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Cyclic AMP response element binding (CREB) protein acts as a positive regulator of *SOX3* gene expression in NT2/D1 cells

Natasa Kovacevic-Grujicic, Marija Mojsin, Jelena Popovic, Isidora Petrovic, Vladanka Topalovic & Milena Stevanovic*
Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, PO BOX 23, 11010 Belgrade, Serbia

***SOX3* is one of the earliest neural markers in vertebrates, playing the role in specifying neuronal fate. In this study we have established first functional link between CREB and human *SOX3* gene which both have important roles in the nervous system throughout development and in the adulthood. Here we demonstrate both *in vitro* and *in vivo* that CREB binds to CRE half-site located -195 to -191 within the human *SOX3* promoter. Overexpression studies with CREB or its dominant-negative inhibitor A-CREB indicate that this transcription factor acts as a positive regulator of basal *SOX3* gene expression in NT2/D1 cells. This is further confirmed by mutational analysis where mutation of CREB binding site results in reduction of *SOX3* promoter activity. Our results point at CREB as a positive regulator of *SOX3* gene transcription in NT2/D1 cells, while its contribution to RA induction of *SOX3* promoter is not prominent. [BMB Reports 2014; 47(4): 197-202]**

INTRODUCTION

SOX proteins (*SRY*-related HMG-*box*) belong to family of transcription factors that act as key regulators of cell fate decisions in numerous developmental processes (1, 2). They perform their function either as classical transcription factors or as architectural components of chromatin (1). *SOX3*, a member of the *SOXB1* subfamily (3), is one of the earliest neural markers in vertebrates, playing the role in specifying neuronal fate (4). This gene is expressed in the central nervous system (CNS) throughout development and is implicated in the genetic cascades that direct brain formation (4, 5).

CREB (cyclic AMP response element binding protein) is a transcription factor that is well-known as both positive and negative regulator of gene transcription (6, 7). This transcription factor can stimulate both basal and inducible transcription of its target genes through its bipartite *trans*-activation domain. CREB is widely distributed in most tissues and has an important role in regulating cell proliferation and differentiation of many different cell types, including neuronal cells (8, 9). Mice lacking *Creb* and *Crem* display generalized cell death in the nervous system during development, whereas postnatal disruption of these factors leads to progressive neurodegeneration in the hippocampus and dorsolateral striatum (10).

Considering that both CREB and *SOX3* have important roles in the nervous system throughout development and in the adulthood we considered to investigate their functional relationship. In our previous work, we have characterized *SOX3* promoter and demonstrated that numerous transcription factors, as well as liganded RXRα are involved in the regulation of *SOX3* gene expression in embryonal carcinoma NT2/D1 cells (11-15). In this study, we have extended our work by demonstrating that CREB *trans*-activates *SOX3* promoter activity through CRE half-site motif located within its promoter region. We provide the first evidence that CREB acts as a positive regulator of *SOX3* gene expression in NT2/D1 cells.

RESULTS AND DISCUSSION

DNaseI protection analysis of the human *SOX3* promoter region

In silico analysis of the *SOX3* optimal promoter region revealed the presence of two CRE half-sites positioned -296 to -292 (CRE half-site 1) and -195 to -191 (CRE half-site 2) relative to *tsp* (Fig. 1A). Comparative analysis of mammalian orthologue sequences revealed that CRE half-site 2 is conserved both in sequence and position between analyzed *SOX3* orthologues, while CRE half-site 1 is conserved among primates only (Fig. 1A).

In order to test whether putative CRE half-sites within *SOX3* promoter region specifically bind nuclear proteins from NT2/D1 cells, we performed DNase I footprinting analysis. We mapped the protected regions on the coding strand at -316 to -279 bp and at -210 to -184 bp relative to *tsp* (Fig. 1B and C). Importantly, both regions protected by NT2/D1 nuclear proteins encompassed two CRE half-sites identified in this study.

*Corresponding author. +381-11397-6212; Fax: +381-11397-5808;
E-mail: natasakovacevic@imgge.bg.ac.rs

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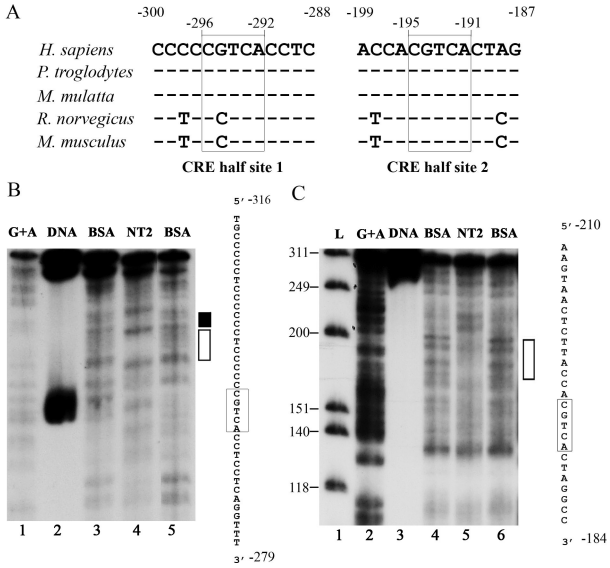

Fig. 1. DNase I footprinting analysis of human *SOX3* promoter region. (A) Alignment of human *SOX3* promoter regions containing putative CRE half-sites 1 and 2 with mammalian orthologue sequences. Numbers above the alignment indicate end positions of the human *SOX3* promoter regions and of predicted CRE half-sites relative to tsp. CRE half-site motifs are boxed. (B) and (C) *Kpn*I/*Xba*I fragments from clones F19R14 (12) and F18R12 (13) were used as probes and digested with DNase I in the presence of 25 μg of nuclear extracts from NT2/D1 cells (NT2) or bovine serum albumine (BSA) as indicated. The nucleotide sequences of the protected regions were determined by comparison with Maxam–Gilbert reaction (G+A). The protected regions encompassing putative CRE half-sites 1 and 2 are marked with white rectangles on the right-hand side. Sequences and positions of corresponding protected regions are indicated and putative CRE half-sites 1 and 2 are boxed. DNA, untreated DNA; L, DNA ladder. Black rectangle represents previously identified protected region within *SOX3* promoter (12).

CREB binds to CRE half-site located -195 to -191 within the human *SOX3* promoter both *in vitro* and *in vivo*

In order to determine if CREB protein specifically binds to protected CRE half-sites we performed EMSA competition and supershift assays. Incubation of F7R7 probe, encompassing CRE half-site 1, with NT2/D1 nuclear extracts resulted in the appearance of three distinct DNA-protein complexes that were specifically competed with unlabeled F7R7 probe (Fig. 2B, lanes 2 and 3). Competition with unlabeled MUT1 and consensus CRE probes (Fig. 2B, lanes 4 and 5) excluded the possibility that CREB participates in complex formation with CRE half-site 1.

Incubation of F3R3 probe, encompassing CRE half-site 2, with NT2/D1 nuclear extracts resulted in the appearance of a distinct DNA-protein complex that was specifically competed with unlabeled F3R3 probe (Fig. 2B, lanes 7 and 8). While MUT2 failed to compete against complex formation (Fig. 2B, lane 9), consensus CRE probe efficiently competed for protein binding with F3R3 probe (Fig. 2B, lane 10), indicating that CREB protein might be involved in complex formation on CRE half-site 2. When MUT2 was used as a probe, formation of a specific complex was impaired (Fig. 2B, compare lanes 7 and 12).

In order to verify that CREB protein specifically binds to CRE half-site 2 and not to the CRE half-site 1, we have performed supershift assays using anti-CREB antibody (Fig. 2C, lanes 3 and 7). This assay resulted in formation of supershifted complex with F3R3 probe only (Fig. 2C, lane 7), while unrelated antibody used as a control (anti-Pax 2/6) did not cause any changes in complex formation (Fig. 2C, compare lanes 6 and 8). Increase in the intensity of DNA-protein complex observed here upon addition of anti-CREB antibody is most likely result of non-specific stability effect, as described previously (16).

Finally, to assess whether CREB binds to the *SOX3* promoter *in vivo*, a chromatin immunoprecipitation (ChiP) assay was performed. DNA precipitated with anti-CREB antibodies yielded specific PCR product whereas anti-actin antibody, used as a negative control, failed to do so (Fig. 2D). Taken together, our results demonstrate that CREB interacts with *SOX3* promoter both *in vitro* and *in vivo*.

CREB upregulates *SOX3* expression in NT2/D1 cells

In order to test if CREB protein has an effect on *SOX3* protein expression *in vivo*, we performed Western blot analysis on WCLs from NT2/D1 cells transfected either with empty or pcDNA3CREB expression construct. CREB overexpression leads to an increase in *SOX3* protein level (Fig. 3A), indicating that this transcription factor acts as a positive regulator of *SOX3* gene activity. Also, we have analyzed the effect of dominant-negative inhibitor of CREB (A-CREB) on *SOX3* protein expression. This inhibitor acts by interacting with the basic region of CREB forming a coiled-coil extension of the leucine zipper and thus preventing CREB from binding to DNA (17). After overexpression of A-CREB in NT2/D1 cells, no significant change in *SOX3* protein level was observed (Fig. 3A).

CREB upregulates *SOX3* promoter activity in NT2/D1 cells

In order to confirm the functional role of CREB in transcriptional regulation of the *SOX3* promoter activity, we have performed transient co-transfection experiments with CREB expression vector. Overexpression of CREB resulted in a significant increase of *SOX3* promoter activity by approximately 7.5-fold (Fig. 3B). Furthermore, when dominant-negative inhibitor of CREB was overexpressed, activity of F19R30 construct was reduced to approximately 65% (Fig. 3B). The functional significance of CRE half-site 2 for *SOX3* promoter activity was analyzed by introducing mutation into this binding site within F19R30 promoter-reporter construct (F19R30MUT2) (Fig. 3C). Mutation of CRE half-site 2 led to reduction in *SOX3* promoter activity to 53% compared to the *wild-type* counterpart (Fig. 3C). Furthermore, the response of F19R30MUT2 to CREB overexpression was decreased approximately to 60% compared to its *wt* counterpart (Fig. 3D). The residual promoter activity is probably the result of synergistic activity of

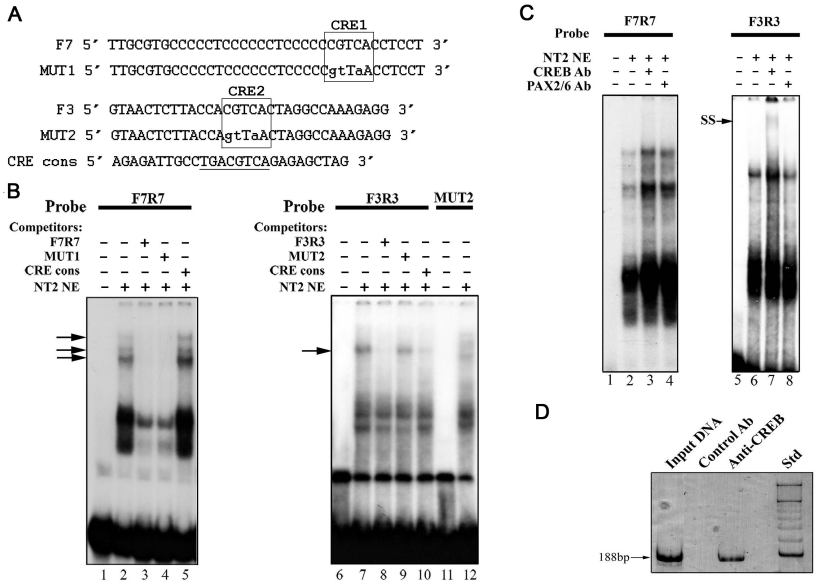

Fig. 2. CREB binds to CRE half-site 2 within the *SOX3* promoter region *in vitro* and *in vivo*. (A) Sequences of *wt*, mutated probes and CRE consensus used in EMSA assays. CRE half-sites 1 and 2 are boxed. Mutated nucleotides are in lowercase and CRE consensus is underlined. (B) Radiolabeled F7R7 and F3R3 probes, encompassing putative CRE half-sites 1 and 2, and NT2/D1 nuclear extracts (NE) were used in EMSA (lanes 2-5 and 7-10, respectively). DNA-protein complexes were competed with a 100-fold molar excess of *wt*, mutated oligonucleotides or consensus CRE as indicated at the top. Radiolabeled MUT2 probe with mutated putative CRE half-site 2 and NT2/D1 nuclear extracts were used in EMSA (lane 12). The arrows indicate shifted complexes. (C) Supershift assays with F7R7 and F3R3 probes and NT2/D1 nuclear extracts (lanes 2-4 and 6-8) using anti-CREB and anti-Pax 2/6 antibodies, as indicated. SS and arrow indicate supershifted complex. (D) ChIP analysis was performed using anti-CREB and control antibodies, as indicated. Input DNA represents PCR amplification of 0.5% of unprecipitated chromatin. PCR product is marked by molecular size and arrow. Std, 50 bp DNA Ladder.

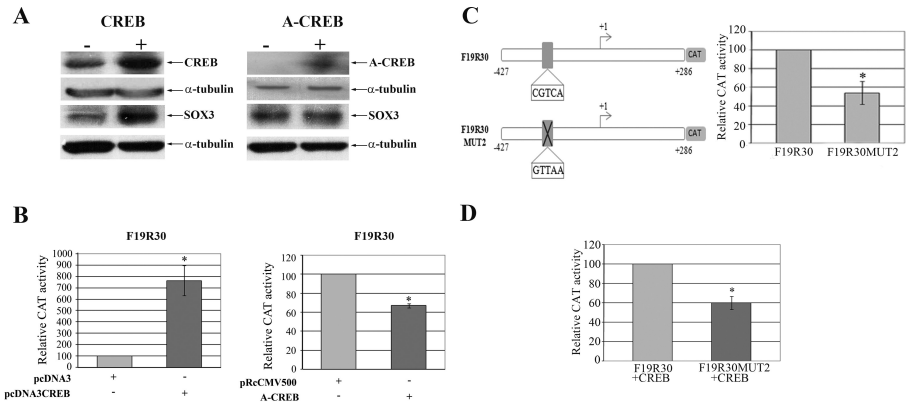

Fig. 3. CREB is a positive regulator of *SOX3* expression and promoter activity in NT2/D1 cells. (A) Western blot analysis of WCLs from NT2/D1 cells transfected with either empty corresponding vector or CREB or A-CREB expression vectors, as indicated. Arrows indicate the positions of CREB, A-CREB, *SOX3* and α-tubulin. The level of α-tubulin was used as a control for equal amounts of input proteins. (B) CREB upregulates, whereas its dominant-negative inhibitor A-CREB downregulates *SOX3* promoter activity. NT2/D1 cells were co-transfected with F19R30 promoter-reporter construct together with either empty corresponding vectors (pcDNA3 or pRcCMV500) or CREB or A-CREB expression constructs, as indicated. (C) Effect of CRE half-site 2 mutation on *SOX3* promoter activity. NT2/D1 cells were transfected with *wt* or mutated promoter-reporter constructs and analyzed for promoter activity. (D) CRE half-site 2 mutation reduced the response of F19R30MUT2 on CREB overexpression. NT2/D1 cells were co-transfected with wt or mutated promoter-reporter constructs together with CREB expression vector. (B), (C) and (D) The normalized CAT activities were calculated as percentage of the activity of F19R30 construct, which was set as 100%. Data are presented as the means ± S.E.M. of at least three independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. *$P < 0.05$.

other transcription factors previously demonstrated to have role in *SOX3* transcriptional regulation. Our functional assays underscore CREB as a potent positive regulator involved in maintaining the basal *SOX3* promoter activity. It was reported that CREB can stimulate basal transcription of target genes through its constitutive activation domain that interacts with the TATA binding protein associated factor (18, 19).

SOX3 is not the only *SOX* gene reported to be regulated by CREB transcription factor. It was shown that CREB specifically interacts with CRE half-site within *SOX9* proximal promoter and that mutation of this site results in downregulation of *SOX9* promoter activity (20). Also, we previously reported that CREB acts as a negative regulator of *SOX18* promoter activity (7).

The role of CREB in RA-induced *SOX3* promoter activity

It was demonstrated that RA induces NT2/D1 cells toward neuronal differentiation (21) and these cells represent an appropriate *in vitro* model system to study early steps of neuronal differentiation. Our previous work showed that induction of NT2/D1 cells by RA during first 48 h is accompanied by upregulation *of SOX3* gene expression (13).

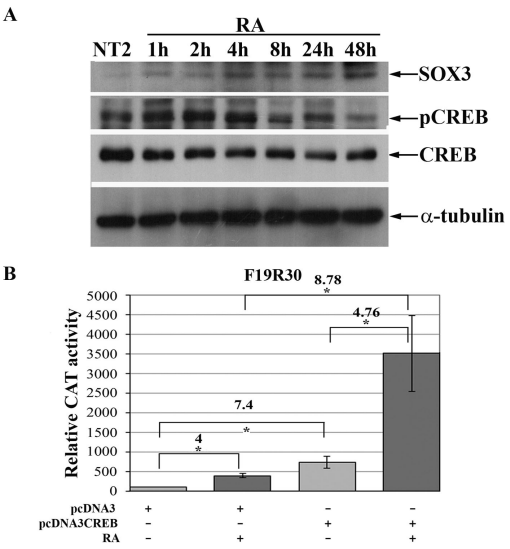

Fig. 4. The role of CREB in RA-induced *SOX3* promoter activity. (A) Effects of RA induction on phosphorylation status of CREB in NT2/D1 cells. Western blot analysis of *SOX3*, CREB and pCREB expression in uninduced (NT2) and NT2/D1 cells treated by RA for 1, 2, 4, 8, 24 and 48 h. Western blots were performed with antibodies against *SOX3*, pCREB, total CREB and α-tubulin, as indicated. Arrows indicate the positions of detected proteins. (B) The effect of CREB overexpression on *SOX3* promoter activity during early phases of RA induction of NT2/D1 cells. NT2/D1 cells were transiently co-transfected with F19R30 promoter-reporter construct together with either empty pcDNA3 or CREB expression construct and analyzed for promoter activity. The normalized CAT activities were calculated as a percentage of the activity of the F19R30 construct co-transfected with empty pcDNA3 vector in untreated cells, which was set as 100%. Data are presented as the means ± S.E.M. of five independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. *$P < 0.05$.

In order to investigate the potential role of CREB in upregulation of *SOX3* during early phases of neuronal differentiation of NT2/D1 cells, we analyzed the effect of CREB overexpression on *SOX3* promoter activity upon treatment with RA. Induction of NT2/D1 by RA yielded an approximately 4-fold increase in reporter gene activity (Fig. 4B) which is in accordance with our previous data (12, 15). Overexpression of CREB in RA-treated NT2/D1 cells led to small increase in *SOX3* promoter activity compared to the effect of CREB alone (8.78-fold versus 7.4-fold, Fig. 4B). It is important to point out that RA inducibility of the *SOX3* promoter in the presence of CREB was not considerably increased (compare 4- and 4.76-fold, Fig. 4B). These results point to CREB as a positive regulator of *SOX3* gene transcription in uninduced NT2/D1 cells, while its contribution to RA induction of *SOX3* promoter is not prominent.

Numerous data suggest that SOX3 and CREB play important roles in vertebrate neurogenesis. *SOX3* is expressed transiently by proliferating and differentiating neural progenitors in the neurogenic regions of the neonatal and adult mouse forebrain (23) suggesting that SOX3 continues to regulate proliferation and survival of neural progenitor cells throughout life. Several studies show that CREB is constitutively activated (phosphorylated) in dividing immature neural cells in neurogenic regions of both embryonic and adult vertebrate brains (24, 25). Modulation of CREB activity during early zebrafish development revealed the important role of CREB in embryonic brain development and in neural proliferation in adult brain (24).

In this study we have established first functional link be-

tween CREB and human *SOX3* gene which both have important roles in the regulation of nervous system development and adult neurogenesis.

MATERIALS AND METHODS

Sequence analysis of the *SOX3* promoter region

Databases searches and sequences alignment were performed using NCBI and ClustalW software. Sequence of human *SOX3* promoter (extracted from sequence under accession number AL121875.10) was aligned with orthologue sequences of *Pan troglodytes* (AC149044.1), *Macaca mulatta* (NW_00121 8193.1), *Rattus norvegicus* (NW_048052.2) and *Mus musculus* (NT_039706.6). MatInspector Release professional 7.4.5 program (26) was used in order to identify potential transcription factor binding sites within promoter regions of *SOX3* orthologues.

Preparation of nuclear extracts and DNase I footprinting

Nuclear extracts from NT2/D1 cells were prepared as described (27). DNase I footprinting was performed using SureTrack Footprinting Kit (Amersham Pharmacia Biotech) and F19R14 and F18R12 probes as described (12, 13). Labeled ΦX174/HinfI DNA ladder and Maxam and Gilbert G+A sequencing reaction were used to identify protected nucleotides within the probes.

Electrophoretic mobility shift assay (EMSA) and supershift assay

The *wild-type* or mutated oligonucleotides used in EMSA and supershift studies are listed in Supplementary Table S1. Radiolabeling of probes and binding reactions were carried out as described (11). For supershift assays, nuclear extracts were pre-incubated with corresponding antibodies (CREB-1 (24H4B) and PAX-2/6 (N19) - Santa Cruz Biotechnology) for 30 minutes at room temperature.

Chromatin immunoprecipitation (ChIP)

Chromatin isolation from NT2/D1 cells and ChIP assays were performed as described (28). Aliquots of cross-linked chromatin were precipitated with either 10 μg of anti-CREB antibody (48H2, Cell Signaling Technology) or 10 μg of anti-actin antibody (H-196, Santa Cruz) as negative control. Primers flanking CRE half-site 2 within human *SOX3* promoter region that were employed in PCR analysis of immunoprecipitated DNA are listed in Supplementary Table S1. PCR protocol included initial denaturation at 95°C for 10 min and 50 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis in 10% native polyacrylamide gels and visualized using PlusOne™ DNA Silver Staining Kit (GE Healthcare).

Western blot analysis

Whole cell lysates (WCL) were prepared 48 h after transient transfection of NT2/D1 cells with 10 μg of either empty pcDNA3 or pcDNA3CREB expression vector and 40 μl of FuGENE® HD Transfection Reagent (Roche Diagnostics) as recommended by manufacturer. When dominant-negative inhibitor of CREB was used, co-transfections were performed with 5 μg of either empty pRcCMV500 or A-CREB expression vector as described (12). In induction experiments, WCLs were prepared from either untreated or NT2/D1 cells treated with 10 μM RA (Sigma-Aldrich) for different periods of time (1, 2, 4, 8, 24 and 48 h) with addition of NaF (50 mM final). Western blots were performed as described (13) using anti-*SOX3* (H-135, Santa Cruz), anti-α-tubulin (DM1A, Calbiochem), anti-CREB (48H2) and anti-phospho-CREB (Ser133) (Cell Signaling Technology) antibodies.

Transfection and reporter gene analysis

Transfections of NT2/D1 cells with *wt* (F19R30) (11) or mutated *SOX3* promoter-reporter constructs were performed as described (12). For co-transfection experiments, 5 μg of either empty pcDNA3 or pcDNA3CREB expression construct or 2.5 μg of either empty pRcCMV500 or A-CREB expression vector were used. In the induction experiments, cells were stimulated by 10 μM RA and assayed for β-gal and CAT activities after 48 h. β-gal and CAT assays were performed as described (11).

Site-directed mutagenesis

For generation of mutant *SOX3* promoter construct (F19R30 MUT2) site-directed mutagenesis was performed by PCR using F19R30 promoter construct as template according to the protocol of QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) with MUT2 primer listed in Supplementary Table S1. Mutated construct was sequenced to confirm that no other mutations occurred during PCR amplification.

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