

THE HUMAN SOX18 GENE: EXPRESSION ANALYSIS AND CHARACTERIZATION OF ITS 5' FLANKING REGION

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Abstract — The aim of this study was to establish an adequate in vitro model system for studying transcriptional regulation of the human SOX18 gene. The paper presents an analysis of expression of this gene in cultured cell lines and characterization of its 5' flanking region. Using RT-PCR, Northern and Western blot analysis, we demonstrated SOX18 expression in HeLa cells, indicating that this cell line provides a suitable model system for studying transcriptional regulation of the given gene. We also cloned, sequenced and for the first time characterized the human SOX18 5' flanking region. It is shown that the region 892 bp in size immediately upstream from the start codone harbors regulatory elements sufficient for transcription and represents an SOX18 promoter region.

Key words: SOX18 gene, HeLa cell line, 5' flanking region, promoter, CpG island

UDC 577.21

INTRODUCTION

SOX proteins constitute a large family of diverse and well-conserved transcription factors implicated in the control of various developmental processes (Pevny and Lovell-Badge, 1997). SOX proteins carry a DNA-binding HMG domain and display properties of both classical transcription factors and architectural components of chromatin (Pevny and Lovell-Badge, 1997). They perform their functions in a complex interplay with other transcription factors in a manner highly dependent on cell type and promoter context (Kamachi et al., 2000).

Sox18/SOX18 is a member of the SOX gene family which is transiently expressed in nascent endothelial cells during embryonic development and adult neovascularization, suggesting that *Sox18* may constitute a major determining gene for endothelial cell specification or differentiation (Downes and Koopman, 2001). Furthermore, murine *Sox18* has been shown to be involved in the induction of angiogenesis during wound healing and tissue repair (Darby et al., 2001). It has also been demonstrated that interference with its function inhibits blood

vessel formation and subsequent tumor growth (Young et al., 2006).

Human SOX18 was previously shown to be expressed in a variety of fetal and adult tissues (Stanojčić and Stevanović, 2000) and recently it was reported that SOX18 plays a role in atherosclerosis in humans (García-Ramírez et al., 2005).

The importance of SOX18 in vascular development is revealed by the vascular defects caused by *Sox18/SOX18* mutations in mice and humans. Mutations in *Sox18* underlie the mutant phenotype of "ragged" mutant mouse (Downes and Koopman, 2001), and mutations in human SOX18 are associated with the hypotrichosis-lymphedema-telangiectasia syndrome (Irthum et al., 2003).

Mounting evidence indicate that the *Sox18/SOX18* gene is an important regulator of vascular development, playing a role in endothelial cell specification or differentiation, atherogenesis, and angiogenesis. Nevertheless, little is known about the transcriptional regulation of human SOX18 gene itself, and isolation and characterization of its promoter

have not been reported. As the first step towards understanding molecular mechanism(s) underlying the regulation of human *SOX18* expression, the aim of this study was to establish an adequate model system for analysis of its transcriptional regulation and to clone and give the first characterization of the 5' flanking region of this gene.

MATERIALS AND METHODS

Cell culture

HeLa (ATCC CCL 2) and HepG2 (ATCC HB 8065) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1 x MEM nonessential amino acids at 37°C in 5% CO₂. NT2/D1 (Andrews, 1984) cells were maintained as described previously (Kovacevic-Grujicic et al., 2005).

RT-PCR analysis

The reverse transcription reaction was achieved using 5 µg of total RNA with polyT reverse primer by SuperScript™ RnaseH⁻ reverse transcriptase (Invitrogen). The PCR reaction was achieved with the following *SOX18* specific primers:

Forward primer F3: 5'GAGCCGGGGCGCTATGGCCTC3' (+133)

Reverse primer R8: 5'AGGCCGTCCAGAGGC GAGCGCTC3' (+684)

The numbers indicated in parentheses correspond to the distance in nt from the 5' end of the sequence to the ATG that represents the translation start site. Products were analyzed using 1% agarose gel stained with ethidium bromide.

Northern blot analysis

Messenger RNAs from HeLa, HepG2, and NT2/D1 cells were blotted on Hybond™ N+ membrane (Amersham Pharmacia Biotech). The DNA probe was generated by *HindIII/PstI* digestion of *SOX18* cDNA clone ICRFp507M16113 ([AJ243896](#)) and radiolabeled with [³²P] dCTP using 10 U of the Klenow fragment (Amersham Pharmacia Biotech) according to the procedure of Mega Prime

Labeling Systems (Amersham Pharmacia Biotech). Hybridization was performed overnight at 65°C in a buffer containing 0.5 M Na₂HPO₄ (pH 7.2), 7% SDS, and 1 mM EDTA, followed by three washes for 5 minutes at 65°C in washing buffer (40 mM Na₂HPO₄, 1% SDS).

Western blotting

HeLa cells were collected with 1x trypsin, washed twice in 1x PBS buffer and lysed with lysis buffer containing 50M m Tris HCl (pH 8.0), 150 mM NaCl, 1% NP40, 5 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. Proteins were separated in by SDS-PAGE in 10% gel using a BioRad minigel apparatus and then electrotransferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked in 10% non-fat milk for 1 h, then incubated by the incubation with primary *SOX18* antibody (sc-20100) for 1 h at room temperature. Complexes were detected with horseradish peroxidase-linked secondary antibody and an ECL detection kit (Amersham Pharmacia Biotech). Actin antibody (sc-10731) was used as a positive control.

Cloning of the 5' regulatory region of the SOX18 gene and generation of the CAT reporter construct

The 5' flanking region of the *SOX18* gene was subcloned from the genomic λ clone as the *PstI-PvuII* fragment 892 bp in size that encompasses the region immediately upstream from the translation start point (ATG codone) (Fig. 2). This fragment was first cloned in the pBSKS+ vector and subsequently released by *HindIII-BamHI* and cloned in pBLCAT6 to generate clone 892pCAT6.

In silico analysis of the SOX18 5' upstream regulatory region

Analysis of GC content was performed using the EMBOSS CpGplot program (<http://www.ebi.ac.uk/emboss/cpgplot/>) and promoter prediction accomplished by PromoterInspector search (www.genomatix.de/).

Transient transfection assays

HeLa cells were transfected using the calcium-

phosphate precipitation method. Cells (1.2×10^6) were seeded in a 10 cm dish (two dishes per transfection) and transfected with 20 μg of the *SOX18* promoter construct together with 6 μg of the pCH₁₁₀ vector (Amersham Pharmacia Biotech) and 8 μg of pBluescript (Stratagene) for 48 h. β -gal assays were performed with the β -galactosidase enzyme assay system (Promega) and chloramphenicol acetyltransferase (CAT) activities were determined using the CAT enzyme linked immunosorbent assay (CAT ELISA, Roche). The promoter-less vector pBLCAT6 was used as a negative control and vector pBLCAT5 was used as a positive control (containing the thymidine kinase-*tk* promoter). Statistical significance was determined by Student's t-test, a difference of $p < 0.05$ being considered significant.

RESULTS

Endogenous expression of the *SOX18* gene in cultured cells

Considering that the human *SOX18* gene has an essential role in early development, as well as in adult tissues, we set out to establish an adequate *in vitro* model system for studying its transcriptional regu-

lation. Accordingly, we have investigated the *SOX18* expression profiles in different cultured cell lines. In so doing we tested by RT-PCR three different permanent cell lines: the cervix adenocarcinoma-HeLa cell line, hepatocellular carcinoma-HepG2 cell line, and embryonal carcinoma- NT2/D1 cell line. Since the human *SOX18* gene contains an intron within the HMG box region (196 bp in size), we used primers designed to encompass this region. These primers give amplification products of different size on genomic DNA (678 bp in size, Fig. 1A, lane 1) or on cDNA obtained by reverse transcription (482 bp in size, Fig. 1A, lanes 2, 3 and 4). By applying RT-PCR, we showed that *SOX18* gene is expressed in all analyzed cell lines (Fig. 1A). Further, in order to quantify *SOX18* expression in those cell lines, we performed Northern blot analysis. This analysis showed among the tested cell lines, HeLa cells expressed the highest level of *SOX18*, which is demonstrated by the presence of a transcript approximately 1800 nt in size (Fig. 1B). Length of the transcript is in agreement with previously reported data (Azuma et al., 2000). In contrast, no expression was detected in NT2/D1 and HepG2 cells, suggesting that the level

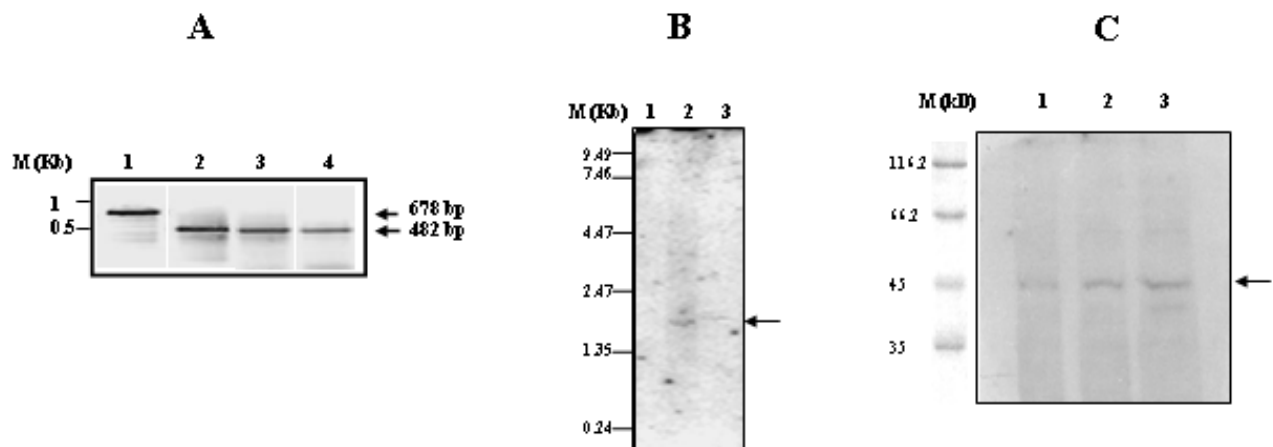


Fig. 1. *SOX18* expression analysis in cultured cell lines.

A: Expression analysis by RT-PCR. PCR on genomic DNA (lane 1); and PCR on cDNA from HepG2 (lane 2), HeLa (lane 3) and NT2/D1 (lane 4) cells. M - 1Kb ladder.

B: Northern blot analysis. Two micrograms of mRNA from HepG2 (lane 1), HeLa (lane 2), and NT2/D1 (lane 3) cells were hybridized with a *SOX18* specific probe. The *SOX18* transcript is marked by an arrow. M- 0.24-9.5 Kb RNA ladder.

C: Western blot analysis. HeLa protein extracts (10, 25, and 50 μg , lanes 1, 2, and 3, respectively) were separated on SDS gel and the presence of *SOX18* protein was detected by specific anti-*SOX18* antibody. M - Protein molecular weight marker.

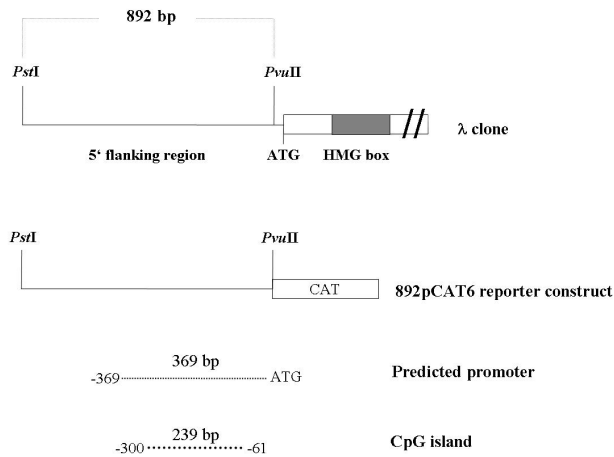


Fig. 2. Schematic representation of the *SOX18* 5' flanking region and relative positions of restriction sites used for generation of the promoter construct.

The predicted promoter obtained by PromoterInspector search is indicated by a dashed line, while a CpG island predicted by EMBOSS CpGplot program is indicated by a dotted line. CAT- chloramphenicol acetyl transferase reporter gene. ATG-translation start site

of expression of the *SOX18* gene in these cell lines is much lower and can not be detected by Northern blot analysis (Fig. 1B).

Further, in order to demonstrate expression of the *SOX18* protein in HeLa cells, we performed Western blot analysis using specific anti-*SOX18* antibody. Western blotting revealed the presence of the *SOX18* protein, approximately 45 kD in size, in HeLa cells (Fig. 1C). Since we demonstrated expression of the *SOX18* gene on both the RNA and the protein level in HeLa cells, in our subsequent studies we used this cell line as a model system for studying the mechanisms involved in transcriptional regulation of *SOX18* expression.

Cloning and in silico analysis of the 5' flanking region of the human SOX18 gene

In order to understand the mechanisms involved in regulation of *SOX18* gene expression, we first cloned and *in silico* analyzed the 5' flanking region of this gene. The 5' flanking region of the human *SOX18* gene was subcloned from the genomic λ clone as a *Pst*I-*Pvu*II fragment (892 bp) and further characterized by restriction mapping and sequencing (Fig. 2).

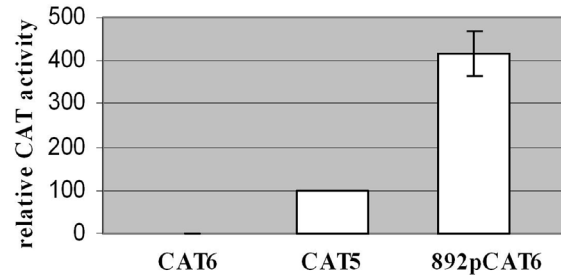


Fig. 3. Promoter activity of the 892pCAT6 reporter gene construct in HeLa cells. Normalized CAT activities were evaluated as a percentage of the pBLCAT5 control vector, which was set as 100%, and are presented as the means \pm S. D. of at least three independent experiments. Statistical significance was determined by Student's t test, a difference of $p < 0.005$ being considered significant.

The obtained sequence was subjected to PromoterInspector search (www.genomatix.de/), and the region 369 bp upstream from ATG was identified as a potential promoter region of the *SOX18* gene (Fig. 2). Further, the 892 bp sequence from the *SOX18* 5' flanking region was analyzed using the EMBOSS CpGplot program (<http://www.ebi.ac.uk/emboss/cpgplot/>). One CpG island of 239 bp in size was identified in the region positioned -300 to -61 relative to ATG, which overlaps with the predicted promoter region (Fig. 2).

Transcriptional activity of the SOX18 5' flanking region

In order to identify the region responsible for regulation of *SOX18* expression, we analyzed the ability of the cloned *Pst*I-*Pvu*II 5' flanking fragment 892 bp in size to drive expression of the *cat* reporter gene. This region was cloned into the promoterless *cat* reporter vector pBLCAT6 and transfected in HeLa cells. The 892pCAT6 construct displayed high reporter activity compared to both the negative (pBLCAT6) and the positive (pBLCAT5) control in which expression of the *cat* reporter gene is driven by the herpes simplex virus thymidine kinase promoter (HSV-TK) (Fig. 3).

Our results indicate that the fragment 892 bp upstream from ATG (clone 892pCAT6) harbors regulatory elements sufficient for transcription and represents the *SOX18* promoter region.

DISCUSSION

In the past few years, crucial steps have been taken in understanding the biological function of the SOX18 protein. Mounting evidence indicates that the *Sox18/SOX18* gene is an important regulator of vascular development, playing a role in endothelial cell specification or differentiation, atherogenesis and angiogenesis (Downes and Koopman, 2001). Its function has recently been linked with some pathophysiological processes, including atherosclerosis and tumor angiogenesis (Garcia-Ramirez et al., 2005; Young et al., 2006). Although involvement of the *Sox18/SOX18* gene in many vital processes during development and in adult life has been reported, little is known about the mechanism governing involved in regulation of its expression. Much previous research was focused on *SOX18* expression analyses in different tissues *in vivo*, but an *in vitro* model system that would permit the study of transcriptional regulation and functional characterization of its promoter has not been reported previously. Accordingly, we set out to establish an adequate *in vitro* model system for studying transcriptional regulation of the human *SOX18* gene. By investigating *SOX18* expression profiles in different cultured cell lines, we showed that HeLa, HepG2, and NT2/D1 cell lines express this gene at a level that can be detected by RT-PCR (Fig. 1A). However, Northern blot analysis (Fig. 1B) demonstrates that *SOX18* expression can be detected in HeLa cells only, suggesting that this cell line represents an appropriate *in vitro* model system for studying transcriptional regulation of the human *SOX18* gene. Further, by Western blot analysis revealed the presence of the SOX18 protein in HeLa cells (Fig. 1C) indicating that this cell line contains all factors necessary for protein expression of the given gene.

In order to understand the mechanisms involved in regulation of *SOX18* gene expression, our next goal was to clone and give the first characterization of the 5' flanking region of the human *SOX18* gene. To this end, we cloned the *PstI-PvuII* fragment 892 bp in size encompassing the region immediately upstream from the translation start codone of the gene in question. This fragment from the *SOX18* 5'

flanking region was further characterized by restriction mapping and sequencing. *In silico* analysis of the obtained sequence performed by PromoterInspector search revealed the presence of a potential promoter of the *SOX18* gene within the *PstI-PvuII* fragment in the region positioned 369 bp upstream from the translation start codone (Fig. 2). We therefore tested the transcriptional activity of this fragment by cloning it into the promoter-less *cat* reporter vector pBLCAT6 and by transfecting it into HeLa cells. The construct displayed high reporter activity, indicating that the fragment 892 bp immediately upstream from ATG harbors regulatory elements sufficient for transcription and represents the *SOX18* promoter region (Fig 3).

The human *SOX18* gene is an important factor in early stages of embryonic development, and there is strong evidence indicating that GC-rich *cis* elements play an essential role in the early embryonic development of mammals (Marin et al., 1997). The sequence of the *SOX18* 5' flanking region was therefore analyzed *in silico* by applying the EMBOSS CpGplot program. When this program was applied, one CpG island 239 bp in size that overlaps with the predicted promoter region was identified in the region positioned -300 to -61 relative to ATG (Fig. 2). Since the GC-rich *cis*-regulatory elements and their cognate binding proteins have been strongly implicated in developmental gene regulation, further work is needed in order to identify regulatory elements within the GC-rich region of the *SOX18* promoter that are essential for its transcriptional regulation.

To conclude, in this paper we have presented the expression analysis, cloning, and first characterization of the 5' flanking region of the human *SOX18* gene. We have also shown that the region 892 bp in size immediately upstream from the start codone harbors regulatory elements sufficient for transcription and represents the *SOX18* promoter region. Given the critical role of *SOX18* during embryonic development and adult neovascularization, transcriptional regulation of this human gene requires further analysis.

Acknowledgment — This work was supported by the Ministry of

Science of the Republic of Serbia (Grant No. 143028).

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ХУМАНИ SOX18 ГЕН: ЕКСПРЕСИОНА АНАЛИЗА И КАРАКТЕРИЗАЦИЈА УЗВОДНОГ РЕГУЛАТОРНОГ РЕГИОНА

ИСИДОРА ПЕТРОВИЋ и МИЛЕНА СТЕВАНОВИЋ

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Циљ овог рада био је успостављање одговарајућег *in vitro* модел система за проучавање транскрипционе регулације експресије хуманог SOX18 гена. У овом раду приказана је експресиона анализа SOX18 гена у перманентним ћелијским линијама као и прва карактеризација узводног регулаторног региона овог гена. Применом RT-PCR, Northern и Western blot есеја, установљено је да је SOX18 ген експримиран у HeLa

ћелијској линији и да ове ћелије представљају адекватан модел систем за проучавање транскрипционе регулације експресије овог гена. Такође, у овом раду је приказано и клонирање и прва карактеризација узводног регулаторног региона SOX18 гена. Показано је да регион 892 бп узводно од АТГ кодона садржи регулаторне елементе неопходне за транскрипцију и да овај регион садржи промотор SOX18 гена.