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TRANS-ACTIVATION OF THE HUMAN *SOX3* PROMOTER BY MAZ IN NT2/D1 CELLS

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Abstract — In this study, we examine the role of three highly conserved putative binding sites for *Myc*-associated zinc finger protein (MAZ) in regulation of the human *SOX3* gene expression. Electrophoretic mobility shift and supershift assays indicate that complexes formed at two out of three MAZ sites of the human *SOX3* promoter involve ubiquitously expressed MAZ protein. Furthermore, in cotransfection experiments we demonstrate that MAZ acts as a positive regulator of *SOX3* gene transcription in both undifferentiated and RA-differentiated NT2/D1 cells. Although MAZ increased both basal and RA-induced promoter activity, our results suggest that MAZ does not contribute to RA inducibility of the *SOX3* promoter during neuronal differentiation of NT2/D1 cells.

Key words: SOX3 gene, MAZ protein, NT2/D1 cells, promoter, neuronal differentiation

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INTRODUCTION

The *Sox* proteins comprise a group of transcription factors that act as key regulators of cell fate decisions in diverse developmental events (Pevny and Lovell-Badge, 1997; Wegner, 1999; Kamachi et al., 2000). Sox transcription factors show both classical and architectural modes of action (Pevny and Lovell-Badge, 1997), either activating or repressing specific target genes through interaction with different partner proteins in a manner highly dependent on cell type and promoter context (Kamachi et al., 2000; Wilson and Koopman, 2002). They are characterized by the presence of a DNA-binding HMG domain (Pevny and Lovell-Badge, 1997; Wegner, 1999) and classified into 10 groups (A - J) according to characteristics of the HMG domain and extra-HMG domain sequences (Bowles et al., 2000).

Together with *Sox1* and *Sox2*, *Sox3* belongs to the *SoxB1* subgroup of transcriptional activators (Kamachi et al., 1995; Kamachi et al., 1998; Uchikawa et al., 1999; Kamachi et al., 2000) that are expressed by proliferating progenitors of the developing CNS (Uwanogho et al., 1995; Collignon et al., 1996; Penzel et al., 1997; Uchikawa et al., 1999; Wood and Episkopou, 1999). The *Sox3/SOX3* gene is implicated in the control of nervous system development and is considered to be one of the earliest neural markers in vertebrates, playing a role in specifying neuronal fate (Brunelli et al., 2003). Chick *Sox1, Sox2 and Sox3* are critical determinants of neurogenesis, keeping neural cells undifferentiated by counteracting the activity of proneural proteins (Bylund et al., 2003).

Evidence for developmental importance of the *SOX3* gene also comes from mutational analysis in humans. Mutations in the *SOX3* gene are associated with X-linked mental retardation with growth hormone deficiency (Laumonnier et al., 2002). Furthermore, Woods et al. (2005) showed that *SOX3* is critical for normal development of the hypothalamopituitary axis in humans. Both overdosage (by duplication) and underdosage (by poly-alanine tract expansion) cause infundibular hypoplasia and hypopituitarism (Woods et al., 2005).

We previously cloned and functionally characterized the human *SOX3* promoter. At that times

we determined the transcriptional start point (*tsp*), basal promoter region, positive regulatory elements necessary for its optimal promoter activity, and some transcription factors involved in regulation of its expression (Kovacevic-Grujicic et al., 2005). We further showed that the TATA box, Sp1, USF, and NF-Y binding sites within the basal promoter of the human *SOX3* gene are of functional importance for its constitutive expression in embryonal carcinoma NT2/D1 cells (Kovacevic-Grujicic et al., 2005). Additionally, we showed that the *SOX3* gene is up-regulated during RA-induced neuronal differentiation of pluripotental embryonal carcinoma (EC) cell line NT2/D1 (Stevanovic, 2003). We also demonstrated that an atypical RA/RXRα response element and multiple CCAAT box control elements are, at least in part, responsible for up-regulation of *SOX3* gene expression (Mojsin et al., 2006; Krstic et al., 2007). Despite the studies mentioned above, only a limited number of transcription factors that interact with the *SOX3* promoter region have been identified and functionally characterized so far, and little is known about its developmental and tissue-specific regulation of expression.

Myc-associated *z*inc finger protein (MAZ) is a transcription factor that contains a proline-rich domain, three poly-alanine repeats, and six C2H2-type zinc finger motifs (Bossone et al., 1992). It was first identified as a transcription factor bound to the *c-myc* gene both at the GA box (GGGAGGG) of the ME1a1 site and at the P2 attenuator region within the first exon of this gene, as well as to a sequence that is involved in the termination of transcription of the complement 2 gene (C2) (Bossone et al., 1992). MAZ plays a role in control of the initiation of transcription of genes for the adenovirus major late protein (Parks and Shenk, 1997), CD4 (Duncan et al., 1995), the serotonin 1a receptor (Parks and Shenk, 1996), and the hematopoietic transcription factor (Bockamp et al., 1995). Additionally MAZ is involved in termination of transcription between closely spaced human complement genes and in termination of transcription within the intron of the mouse *IgM-D* gene (Ashfield et al., 1994). Thus, MAZ appears to be a transcription factor with a dual role in the initiation and termination of transcription. MAZ is expressed ubiquitously but at different levels in different human tissues, and its expression appears to be regulated in a cell cycle-dependent manner by the MAZ protein itself (Song et al., 1998).

We here establish that the human *SOX3* promoter region contains three putative binding sites for MAZ that are conserved among primates. Additionally, we demonstrate that two out of three potential MAZ-binding sites within the human *SOX3* promoter can specifically bind MAZ protein present in NT2/D1 nuclear extracts. Furthermore, we show that exogenously added MAZ leads to an increase in *SOX3* promoter activity in both undifferentiated and RA-differentiated NT2/D1 cells.

MATERIALS AND METHODS

In silico analysis of SOX3/Sox3 promoter regions

The MatInspector Release 7.4.5 professional program (http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl) was used to search the matrix family library database to identify putative MAZ binding sites in the human *SOX3* promoter region spanning the nucleotides -427 to +286 relative to *tsp*. Sequence alignments of the corresponding *SOX3/Sox3* promoter regions between human, chimpanzee, rhesus macaque, rat and mouse were performed using ClustalW software (EMBL-EBI).

Preparation of nuclear extracts and bacterially expressed recombinant protein

Nuclear extracts from uninduced and RA-induced NT2/D1 cells were prepared according to standard procedure (Dignam et al., 1983). The recombinant GST-MAZ fusion protein was expressed in the BL21 *E. coli* strain and purified following manufacturer instructions (Amersham Pharmacia).

Electrophoretic mobility shift analysis

The following oligonucleotides were used in EMSA and supershift studies:

F M1 - 5’ TTGCGTGCCCCCTCCCCCCTCCCCC CGTCACCTC 3’ (-321)

R M1 - 5' GGAGGTGACGGGGGGAGGGGGGAG GGGGCACGCAA 3' (-287)

F M2 - 5' CTCCGGGTTGCGAGGGGCGGACCA AGCCCCAA 3' (-63)

R M2 - 5' GGGTTGGGGCTTGGTCCGCCCCTCG CAACCCGGAG 3' (-29)

F M3 - 5' GCTCCAGCCCCGGGAGCG 3' (+190)

R M3 - 5' GAGTTCAGTCTCCAGAAGGCTG 3' (+279)

The positions of 5' ends of primers are numbered relative to *tsp*. EMSA probes M1 and M2 were generated by annealing corresponding forward (F) and reverse (R) oligonucleotides. Since R oligos contained 1-3 unpaired G nucleotides at the 5' ends, double-stranded oligonucleotides were labeled by the fill-in reaction using the Klenow fragment and [α-^{32}P] dCTP. The M3 probe was generated by PCR using corresponding primers and end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP.

For EMSA experiments, a weighed portion (840 ng) of recombinant MAZ protein or 2.5 μg of nuclear extracts prepared from uninduced and RA-induced NT2/D1 cells was incubated for 30 min at 37˚C with 1 ng (M1 or M2) or 4.6 ng (M3) of ^{32}P-labeled oligonucleotide probes in a binding buffer consisting of 10 mM HEPES (pH 7.9), 15 mM KCl, 0.25 mM EDTA, 0.25 mM $ZnSO_4$, 3% glycerol, 0.25 mM DTT, and 50 ng/μl of poly (dI-dC) in a total volume of 20 μl. In the supershift assays, 4 μg of antibody against MAZ (kindly supplied by Dr. Kenneth B. Marcu) was added to the reaction mixtures and incubated for 30 min at room temperature before addition of the corresponding probes.

Cell culture, transfection, and reporter gene analysis

NT2/D1 cells were maintained as described elsewhere (Andrews, 1984). For each transfection, a total of 1.2 × 10^6 NT2/D1 cells were seeded into a 10-cm dish and 5 μg of the F19R30 *SOX3* promoter reporter construct, together with 5 μg of empty pCDNA3 (Invitrogen) or the pCDNA3-MAZ expression vector and 3 μg of the pCH110 vector (Amersham Pharmacia Biotech), were cotransfected using the calcium phosphate precipitation method as described by Nikcevic et al. (2006). On the following day, the cells were induced by 10 μM all-trans RA (Sigma) for 48 h. The pCH110 vector expressing β-gal was used to adjust for differences in transfection efficiency. β-gal and CAT assays were performed as described by Kovacevic-Grujicic et al. (2005). Normalized CAT activities were calculated as percentages of the F19R30 construct activity in uninduced NT2/D1 cells transfected with empty pCDNA3 (which was set as 100%) and are presented as the means ± SD of four independent experiments. Mean values of relative CAT activities were compared with the paired sample *t*-test (Microsoft Excel) and a *p* value of less than 0.05 was considered significant.

RESULTS

The human SOX3 promoter region contains three putative MAZ-binding sites

Previous *in silico* analysis of *SOX3* promoters in mammalian orthologs revealed many conserved putative binding sites for various transcription factors, including MAZ (Kovacevic-Grujicic et al., 2007). As shown in Fig. 1A, MatInspector analysis revealed three putative MAZ-binding sites within the optimal promoter region of the human *SOX3* gene positioned from -304 to -301 (annotated as MAZ 1), -52 to -49 (annotated as MAZ 2), and +213 to +216 (annotated as MAZ 3) relative to *tsp*. Comparative analysis of these putative human *SOX3* regulatory regions with corresponding flanking sequences from chimpanzee (*Pan troglodytes*) and rhesus macaque (*Macaca mullata*) revealed that all three putative MAZ-binding sites are conserved with respect to their positions (Fig. 1A), while MAZ 2 and 3 are conserved with respect to the sequence as well. Within the analyzed primate promoter sequences, a single nucleotide substitution was detected in the rhesus macaque sequence homologous to MAZ 1 (Fig. 1A). Although the analyzed MAZ-binding sites from various promoters are characterized by the sequence CCCTCCC or GGGAGGG (Bossone et al., 1992; Des Jardins and Hay, 1993; Duncan et al., 1995; Okamoto et al., 2002), it has also been reported that MAZ is able to bind to other GC rich

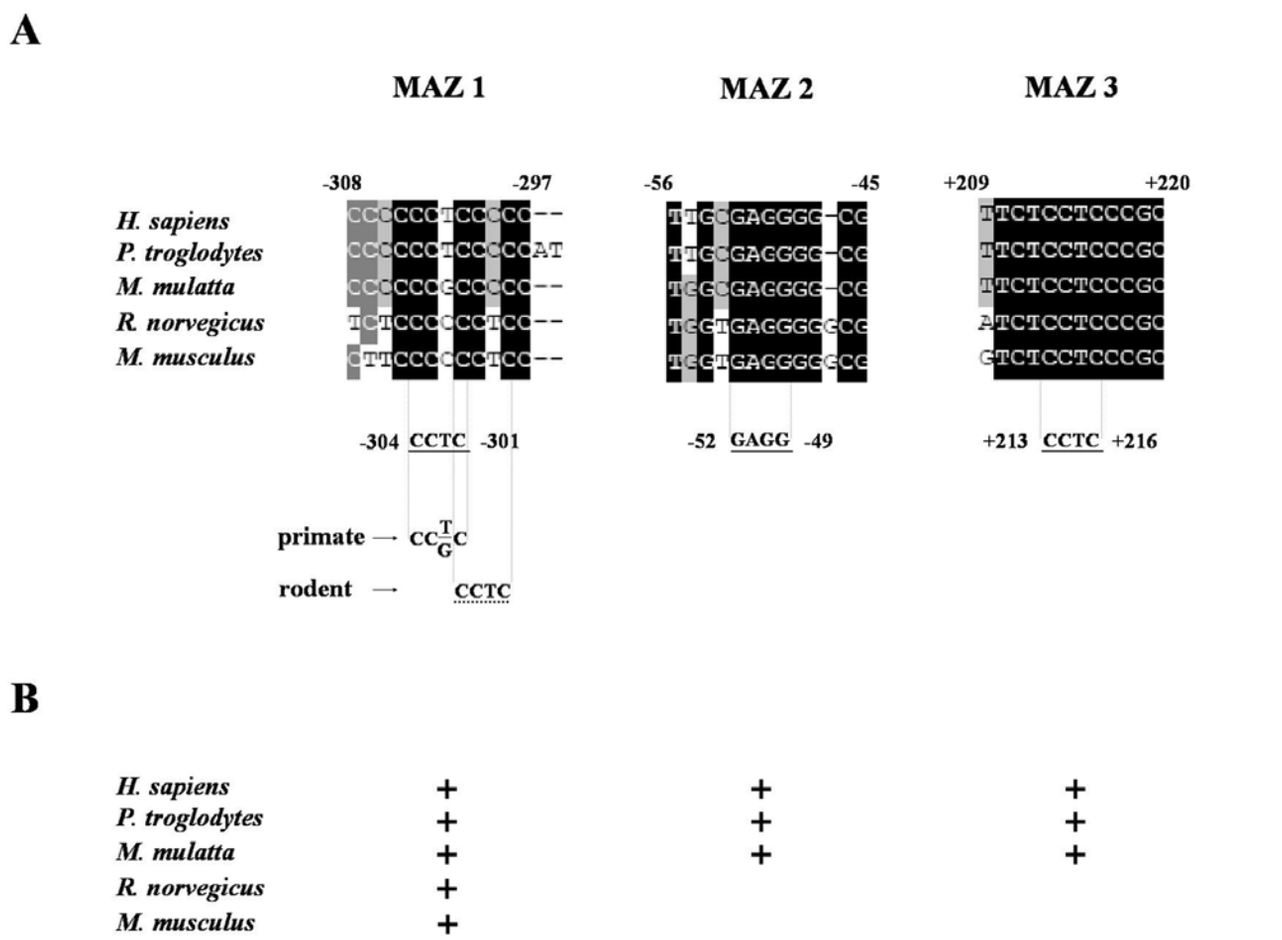

Fig. 1. (A) Alignment of mammalian *SOX3/Sox3* promoter regions containing putative MAZ binding sites predicted by MatInspector. Numbers above the alignment indicate the end positions of the presented *SOX3* promoter regions relative to *tsp*. Putative core MAZ-binding sites in the human *SOX3* promoter are underlined by solid lines and their positions relative to *tsp* are indicated. Putative core MAZ-binding sites in rodent *Sox3* promoter regions are underlined by a dotted line. (B) Schematic overview of the putative MAZ-binding sites predicted by the MatInspector program in *SOX3/Sox3* promoter regions of man (*Homo sapiens*), chimpanzee (*Pan troglodytes*), rhesus macaque (*Macaca mullata*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*). + denotes the presence of putative MAZ-binding site.

sequences such as CCGCCC (Song et al., 2001). Accordingly, it is very likely that the same sequence present in the putative MAZ 1 site of the rhesus macaque *Sox3* promoter might be functional.

While putative MAZ 1, 2, and 3 are conserved in both position and sequence among primates, MatInspector analysis showed that only the putative MAZ 1 site is preserved with respect to its position and sequence in rat (*Rattus norvegicus*) and mouse (*Mus musculus*) *Sox3* promoter regions (Fig. 1A and B). The rodent putative MAZ 1 site is positioned three nucleotides downstream from the primate MAZ 1 site (Fig. 1A). Although the MatInspector program did not recognize the presence of two other MAZ-binding sites in the rodent *Sox3* promoter regions that would correspond to putative MAZ 2 and MAZ 3 in primates, alignment analysis showed 100% nucleotide identity within the core MAZ-binding sites of corresponding regions in primates and rodents (Fig. 1A). The observed conservation of MAZ-binding sites within *Sox3/SOX3* promoter

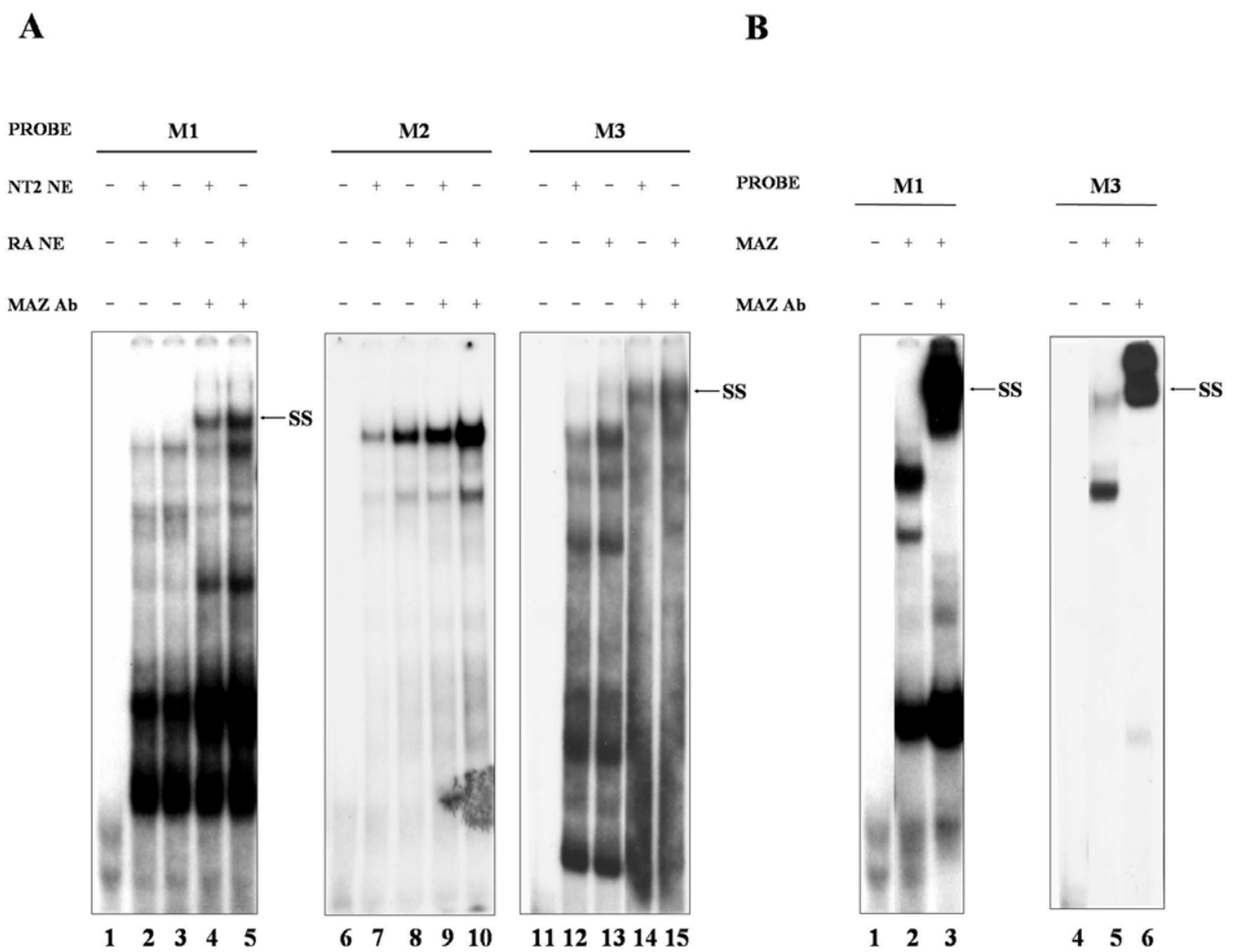

Fig. 2. EMSA with oligonucleotide probes containing putative MAZ binding sites from the human *SOX3* promoter. (A) EMSAs were performed with radioactively labeled probes M1, M2 and M3 and nuclear extracts from untreated (lanes 2, 4, 7, 9, 12, and 14) and RA-treated (lanes 3, 5, 8, 10, 13 and 15) NT2/D1 cells. Supershift analyses were performed by adding anti-MAZ antibody (lanes 4, 5, 9, 10, 14, and 15). Supershifted complexes (SS) are marked with arrows. (B) EMSAs were performed with radioactively labeled probes M1 and M3 and recombinant MAZ protein (lanes 2, 3, 5, and 6). Supershift analyses were performed by adding anti-MAZ antibody (lanes 3 and 6). Supershifted complexes (SS) are marked with arrows.

regions in mammals suggests the existence of a common transcriptional regulatory mechanism that relies on this transcription factor.

Binding of the MAZ transcription factor to putative binding sites in the human SOX3 promoter

Since putative transcription factor-binding sites that have high core similarity and are located within conserved regions could have relevance in transcriptional regulation, we chose to investigate the role of the MAZ transcription factor in transcriptional regulation of human *SOX3* gene expression. To judge from MatInspector and alignment analysis, we generated oligonucleotide probes M1 (-321 to -287 relative to *tsp*), M2 (-63 to -29), and M3 (+190 to +279) that include the predicted conserved MAZ-binding sites from the human *SOX3* pro-

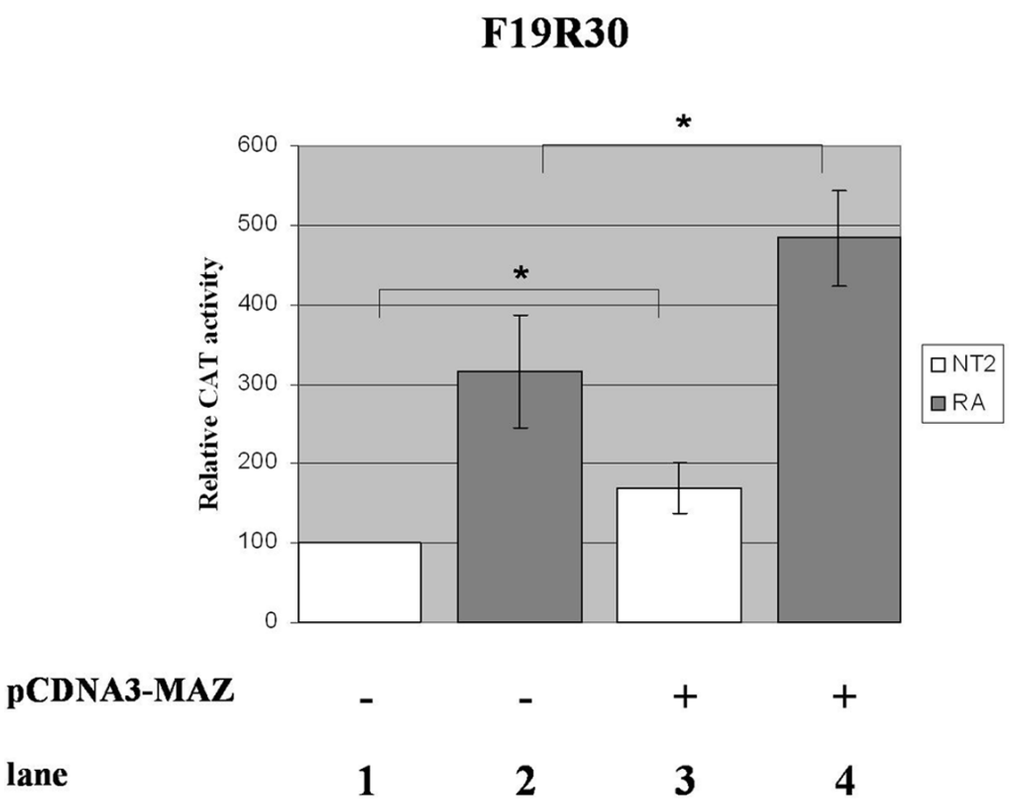

Fig. 3. Effect of MAZ overexpression on *SOX3* promoter activity. NT2/D1 cells were transiently cotransfected with the F19R30 *SOX3* promoter construct together with either empty pCDNA3 or the pCDNA3-MAZ expression vector. Normalized CAT activities were calculated as percentages of the F19R30 activity in uninduced NT2/D1 cells transfected with empty pCDNA3 (which was set as 100%) and are presented as the means ± SD of four independent experiments. Mean values of relative CAT activities were compared with the paired sample *t*-test (Microsoft Excel). Values of $p < 0.05$ are indicated by asterisks.

moter region. In order to test whether these regions specifically interact with nuclear proteins prepared from uninduced and RA-induced NT2/D1 cells, we performed electrophoretic mobility shift assay. As shown in Fig. 2A, incubation of all three probes with nuclear extracts resulted in the appearance of several complexes (Fig. 2A, lanes 2, 3, 7, 8, 12, and 13). The shifted complexes gave the same binding patterns with nuclear extracts prepared from both stem and RA-induced NT2/D1 cells.

In order to test the presence of MAZ in protein/DNA complexes, we used antibody against MAZ. Addition of this specific antibody resulted in the formation of supershifted complexes with probes M1 and M3 (SS, Fig. 2A, lanes 4, 5, 14, and 15), but not with the M2 probe (Fig. 2A, lanes 9 and 10). In addition, the ability of probes M1 and M3 to bind MAZ was further confirmed by using purified recombinant MAZ protein (Fig. 2B, lanes 2 and 5). Complexes obtained by binding of recombinant MAZ were completely supershifted in the presence of antibody against MAZ (Fig. 2B, lanes 3 and 6). From these experiments we conclude that both recombinant and endogenous MAZ specifically interact with probes M1 and M3 containing putative MAZ binding sites from the human *SOX3* promoter.

It is interesting to point out that while the MatInspector program predicted a potential MAZ-binding site within the M2 probe (-63 to -29) of the human *SOX3* promoter, anti-MAZ antibody failed to detect the presence of MAZ in formed DNA/protein complexes. This result shows that MAZ protein cannot recognize the predicted MAZ 2 binding site within the *SOX3* promoter.

Trans-activation of the human SOX3 promoter by MAZ in NT2/D1 cells

Since we demonstrated specific MAZ binding to two conserved binding sites within the *SOX3* promoter, we wanted to test the effect of MAZ overexpression on *SOX3* promoter activity in NT2/D1 cells. Accordingly, a pCDNA3-MAZ expression vector containing a full-length coding sequence of the human MAZ protein was cotransfected with the F19R30 *SOX3* promoter construct (spanning the sequence from -427 to +286 relative to *tsp*) (Kovacevic-Grujicic et al., 2005) into NT2/D1 cells.

We previously found that neural induction of NT2/D1 cells by RA is accompanied by up-regulation of *SOX3* gene expression at both the mRNA and protein levels (Stevanovic, 2003; Mojsin et al., 2006). It was previously reported that MAZ is essential for ME1a1 site-mediated expression of the *c-myc* gene during neuroectodermal differentiation of mouse P19 EC cells (Komatsu et al., 1997). Furthermore, Okamoto et al., (2002) identified a GC-rich element within promoter of N-methyl-D-aspartate (NMDA) receptor subunit type 1 (NR1) which is recognized by MAZ and Sp1 and is responsible for induction of this promoter during neuronal differentiation of P19 cells. Accordingly, in order to investigate the potential role of MAZ in *SOX3* up-regulation during neuronal differentiation of NT2/D1 cells, we also analyzed the effect of MAZ overexpression on *SOX3* promoter activity in the presence of 10 μM RA.

As shown in Fig. 3, activity of the F19R30 *SOX3* promoter construct is increased approximately 3.0-fold in RA-treated versus untreated NT2/D1 cells (lane 2 versus 1), which is in accordance with data obtained previously for this *SOX3* promoter construct (Krstic et al., 2007). *Trans*-activation of the human *SOX3* promoter by MAZ was demonstrated in both uninduced and RA-induced NT2/D1 cells. Overexpression of MAZ led to 1.7-fold increase of *SOX3* promoter activity in untreated (lane 3 versus 1, Fig. 3) and 1.5-fold increase in RA-treated (lane 4 versus 2, Fig. 3) NT2/D1 cells. Taken together, our results point to MAZ as a positive regulator of *SOX3* gene transcription in both undifferentiated and RA-differentiated NT2/D1 cells. Although MAZ increased both basal and RA-induced promoter activity, the factor of RA induction in the absence or presence of exogenously added MAZ (lane 2 versus 1 and lane 4 versus 3, Fig. 3) remained approximately the same, suggesting that MAZ does not contribute to RA inducibility of the *SOX3* promoter during neuronal differentiation of NT2/D1 cells.

In conclusion, the data presented in this paper suggest that MAZ up-regulates *SOX3* promoter activity through conserved binding sites within the *SOX3* promoter region. Further experiments are needed in order to determine if MAZ acts through a single (MAZ 1 or 3) or both (MAZ 1/3) putative-MAZ binding sites identified and characterized in this study. Although the mechanism underlying this activation remains to be established, the ability of MAZ to act as an activator probably reflects its interaction with other, not yet identified, transcriptional factors involved in development and tissue-specific regulation of *SOX3* gene expression.

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TRANS-АКТИВАЦИЈА ХУМАНОГ *SOX3* ПРОМОТОРА MAZ ПРОТЕИНОМ У NT2/D1 ЋЕЛИЈАМА

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У овом раду проучавана је улога три високо конзервисана потенцијална места везивања за "*Myc*-associated zinc finger protein" (MAZ) у регулацији експресије хуманог *SOX3* гена. Есеји измењене електрофоретске покретљивости у присуству антитела на MAZ указују да комплекси који се формирају на два од три проучавана места у оквиру *SOX3* промотора садрже MAZ протеин. Такође, у експериментима котрансфекције смо показали да MAZ има улогу позитивног регулатора транскрипције *SOX3* гена, како у недиференцираним, тако и у диференцираним NT2/D1 ћелијама. Иако је MAZ повећао и базалну и ретиноичном киселином индуковану промоторску активност, наши резултати указују да овај транскрипциони фактор не доприноси индуцибилности *SOX3* промотора током неуралне диференцијације у присуству ретиноичне киселине.