06 4462 854.
E-mail address: elena.vicini@uniroma1.it (E. Vicini).

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of conservation (Grimm et al., 1998; Matsushita et al., 1997). It was later shown that an additional exon as well as alternative intronic promoters, are present in murine *gdnf* (Tanaka et al., 2000a). Transcription from alternative promoters generates transcripts harboring alternative 5′-UTR ends. A similar situation is present in the human gene that harbors several 5′-untranslated (5′-UTR) exons (Airavaara et al., 2011). At present, data on the genomic regulation of *gdnf* in Sertoli cells are lacking.

The aim of the present study was to characterize the promoter region(s) of the murine *gdnf* active in Sertoli cells. By in-silico analysis, we identified putative regulatory elements of the *gdnf* promoter that are highly conserved in mouse, rat and human GDNF genes, suggesting conserved functions. We have identified the transcriptional start sites and some of the regulatory regions, as well as the presence of cAMP responsive elements (CRE) that are important for basal and cAMP-induced expression of the murine *gdnf* in Sertoli cells. Cloning of the mouse GDNF promoters provides the basis for future studies aimed at the characterization of specific sequences required for modulation of transcriptional activity.

2. Material and methods

2.1. Animals

CD1 and C57BL/6 mice were used in the experimental procedures. The animals were housed in a standard facility in accordance with guidelines for animal care at the University of Rome "La Sapienza". All procedures were approved by the Department of Health Animal Care and Use Committee.

2.2. Isolation and in vitro cultures of the seminiferous epithelial stages

Adult C57BL/6 mice were used for the procedure as previously described (Grasso et al., 2012). Briefly, the testes were removed, washed in a PBS solution and then decapsulated. To discriminate the different stages of the cycle of the seminiferous epithelium, the seminiferous tubules were dissected on a transilluminating dissection microscope (Parvinen and Vanha-Perttula, 1972). Three groups of stages were dissected: stages II–VI (strong spot), stages VII–VIII (dark zone) and stages IX–XI (pale zone). 30 mm Of pieces of seminiferous tubules of each group of stages were seeded in 24-well culture plates with DMEM supplemented with antibiotics, L-glutamine, non essential aminoacid, Hepes pH 7.7, gentamicin in the absence or in the presence of 100 ng/ml FSH (NIADDK-ovine-FSH). Seminiferous tubules were incubated for 24 h at 32 °C and 5% CO_2 and used for RNA isolation and Real Time PCR experiments as described below.

2.3. Sertoli cell cultures

Primary Sertoli cells were obtained from 17-day-old CD1 mice (14–20 mice for each experiment) by using a two-step enzymatic digestion as previously reported (Vicini and Conti, 1997). Briefly, isolated testes were washed twice with Hank's solution (Sigma, Milano, Italy), and the tunica albuginea was removed. Testes were chopped, and then immersed in 0.002% DNase I (Roche, Monza, Italy), 0.25% Trypsin (Difco BD, Milano, Italy) in Hank's and agitated at 32° for 15 min. Enzymatic digestion was blocked by adding 10% FBS, and the cellular suspension was settled by unitary gravity for 4 min, and then washed three times with fresh Hank's solution. The second digestion was performed with 0.002% Dnase I (Roche, Monza, Italy) and 0.2% Collagenase A (Roche, Monza, Italy) in Hank's for 20 min at 32 °C. The cellular suspension was then settled by unitary gravity, washed with fresh Hank's solution, collected in a 15 ml polystyrene tube and centrifuged at 320 rpm for 2 min. The cell pellet was resuspended in MEM 1X (Gibco), supplemented with glutamine, Hepes, non-essential amino acids and an antimicrobial solution, and then plated. For transfection and RNA interference experiments, cells were plated in 6-multi-well dishes; for RNA extraction, cells were plated in 60 mm dishes. Cells were maintained at 32 °C and 5% CO_2. To remove germ cells, a hypotonic treatment was performed after 48 h, as described (Galdieri et al., 1981). The purity of Sertoli cell cultures was assessed by morphological analysis and by immunolocalization of α-SMA (smooth muscle actin), a marker of peritubular myoid cells. Purity of Sertoli cells was routinely higher than 95%.

2.4. RNA extraction and real time PCR

Total RNA from cultured segments of seminiferous tubules or Sertoli cell cultures was isolated using TRIzol reagent (Life Technologies, Monza, Italy) according to the manufacturer's instructions. RNA concentration was measured using NanoDrop 1000 (ThermoFisher Scientific, Milano, Italy). RNA quality was assessed by agarose gel electrophoresis and by calculating the ratio of the absorbance at 260 and 280 nm (A260/280) that was routinely comprised between 1, 9 and 2. One microgram of total RNA was used for cDNA synthesized using Transcriptor reverse transcriptase and Random Hexamers (Roche, Monza, Italy). In the control samples, reverse transcriptase was omitted to monitor genomic DNA contamination. The list of genes analyzed and respective primers used are reported in Supplementary Table 1. Primer pairs were designed using IDT SciTools software (http://eu.idtdna.com) and nucleotide sequences available at NCBI databases (Supplementary Table 1). One μl of cDNA was subjected to real-time analysis with 0.3 μM of both forward and reverse primers and 10 ml of 2X Master mix (FluoCycle SybrGreen Kit; Euroclone, Milano, Italy) to a final reaction volume of 20 ml. Reactions were performed in triplicate for each sample on 7500 Real-Time PCR Systems (Applied Biosystems, Life Technologies, Monza, Italy). Conditions for quantitative real-time PCR were 95 °C for 5 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. A melting curve analysis was performed as a quality control for the dissociation of double-stranded DNA during heating. The analysis was carried out at the end of last cycle by increasing the temperature stepwise from 65 °C to 95 °C in 0.2 °C increments. Blank controls were assayed in each reaction and for each primer pair to detect reagent contamination. The cDNA levels were standardized by normalizing to a β-actin control. Expression levels of interest genes were also normalized against two other reference genes, GAPDH and 18S, giving similar results (data not shown). Relative gene expression data was evaluated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Each real-time PCR assay was repeated at least two times, using duplicate samples in three different experiments. The mean values with standard error of the mean (SEM) were used for comparison.

2.5. 5′RACE (rapid amplification of cDNA ends)

The genomic organization of the mouse *gdnf* gene was obtained from the ENSEMBL public database (ENSMUSG0000022144). Sertoli cells were treated for 24 h in the presence of 1 mM $(Bu)_2$cAMP. Total RNA was extracted using TRIzol (Life Technologies, Monza, Italy). To obtain full-length 5′ ends, the 5′RACE procedure was conducted with a GeneRacer Kit (Life Technologies, Monza, Italy). First, 5 μg of total RNA was subjected to de-phosphorylation by calf intestinal phosphatase (CIP), and then it was treated with tobacco acid pyrophosphatase (TAP) to remove the 5′cap. Once the 5′cap structure was removed, total RNA was ligated to a 5′-specific oligo adapter, producing known priming sites for the 5′RACE procedure.

Oligo-capped RNA was retro-transcribed into cDNA with random primers, using the Superscript III RT kit (Life Technologies, Monza, Italy). One μl of the cDNA was used for subsequent amplifications with the forward primer (GENE RACER 5′ primer, Life Technologies, Monza, Italy) and a reverse GDNF-specific primer binding either exon II or III. The cycle parameters were: 94 °C for 2 min for the initial denaturation; followed by 30 cycles of 94 °C for 30 s; 65 °C for 30 s; 68 °C for 2 min. One μl of the PCR product was then used for a second nested-PCR, using the adapter forward primer (GENE RACER 5′ NESTED, Life Technologies, Monza, Italy) and a reverse GDNF-specific primer corresponding to exon II. The sequences of primers used are reported in Supplementary Table 1. The cycle parameters were: 94 °C for 2 min for the initial denaturation; followed by 30 cycles of 94 °C for 30 s; 65 °C for 30 s; 68 °C for 2 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide staining. The PCR products were gel-purified, cloned in TOPO® TA vector (Life Technologies, Monza, Italy) and sequenced.

2.6. Plasmid construction

pGL2-Basic, a promoterless luciferase vector (Promega, Milano, Italy), was used to test the various sequences of the mouse GDNF gene for promoter activity. The different fragments of the putative gene promoters were isolated by enzymatic digestion from a genomic BAC clone (RP23–305M4, GenBank access number: AC130656). To identify appropriate restriction sites on the genomic clone, restriction analysis was carried out using Webcutter. The original polylinker of pGL2-Basic was replaced with one harboring restriction sites tailored for the restriction profile of the GDNF gene. Each plasmid was named after the relative position of the insert on the genomic DNA sequence, where we arbitrarily set the +1 position in correspondence to the TSS1. For plasmids −1857/−182, +109/+1002, +505/+1002 and +715/+1002, the genomic fragments were obtained by PCR using flanking primers harboring MluI, PstI, HindIII and BglII sites, and then they were subcloned in the modified pGL2 vector. To obtain internal deletions of the GDNF genomic fragments, the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Milano, Italy) was employed. Briefly we performed a PCR associated technique to create deletion mutants of the construct −1857/+753 using primers carrying the appropriate deletions of the region of interest, following the manufacturer's directions (Agilent Technologies, Milano, Italy). Primer couples (forward and reverse) were designed to keep the mutation in the middle of the primer. After PCR extension, 2–5 μl of the reaction was used to transform XL-10 gold bacteria (Stratagene, Milano, Italy). Several bacterial colonies were selected and sequenced to evaluate introduction of the desired mutation.

2.7. Transfection, luciferase and β-galactosidase assays

Sertoli cell transfection was performed as described, with minor modifications (Vicini and Conti, 1997). Briefly, cells were deprived of the antimicrobial solution for at least 24 h and then transfected with Lipofectamine 2000 (Invitrogen, Monza, Italy), 1.5 μg/ml of the reporter construct and 0.5 μg/ml CMV-βgal to allow normalization to β-galactosidase expression. After 5 h, the medium was aspirated and the cells were washed and fed with complete growth medium. After 20 h, the medium was aspirated and replaced with fresh medium containing or lacking 1 mM $(Bu)_2$cAMP for an additional 24 h. Cells were washed and lysed using 1X Reporter lysis buffer (Promega, Milano, Italy), according to the manufacturer's directions. The cell lysates were centrifuged (16,000*g* for 2 min at 4 °C), and aliquots of the supernatants were assayed. For each sample, luciferase activity was tested in duplicate, mixing 20 μl cell extract with 100 μl Luciferase Assay Reagent (Promega). The light that was produced was measured in an Auto Climat Lumat LB 952 T/16 luminometer (Berthold, Monza, Italy) and expressed as relative light units (RLU). β-Galactosidase assays were performed in duplicate, by adding an equal volume of Assay 2X Buffer (Promega, Milano, Italy) to the cell extracts. The samples were incubated at 37 °C, in parallel with a standard curve of purified β-galactosidase enzyme (Promega, Milano, Italy), until a yellow color developed. β-Galactosidase milliunits in each sample were calculated using the standard curve values. Luciferase activity (RLU) was normalized relative to the β-galactosidase activity (milliunits) to correct for differences in transfection efficiency. Each construct was assayed in duplicate in at least three different experiments.

2.8. Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM). Data were analyzed using a *t*-test to define the significance of the differences between two groups, or, to compare many groups, using a one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test or Dunn test.

3. Results

3.1. FSH modulates gdnf mRNA levels in a stage-dependent fashion

Expression of *gdnf* in primary Sertoli cell cultures is stimulated by FSH (Tadokoro et al., 2002), and in the mouse testis, the levels of GDNF vary with the stages of the epithelial cycle (Caires et al., 2012; Grasso et al., 2012; Hasegawa et al., 2013). We have previously shown that both mRNA and protein GDNF levels are higher at stages II–VI and both decrease at stages VII–VIII and IX–XI (Sato et al., 2011). The expression profile of GDNF during the cycle of the seminiferous epithelium parallels the profile of the FSH-response in the rat, which is high in stages XIII–V and decreases subsequently (Parvinen, 1982; Parvinen et al., 1980). To directly test whether the *gdnf* mRNA level is regulated by FSH in stage-dependent manner, staged seminiferous tubules were cultured for 24 h in the absence or presence of 100 ng/ml FSH (Fig. 1). In stages II–VI, the *gdnf* mRNA was significantly induced compared to control levels ($p < 0.05$), while in stages IX–XI, FSH significantly reduced *gdnf* mRNA compared to the control levels ($p < 0.05$). No significant differences were found in stages VII–VIII. These data show that FSH modulates *gdnf* expression in a stage-dependent fashion.

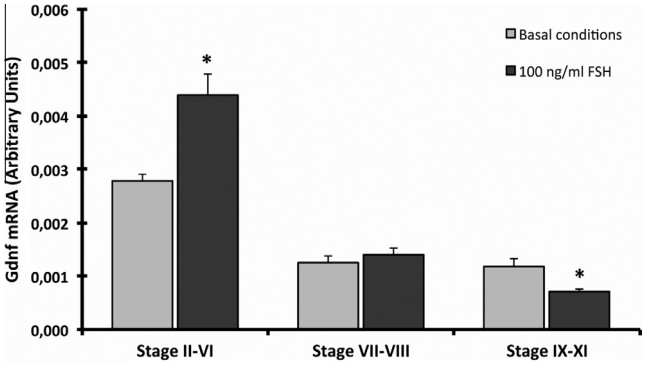

Fig. 1. *Gdnf* expression is modulated by FSH. Isolated seminiferous tubules at different stages of the cycle of the seminiferous epithelium were cultured for 24 h in the presence or absence of 100 ng/ml FSH. Gdnf mRNA levels were evaluated by real-time RT-PCR, normalized to β-actin levels and expressed as arbitrary units. Data are presented as the mean ± SEM from 3 experiments. $^{*}p < 0.05$ vs. basal conditions.

3.2. Identification of the transcriptional start sites (TSS)

Because the murine *gdnf* is a multi-promoter gene whose transcription gives rise to alternative 5′-UTRs (Tanaka et al., 2000a,b), we aimed to identify the TSSs in Sertoli cells by 5′RACE, as detailed in Material and Methods. The primers employed in the 5′RACE were designed to detect all the 5′-UTR variants previously identified in neural and glial cell lines (Tanaka et al., 2000a,b). Three different bands of approximately 1.3 kb, 0.22 kb and 0.18 kb were consistently amplified (Fig. 2A). Sequence analysis and comparison with the genomic organization of the mouse *gdnf* gene (ENSMUSG0000022144) revealed that the three 5′-ends spanned the area of exon 1 (Fig. 2B). In the present study, the first putative AUG has been assigned accordingly to the consensus coding sequence (CCDS) for murine GDNF (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi, accession number: CCDS 27371.1). The first and the second putative transcriptional start sites (TSS1 and TSS2) are localized upstream of the putative initiation of translation, while the third (TSS3) is localized downstream from it. Although our experimental strategy was aimed at detecting all transcripts, including those generated from the alternative intronic promoters upstream of exon 2 and 3 that are active in neural and glial cell lines (Tanaka et al., 2000a,b, 2001), the latter were never detected in Sertoli cells. We have also tested the promoter activity of genomic fragments bearing the intronic promoters 2 and 3 that were subcloned upstream of the coding region of luciferase cDNA and transiently transfected in immature Sertoli cells. These genomic region were not active in immature Sertoli cells (data not shown).

3.3. Determination of evolutionarily conserved potential DNA binding sites

To identify relevant DNA binding sites on *gdnf* for known transcription factors, we performed an in-silico analysis of evolutionarily conserved regions (ECRs) between human, rat and mouse (http://ecrbrowers.dcode.org), followed by the identification of conserved DNA binding sites for known transcription factors (Mulan: http://mulan.dcode.org/) (Loots and Ovcharenko, 2007a,b) (Fig. 3). ECRs browser analysis was performed on human *gdnf* as reference. Fig. 3A shows the alignment of the *gdnf* genomic regions in human, rat and mouse obtained by the in-silico analysis. The different genomic regions are color-coded as detailed in the figure legend. The sequence conservation is rendered by the height of the peaks and is shown for values above 50% to a maximum of 100% (complete conservation). ECRs are identified as genomic sequences having a minimum sequence identity of 70% and a minimum length of 100 bp and are depicted as horizontal pink bars. The different human gene transcripts are shown at the top. The human, rat and mouse genes shared remarkable similarity in terms of intron lengths and conservation within the coding exons (blue peaks). However, the 5′-UTR exons (yellow) included in the mature mRNAs are divergent. While exon 1 in humans is highly conserved among the three species, the other two 5′-UTR exons expressed in human tissues are not included in any mouse or rat mRNAs databases. Notably, the genomic 5′-flanking region of exon 1 (“red peaks” in Fig. 3A) showed very high sequence conservation among the three species, suggesting the presence of a conserved promoter/enhancer. This region has been identified as the most distal *gdnf* promoter active in neural and glial mouse cell lines (Tanaka et al., 2000b, 2001). The analysis of conserved DNA binding sites among the three different species highlighted the presence of several binding sites, including a canonical TATA-box, NF-kB, an androgen receptor (AR), cAMP-responsive elements (CRE) and NRSE-like binding sites (Fig. 3B and C). The first transcriptional start site (TSS1) is located approximately 30 bp downstream from the canonical TATA-box sequence and approximately 900 bp upstream from the putative initiation of translation (Fig. 3B and C). The TSS2 and TSS3 are 100 bp apart from each other. TSS2 is located approximately 70 bp upstream from the putative initiation of translation, while TSS3 is approximately 30 bp downstream from it.

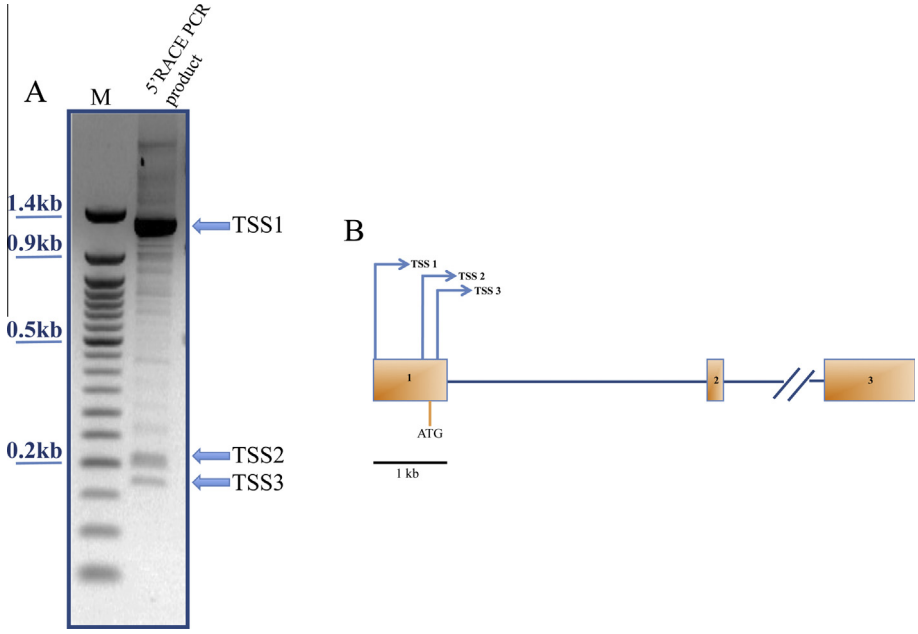

Fig. 2. Identification of the transcriptional start sites (TSSs) of *gdnf* in Sertoli cells. Total RNA was extracted from primary Sertoli cell cultures stimulated with 1 mM $(Bu)_2$cAMP for 24 h. A 5′RACE was performed on 5 μg RNA, as detailed in the Material and Methods. (A) 5′RACE PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide staining. One representative experiment of the four performed is reported. (B) Schematic representation of the murine GDNF gene, according to the ENSEMBL database (ENSMUSG0000022144). The first putative AUG has been assigned according to the consensus coding sequence (CCDS) for murine GDNF (CCDS 27371.1). The localization of the three TSSs on the first exon is marked by arrows. M, DNA molecular weight.

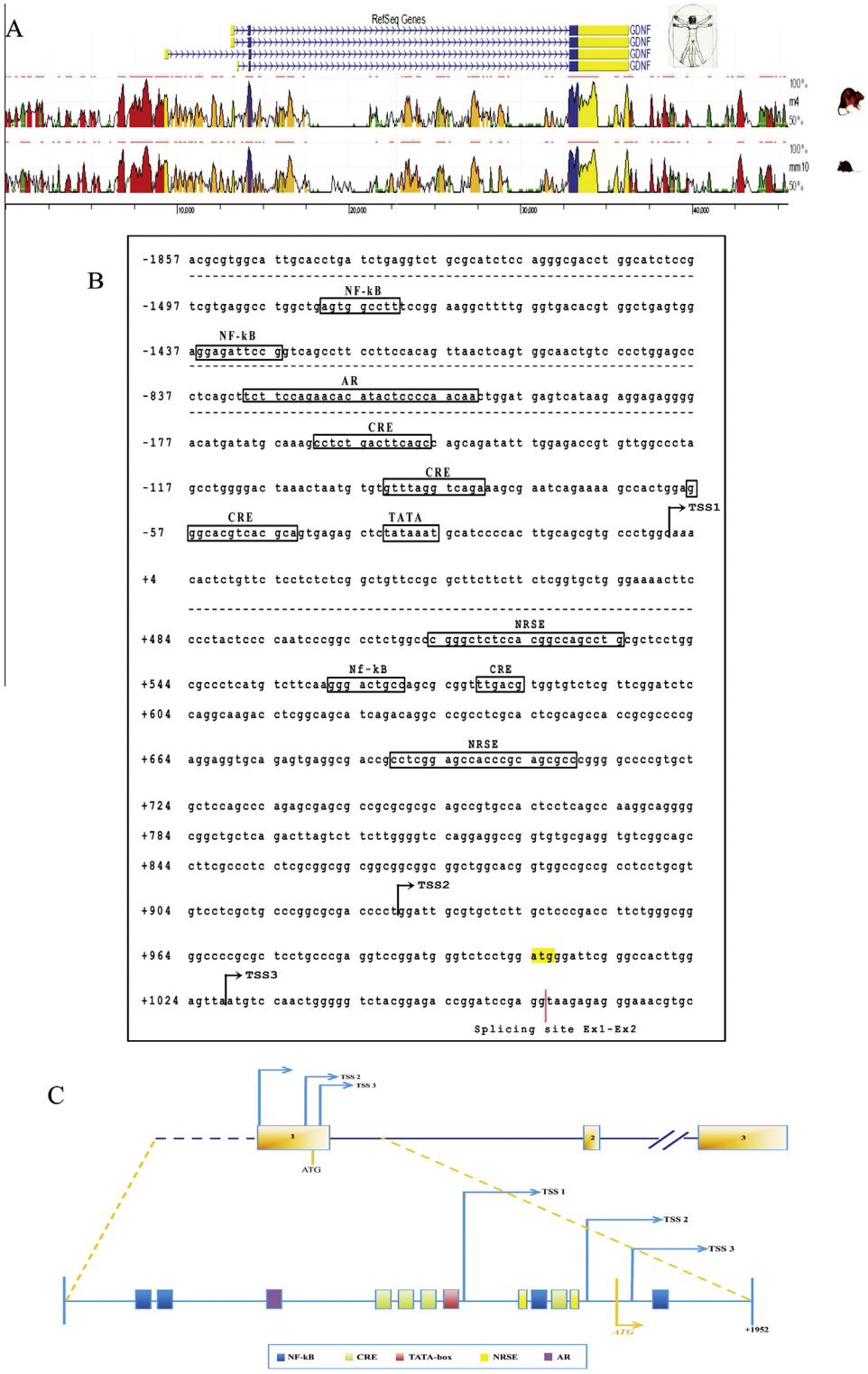

Fig. 3. Identification of evolutionarily conserved, putative DNA binding sites on the promoter region. (A) ECR browser analysis on human was performed to show *gdnf* evolutionarily conserved regions between human, rat and mouse (http://ecrbrowser.dcode.org/). Genomic conservation of coding exons (blue) and introns (salmon), as well as UTRs (untranslated regions, yellow), transposons and simple repeats (green) and intergenic sequences (red), is visualized. The different human gene transcripts are shown at the top. Layer height presents the percentage of identity (sequence conservation). ECRs are identified as regions of high sequence identity against a neutrally evolving background and are indicated as horizontal pink bars (minimum sequence identity 70%, minimum length 100 bp). The highly conserved intergenic regions located near to and upstream of the 5′-end of the gene (red peaks) represent a putative conserved promoter/enhancer (promoter 1). (B) Sequence of the murine genomic region flanking the transcriptional start sites (TSSs) of the *gdnf* mRNA variants expressed in Sertoli cells. Nucleotides are numbered from the TSS1. The first putative ATG has been assigned according to the consensus coding sequence (CCDS) for murine GDNF (CCDS 27371.1) and is marked in yellow. TSSs are marked as arrows. Evolutionarily conserved, potential regulatory elements were found by Mulan analysis, as detailed in Material and Methods. Potential regulatory elements are boxed. Dotted lines represent genomic regions that were void of conserved potential regulatory elements, and they are not shown. The splicing site between exon 1 and 2 is shown with a red bar. (C) Schematic representation of the genomic regions flanking the TSSs. Nucleotides are numbered from the TSS1. TSSs are marked as arrows. Potential regulatory elements are shown as boxes and are color-coded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Analysis of basal and stimulated activity of the putative promoter regions

To determine whether the genomic region around the transcriptional start sites identified by 5′RACE can indeed function as a promoter, several genomic fragments were subcloned upstream of the coding region of luciferase cDNA. The promoter activity was studied by transient transfection and by measuring the luciferase activity in primary Sertoli cell cultures obtained from immature mice. In this model, endogenous *gdnf* levels were firstly down-regulated (8-h treatment) and then up-regulated by a 24-h treatment with 1 mM $(Bu)_2$cAMP, a cell-permeable cAMP analogue (Supplementary Fig. 1). After transfection, cells were left untreated or treated with 1 mM $(Bu)_2$cAMP for 24 h. In view of the presence of more than one transcriptional start site, different constructs containing the proximal (TSS2 and 3) and distal (TSS1) cap sites were used to determine basal and stimulated promoter activity (Fig. 4). Among all the genomic fragments tested, only those containing 5′-flanking regions to TSS1 showed a significant luciferase activity increase over the promoter-less vector in basal conditions. Genomic fragments containing 5′-flanking regions to TSS2 and TSS3 (+109/+1002, +505/+1002, and +715/+1002) showed no significant luciferase activity increase over the promoter-less vector. The luciferase activity of the longest fragment, bearing the TSS1 (−1857/+753), was increased 6.6-fold over the promoter-less vector ($p < 0.001$). Furthermore, deletion of its 3′-region between +753 and +109 induced a significant increase in luciferase activity, when compared to the parental fragment (−1857/+753 vs. −1857/+109, $p < 0.001$) in basal conditions. Further 3′ deletion from +109 to −182, which removed the TSS1, the TATA box and the CRE binding sites (−1857/−182 fragment), completely abolished the luciferase activity in basal conditions, dropping it to the value of the promoter-less vector. These results suggested that: (1) the core promoter region is located between −182 and +109; and (2) a negative regulator of the basal promoter activity is located in the +753 to +109 region. Because *gdnf* mRNA in seminiferous tubules accumulates after stimulation with FSH (Fig. 1), if all the transcriptional units are correctly identified, the activity of at least one construct should be regulated by cAMP. To test this hypothesis, luciferase activity was measured in transfected Sertoli cells treated with 1 mM $(Bu)_2$cAMP (Fig. 4). A significant induction of luciferase activity over basal conditions was detected in cells transfected with the −1857/+753 and −1857/+109 fragments, which bear the CRE binding sites. In the −1857/+753 fragment, $(Bu)_2$cAMP treatment induced a 4.8-fold increase over basal conditions ($p < 0.001$), while in the −1857/+109 fragment, the induction was 3.4-fold over basal conditions ($p < 0.05$).

3.5. Identification of negative regulatory sequences downstream of the TSS1

We next aimed to narrow the region involved in the negative regulation of luciferase activity. The in silico analysis of evolutionarily conserved regions revealed the presence of two putative NRSE-like binding sites in the +753 to +109 region that may be recognized by the transcriptional repressor RE1-Silencing Transcription factor (REST/NRSF). REST may generally repress neuronal genes in non-neuronal tissues (Ooi and Wood, 2007). Therefore, we evaluated the involvement of these specific regions in the negative regulation of the luciferase activity (Fig. 5). The two putative NRSE-like binding sites were deleted to obtain the −1857/+753 Δ(+513/+534, +684/+710) genomic fragment. The promoter activity of the parental and deleted fragments were measured both in basal and $(Bu)_2$cAMP-stimulated conditions. Deletion of the two putative NRSE-like binding sites did not significantly modify the luciferase activity in both conditions. To further exclude a possible involvement of REST/NRSF on the negative regulation of *gdnf* mRNA levels in Sertoli cells, REST levels were down-regulated by short interference RNA (Supplementary Fig. 2). We reasoned that if REST negatively regulates *gdnf* transcription, its down-regulation could lead to an increase of the levels of *gdnf* transcripts. Primary Sertoli cells were transfected with either REST-specific siRNA or with scrambled siRNA as a control, and the transfected cells were left untreated or were treated with 1 mM $(Bu)_2$cAMP. Then, *gdnf* mRNA was evaluated by real-time PCR (Supplementary Fig. 2). In line with the site-mutagenesis experiments, down-regulation of REST in Sertoli cells did not modify the basal or the stimulated *gdnf* mRNA levels compared to the scrambled-treated cells. Altogether, these data indicated that the NRSE-like binding sites are not involved in the negative regulation of promoter activity. However, internal deletion of the region, including the two NRSE-like binding sites, and a CRE and an NF-kB binding site (−1857/+753 Δ(+513/+710)), was able to induce a significant increase in luciferase activity over the −1857/+753 parental construct, in both basal and $(Bu)_2$cAMP-stimulated conditions (3.2-fold and 1.8-fold, respectively) (Fig. 5). This indicated the involvement of this 200-bp region in the negative regulation of luciferase activity.

3.6. Involvement of the CRE binding sites in the regulation of basal and cAMP-stimulated transcription of gdnf

The analysis of conserved DNA binding sites among mouse, rat and human *gdnf* genes revealed the presence of three conserved CRE binding sites, which in the mouse are localized upstream of TSS1 (Supplementary Fig. 3). To directly test their involvement in cAMP-induced activity of genomic fragments, the three CRE sites were deleted in the −1857/+753 fragment (Fig. 6). Deletion of the three CRE sites significantly hampered both the basal and the $(Bu)_2$cAMP-induced activities. Compared to the parental −1857/+753 fragment, activity of the CRE-mutated fragment was reduced to 55% in the basal condition ($p < 0.05$) and to 65% in the $(Bu)_2$-cAMP-stimulated condition ($p < 0.05$). These data show a direct involvement of the CRE binding sites upstream of TSS1 in cAMP-regulated transcription of the murine *gdnf* gene in Sertoli cells.

4. Discussion

GDNF, an important player in the spermatogonial stem cell's niche, regulates several aspects of the spermatogonial stem cell physiology, such as the balance of self-renewal vs. differentiation, cell survival, proliferation, and migration (Hofmann, 2008). The FSH-dependent expression of GDNF by Sertoli cells was first established by Nishimune's group in 2002 (Tadokoro et al., 2002). In an animal model for germ-cell defects (Sl/Sl mice), the injection of a GnRH antagonist induced a reduction of undifferentiated spermatogonia proliferation with a concomitant GDNF suppression. Importantly, in primary Sertoli cell culture, FSH induced a time- and concentration-dependent increase in GDNF expression. FSH also increases the level of expression of GDNF in TM4 cells, a Sertoli cell line (Simon et al., 2007). More recently it was shown that the levels of GDNF varies during the cycle of the seminiferous epithelium in different species, even though conflicting data have been reported on the timing of the GDNF peak (Caires et al., 2012; Grasso et al., 2012; Hasegawa et al., 2013; Johnston et al., 2011; Makela et al., 2011; Sato et al., 2011). In the present study, using in vitro culture of isolated and staged seminiferous tubules, we directly showed that stage-specific expression is modulated by FSH. This supports the idea that, as observed in other systems, the endocrine system may control the stem cell compartment indirectly through the niche (Gancz and Gilboa, 2013; Joshi et al., 2012). It should be noted that male mice knockout for the FSH

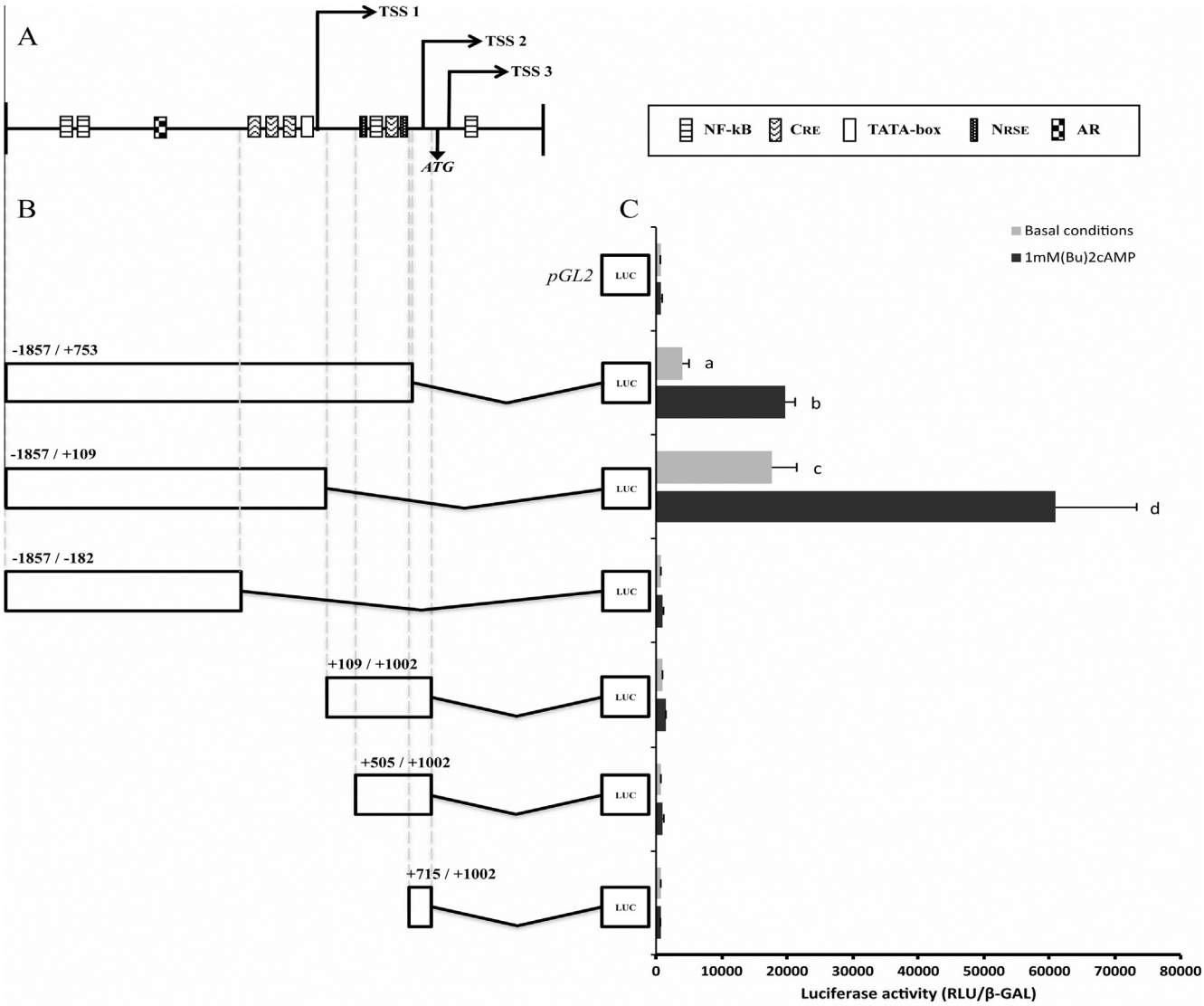

Fig. 4. The basal and stimulated activity of the 5′-flanking region of the murine *gdnf* gene in Sertoli cells. (A) Schematic representation of the genomic regions flanking the TSSs. TSSs are marked as arrows. Nucleotides are numbered from the TSS1. Potential regulatory elements are shown as boxes. (B) Representation of the *gdnf*-luc-plasmids containing different 5′ and 3′ deletion of the −1857/+1002 bp genomic region. C) Sertoli cell cultures were transfected with plasmids indicated in B and treated with vehicle (basal conditions) or with 1 mM $(Bu)_2$cAMP for 24 h. Luciferase activity is expressed as RLU, corrected by β-gal activity. Data are the mean ± SEM from $n = 4$ (individual points determined in duplicates). a Significant difference vs. b ($p < 0.001$); c significant difference vs. d ($p < 0.05$); a significant difference vs. c ($p < 0.05$); b significant difference vs. d, ($p < 0.05$).

receptor (FSHR-null mice) are still fertile, albeit number of germ cells are reduced in FSHR-null mice compared to wild-type littermates (Krishnamurthy et al., 2000). This indicates that FSH/FSHR axis might not be essential for GDNF production in vivo. Because *gdnf* in Sertoli cells is likely regulated by other systemic or locally derived factors, the analysis of the *gdnf* regulatory regions may shed light on the regulation of the mammalian testis niche.

Available evidence for the murine *gdnf* gene indicated the presence of three alternative promoters upstream of exons 1, 2 and 3, respectively, producing cDNAs with different 5′-UTR segments, common coding exons and a long 3′-untranslated region (3′-UTR) (Matsushita et al., 1997; Tanaka et al., 2000a,b, 2001). However, among the promoters, the one localized upstream of exon 1 is more active, most likely supporting the major portion of *gdnf* transcription in the neuronal model (Tanaka et al., 2000a). From the 5′-RACE experiments, we found that all the TSSs identified in Sertoli cells spanned the area of the exon 1. Moreover, genomic fragments carrying the intronic promoters 2 and 3 are not active in immature Sertoli cells. These data strongly suggest that the only active promoter of *gdnf* in Sertoli cells is promoter 1, which sustains all *gdnf* transcription.

In the 5′-RACE experiments, we were able to identify three different TSSs. The most distal, the TSS1, coincides with the TSS identified in the mouse embryo (Matsushita et al., 1997). Although the TSSs upstream of exon 1 in human *gdnf* gene have not been experimentally identified, a putative TSS identified by bioinformatic analysis is localized in the same region as in the mouse (Grimm et al., 1998). The TSS1 is localized approximately 30 bp downstream the TATA-box sequence and approximately 900 bp upstream from the putative first AUG. Transcripts generated from TSS1 therefore have a long 5′-UTR region. In our 5′-RACE experiments, we identified the TSS2 and the TSS3, located approximately 70 bp upstream and 30 bp downstream, respectively, from the putative first AUG. We hypothesized that 5′-flanking regions of these TSSs may contain an alternative promoter region, as was described for TGA-3 cells, a mouse astroglial cell line (Tanaka

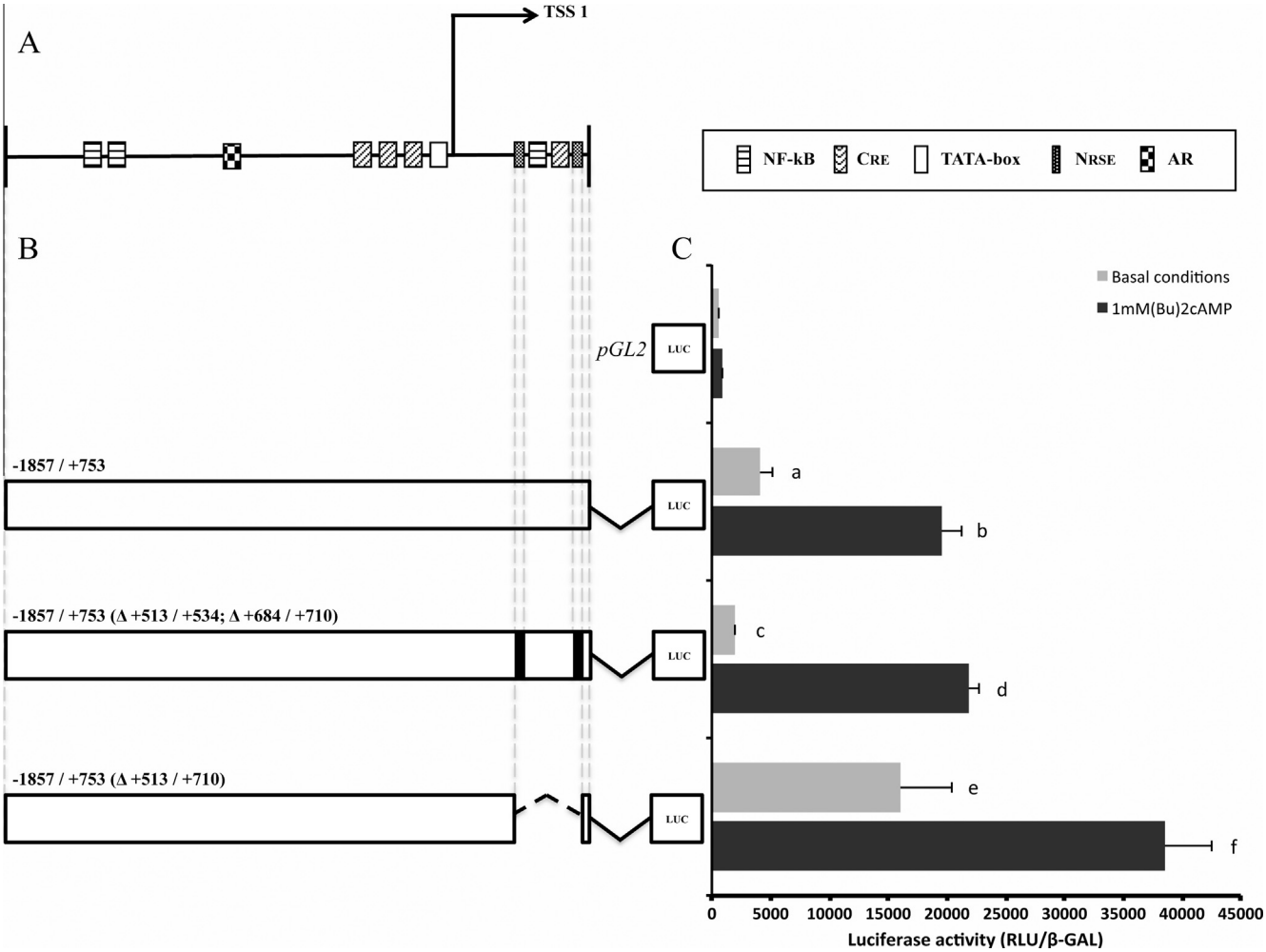

Fig. 5. Identification of a negative regulatory region in the 5′-UTR of gdnf mRNA expressed in Sertoli cells. (A) Schematic representation of the genomic regions flanking the TSS1 (marked by an arrow). Nucleotides are numbered from the TSS1. Potential regulatory elements are shown as boxes. (B) A representation of the *gdnf*-luc-plasmids containing different internal deletions in the −1857/+753 bp genomic region. (C) Sertoli cell cultures were transfected with plasmids indicated in B and treated with vehicle (basal conditions) or with 1 mM $(Bu)_2$cAMP for 24 h. Luciferase activity is expressed as RLU, corrected by β-gal activity. Vertical black lines represent the deletion of single NRSE binding site, while the v-shaped broken line represents the deletion from the +513 bp to the +710 bp genomic region. Data are the mean ± SEM from $n = 4$ (individual points determined in duplicates). a Significant difference vs. b ($p < 0.001$); c significant difference vs. d ($p < 0.001$); e significant difference vs. f ($p < 0.05$); a significant difference vs. e ($p < 0.05$); c significant difference vs. e ($p < 0.05$); b significant difference vs. f ($p < 0.05$); d significant difference vs. f ($p < 0.05$).

et al., 2001). However, these regions did not show significant luciferase activity compared with the promoter-less vector. Because luciferase activity was significantly up-regulated in genomic fragments bearing TSS1 and its surrounding regions, these results suggest that TSS1 is the predominant TSS in Sertoli cells.

In the present study, we found a region of the 5′-UTR of the mouse *gdnf* gene that acts as a suppressor of luciferase activity. This is in line with published data in neural models that show several regions within the 5′-UTR that negatively regulate luciferase activity (Tanaka et al., 2000b, 2001). Tanaka and his group identified a negative regulatory sequence in the region +316/+711 and a possible attenuation of translation due to an interfering ATG at position +551 (Tanaka et al., 2000b, 2001). Interestingly, analysis of the evolutionarily conserved regions among human, mouse and rat *gdnf* genes revealed two conserved NRSE-like sites at the +513/+534 and +684/+710 positions. NRSE is a cis-element that negatively regulates the transcription of genes in non-neuronal tissues that express the NRSF/REST protein (Ooi and Wood, 2007). Here we found that deletion of NRSE-like sites, did not rescue luciferase activity of the −1857/+753 genomic fragment. Moreover, the *gdnf* expression level in primary Sertoli cells did not increase after siRNA treatment of REST/NRSE. Therefore, we excluded a direct involvement of REST/NRSE on the inhibition of luciferase activity. The internal deletion of an approximately 0.25 kb genomic region that includes the two NRSE-like binding sites, and a CREand an NF-kB binding site (−1857/+753 Δ (+513/+710), was able to induce a 7-fold increase in basal luciferase activity over the −1857/+753 parental construct. Because this region is included in the 5′-UTR of the *gdnf* mRNA, the inhibitory effect on luciferase activity could occur at both the transcriptional and translational levels. Additional experiments are needed to clarify the mechanisms of inhibition involved.

By in-silico analysis, we have identified a list of conserved transcriptional binding sites on the 5′-flanking region of the TSS1. Comparisons between human and rodent DNA sequences are widely used to identify regulatory regions. When a strong sequence conservation for the binding of a putative transcription factor is found, the hypothesis can be put forward for a conserved function (Wasserman et al., 2000). However, direct experimental data have revealed that 30–40% of human functional sites are not functional in rodents (Dermitzakis and Clark, 2002). Therefore, it is important to directly test the involvement of sequences that have been iden-

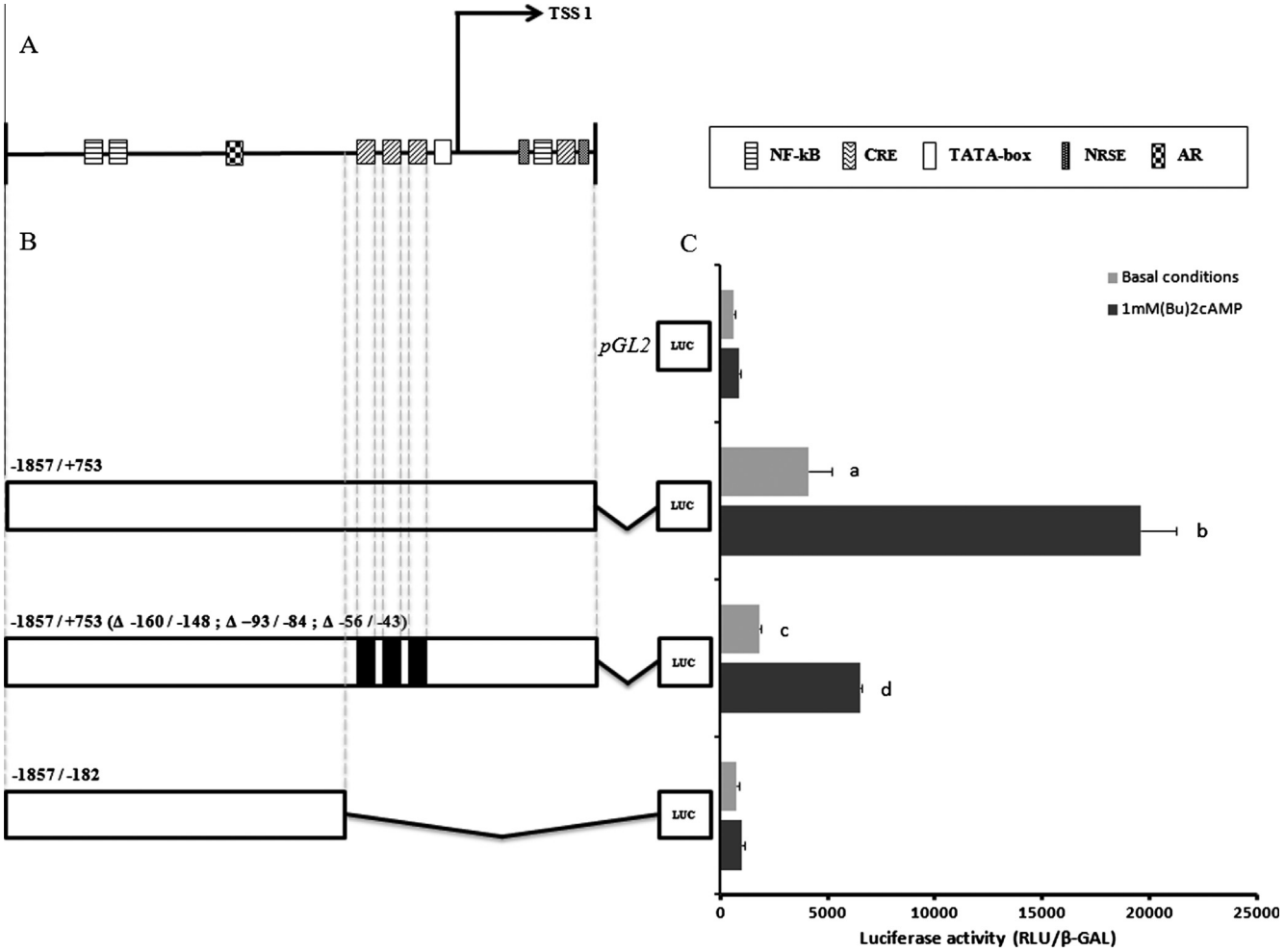

Fig. 6. Involvement of the three CRE sites in the basal and cAMP-induced transcription of murine *gdnf*. (A) Schematic representation of the genomic regions flanking the TSS1 (marked by an arrow). Nucleotides are numbered from the TSS1. Potential regulatory elements are shown as boxes. (B) A representation of the *gdnf*-luc-plasmids containing different internal deletions in the −1857/+753 bp genomic region. (C) Sertoli cell cultures were transfected with plasmids indicated in B and treated with vehicle (basal conditions) or with 1 mM $(Bu)_2$cAMP for 24 h. Luciferase activity is expressed as RLU, corrected by β-gal activity. Vertical black lines represent the internal deletion of single CRE binding site. Data are the mean ± SEM from $n = 4$ (individual points determined in duplicates). a Significant difference vs. b ($p < 0.001$); c significant difference vs. d ($p < 0.05$); a significant difference vs. c ($p < 0.05$); b significant difference vs. d ($p < 0.05$).

tified by in-silico analysis. Among the conserved binding sites, we were interested in the identification of cAMP responsive elements (CRE) that could be implicated in the FSH-induced response of the *gdnf*. It is well known that the transduction pathways activated by FSH lead to the phosphorylation and activation of CREB (cAMP response element binding protein), a transcription factor that binds to CRE (Walker and Cheng, 2005). CREB belongs to the bZIP superfamily of transcription factors, and within this superfamily, CREB and the closely related factors CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1) comprise a subcategory referred to as the CREB family (Don and Stelzer, 2002). The prototypical target sequence for CREB is the palindromic cAMP response element (TGACGTCA), which was first identified in the neuropeptide somatostatin gene (Montminy et al., 1986). Since then, CRE or CRE-like sequences have been identified in hundreds of cellular and viral genes, where they are required for second messenger-directed transcriptional responses (De Cesare and Sassone-Corsi, 2000). Our analysis revealed the presence of three CRE sites, localized just upstream of the TATA-box and TSS1, that were highly conserved among human, mouse and rat *gdnf* genes. Transfection experiments showed that deletion of the three CRE sites not only blunted the cAMP-response but also caused a 55% decrease in basal promoter activity. This indicates that the CREs in this genomic construct may also function as a basal stimulatory element (Quinn et al., 1988). The observation that cAMP induces *gdnf* mRNA expression and the activity of the *gdnf* promoter in Sertoli cells, but not in glioma cells (Matsushita et al., 1997), suggests that the impact of the cAMP/PKA pathway on GDNF expression might be dependent on cell type.

In our in-silico analysis of conserved putative transcription factor binding sites, we found two NF-Kbs in the 5′-flanking region of TSS1 and one NF-kB in the 5′-UTR. The increase in GDNF mRNA upon treatment with TNFα and IL-1β in primary Sertoli cells (Simon et al., 2007), as well as in glioma cell lines (Baecker et al., 1999), suggests the involvement of NF-kB, and this hypothesis awaits further analysis.

In conclusion, we have identified the *gdnf* promoter that is active in Sertoli cells, and, for the first time, we have shown a direct involvement of the CRE binding sites in cAMP-regulated transcription of murine *gdnf* in Sertoli cells. We have also identified several putative transcriptional binding sites on the promoter which are conserved among species. This study provides the basis for future studies aimed at the characterization of specific sequences required for modulation of transcriptional activity. Because of the importance of GDNF for SSC self-renewal and proliferation, the results obtained may give important insights on how hormonal

or paracrine signals modulate the expression of niche-derived factors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2014.07.006.

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