ELSEVIER

Cancer Genetics and Cytogenetics 196 (2010) 124–132

Cancer Genetics and Cytogenetics

Identification and characterization of the human *SLC5A8* gene promoter

Yu Zhang[a,b], Yong Li Bao[a,*], Yin Wu[b], Chun Lei Yu[a,b], Ying Sun[c], Yu Xin Li[b,c,*]

[a]*National Engineering Laboratory for Druggable Gene and Protein Screening, Northeast Normal University, Changchun 130024, China*
[b]*Research Center of Agriculture and Medicine Gene Engineering of Ministry of Education, Northeast Normal University, Changchun 130024, China*
[c]*Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China*

Received 15 July 2009; accepted 8 September 2009

Abstract The human *SLC5A8* gene is a tumor suppressor. Its silencing may contribute to the carcinogenesis and progression of various tumors, which makes this gene an attractive molecular marker and a potential target for diagnosis and therapy. Little is known about transcriptional mechanisms controlling *SLC5A8* gene expression. To better understand the molecular mechanisms regulating *SLC5A8* expression, we characterized the 5′-regulatory region and a part of exon 1. Luciferase reporter assays of deletion mutants of *SLC5A8* promoter demonstrated that a 295-bp region is essential for the basal promoter activity of the *SLC5A8* gene. Further analysis indicated that the CCAAT boxes and GC boxes were involved in positive regulation of *SLC5A8* promoter. Overexpression of two transcription factors, CCAAT/enhancer binding protein beta (C/EBPβ) and specific transcription factor 1 (Sp1), upregulated the activities of the human *SLC5A8* promoter and protein expression, suggesting that both C/EBPβ and Sp1 transcription factors might have functions in *SLC5A8* transcription. Taken together, our results elucidate the mechanism underlying the regulation of *SLC5A8* gene transcription and also define a novel regulatory sequence that may be used to increase expression of the *SLC5A8* gene in cancer gene therapy.

1. Introduction

The *SLC5A8* gene (solute carrier family 5, member 8) encodes a Na^+-coupled cotransporter for short-chain fatty acids (acetate, propionate, and butyrate), lactate, pyruvate, and nicotinate [1–4], and mediates iodide transport from thyrocyte into the colloid lumen in thyroid gland [5].

SLC5A8 was identified as the first plasma membrane transporter postulated to function as a tumor suppressor [6]. Silencing of its expression by epigenetic mechanisms represents an early event in the progression of colorectal cancers. Recently, the expression of *SLC5A8* has been shown to be silenced also in a variety of noncolonic cancer tissues, including thyroid, kidney, stomach, brain, breast, pancreas, and prostate [7–16]. Increasing evidence suggests that silencing of *SLC5A8* may contribute to the carcinogenesis and progression of tumors. Reexpression of *SLC5A8* in *SLC5A8*-deficient colon cancer cell lines induces apoptosis and prevents colony formation [17,18].

The tumor-suppressive function of the SLC5A8 protein is related to its ability to mediate concentrative uptake of butyrate, propionate, and pyruvate, all of which are inhibitors of histone deacetylases; such inhibitors show promise in the treatment of cancer [19,20]. Butyrate is an important energy substrate for normal colonocytes; it induces differentiation in normal colonocyte, but causes apoptosis in colon cancer cells [6,21]. Sodium-coupled pyruvate transport underlies the tumor-suppressive role of *SLC5A8* [12,22]. The acetylation status of histones in chromatin is a key determinant of gene expression and, histone deacetylase inhibitors have been shown to cause growth arrest and apoptosis in a variety of tumors [5]. Recent studies have shown that expression of *SLC5A8* is under the control of the transcription factor CCAAT/enhancer binding protein (C/EBP) [12].

Nonetheless, the mechanisms responsible for the regulation of the expression of *SLC5A8* are not well understood. This need has inspired our interest in *SLC5A8* regulatory mechanisms. The objective of the present study was to identify and characterize the human *SLC5A8* gene promoter.

2. Materials and methods

2.1. Amplification of SLC5A8 gene 5′-flanking region and nucleotide sequencing

Human genomic DNA was extracted by genomic DNA kit (v-Gene Biotechnology Limited, Hangzhou, China).

* Corresponding author. Tel. and fax: +86-431-8509-9502.
E-mail address: baoyl800@nenu.edu.cn (Y.L. Bao) or liyx486@nenu.edu.cn (Y.X. Li).

0165-4608/10/$ – see front matter
doi:10.1016/j.cancergencyto.2009.09.004

To obtain the 5′-flanking region of the *SLC5A8* gene, polymerase chain reaction (PCR) amplification on human genomic DNA (the primers are listed in Table 1) was performed in a 50-μL reaction mixture. After an initial denaturation step at 95°C for 5 minutes, the PCR reactions were performed for 30 cycles at 94°C for 30 seconds, 57°C for 45 seconds, and 72°C for 1 minute 30 seconds, with a final extension of 7 minutes at 72°C. The PCR product was purified using a gel purification kit and cloned into the T/A cloning vector pUCm-T (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China). Positive clones of pT/A − 1645/+396 were isolated and sequenced.

2.2. Plasmids

All *SLC5A8* promoter fragments were cloned into the pGL2 basic vector (Shanghai Sangon Biological Engineering Technology & Services). Construct naming is based on the positions of the promoter fragments relative to transcriptional initiated site. The 2042-bp 5′-blank region of *SLC5A8* gene was generated by digesting the plasmid pT/A − 1645/+396 with *Sac*I and *Hin*dIII endonucleases, and subcloning the released fragment into the *Sac*I/*Hin*dIII site of pGL2 basic vector to obtain plasmid pGL2 − 1645/+396. Subsequently, a series of deleted promoter fragments was obtained either by cleaving with endonucleases or by PCR and subcloned into pGL2 basic.

Ten deletion constructs of the *SLC5A8* promoter region were created. Three of these progressive deletion constructs (pGL2 − 1645/−567, pGL2 − 567/+396, pGL2 − 36/+396) were obtained by cleaving with endonucleases (−567: *Bgl*II; −36: *Kpn*I); the other seven deletion constructs (pGL2 − 294/+396, pGL2 − 294/+1, pGL2 − 284/+1, pGL2 − 273/+1, pGL2 − 188/+1, pGL2 − 165/+1, and pGL2 − 155/+1) were originated from the construct pGL2 − 567/+396. All fragments were amplified by PCR using primers as listed in Table 1. All the primers included the *Bgl*II endonuclease site in the 5′-end and the *Hind*III endonuclease site in the 3′-end. The promoter fragments were then subcloned into the *Bgl*II and *Hind*III sites of pGL2 basic vector and sequenced.

Table 1
Primer pairs used for generating *SLC5A8* promoter

Primers	Sequence
pGL2 − 1645/+396	Forward: cgagctcgccataacttacagtagcc
	Reverse: cccaagcttacgaaggtgccgat
pGL2 − 294/+396	Forward: gaagatctagcacagatttcct
	Reverse: cccaagcttacgaaggtgccgat
pGL2 − 294/+1	Forward: gaagatctagcacagatttcct
	Reverse: cccaagcttgggagcgctct
pGL2 − 284/+1	Forward: gaagatcttcctgctctcc
	Reverse: cccaagcttgggagcgctct
pGL2 − 273/+1	Forward: gaagatcttttacgatcg
	Reverse: cccaagcttgggagcgctct
pGL2 − 188/+1	Forward: gaagatcttatcaaataagg
	Reverse: cccaagcttgggagcgctct
pGL2 − 165/+1	Forward: gaagatctctccaggcgtcc
	Reverse: cccaagcttgggagcgctct
pGL2 − 155/+1	Forward: gaagatctcccttccgtaa
	Reverse: cccaagcttgggagcgctct

The C/EBPβ, Sp1, and *SLC5A8* eukaryote expression vectors were kindly provided by Shizuo Akira (Osaka University), Robert Tjian (University of California at Berkeley), and Thierry Pourcher (University of Nice Sophia Antipolis), respectively.

2.3. Cell culture and transient transfection

Human hepatic carcinoma cells (SMMC7721 cell line), human gastric carcinoma cells (SGC7901), human colorectal carcinoma cells (RKO), human colon adenocarcinoma cells (SW480), mouse fibroblast cells (L929), Chinese hamster ovary cells (CHO), human normal liver cells (L02), and human embryonic kidney 293 T cells (HEK 293 T) were cultured at 37°C, 5% CO_2 enriched atmosphere, in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum (TBD Science, Tianjin, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (Ameresco, Solon, OH).

Transfections were performed with a Gene Comparison system (GENE TRANS, Changchun, China) according to the manufacturer's instructions; 1×10^5 cells were seeded in each well of 24-well plate 24 hours before transfection. The cells were transfected with 1.5 μg of various *SLC5A8* promoter constructs or pGL2 basic vector, and 0.5 μg of pCMV-β-galactosidase plasmid for normalizing transfection efficiency per well, according to the manufacturer's instructions.

2.4. Analysis of luciferase activity

Firefly luciferase activity was measured 48 hours after transfection. Cells were harvested and washed twice with phosphate-buffered saline, then lysed in 85 μL cold lysis buffer (25 mmol/L glycylglycine, pH 7.8; 1% Triton X-100; 15 mmol/L $MgSO_4$; 4 mmol/L ethylene glycol tetraacetic acid [EGTA], and 1 mmol/L dithiothreitol). For luciferase assay, 100 μL 25 mmol/L luciferin (potassium salt, BD Monolight; BD Biosciences−Pharmingen, San Jose, CA) and 5 μL assay cocktail (1 mol/L adenosine triphosphate; 15 mmol/L KH_2PO_4, pH 7.8; 15 mmol/L $MgCL_2$) were added to 45 μL cell lysate, and then the luciferase activities were measured using a FLUOstar OPTIMA fluorescence reader (BMG LABTECH, Offenburg, Germany). β-Galactosidase activity was measured using a β-galactosidase assay system (Promega Biosciences, San Luis Obispo, CA).

2.5. Western blot analysis

The HEK 293 T cells were plated in a 6-well plate in a concentration of 5×10^5 cells in 2 mL of DMEM. After cotransfection with eukaryote expression vector of C/EBPβ or Sp1 for 48 hours, cells were harvested and rinsed twice

with PBS. Cell extracts were prepared with lysis buffer (1% Nonidet P-40; 50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L NaF; 1 mmol/L phenylmethylsulfonyl fluoride; 4 μg/mL leupeptin; 1 μg/mL aprotinin) for 30 minutes, with occasional rocking, followed by centrifugation at 15,000 rpm (21,890×g), for 15 minutes at 4°C. Identical amounts (100 μg of protein) of cell lysate were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were electrophoretically transferred to polyvinylidene fluoride membrane and blocked with 5% fat-free dry milk in TBST (20 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; 0.02% Tween-20) for 1 hour, at room temperature. The membrane was immunoblotted with goat anti-human *SLC5A8* polyclonal antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% milk/TBST.

To assure equivalent protein loading, the membranes were simultaneously incubated with glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:1000) (Kangcheng Biological Company, Shanghai, China) at 4°C, overnight. Membranes were washed three times, incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature, and washed extensively before detection. The membranes were subsequently developed using enzymatic chemiluminescence (ECL) reagent (Beyotime, Shanghai, China) and exposed to film according to the manufacturer's protocol.

2.6. Bioinformatics analysis of SLC5A8 promoter region

The TATA box and putative binding sites of transcription factors within the 5′-regulatory region of *SLC5A8* gene were analyzed with the following software packages: Match 1.0 public (http://www.gene-regulation.com) [23], MAPPER (http://mapper.chip.org) [24], and MatInspector 8.0 (http://www.genomatix.de/matinspector.html) [25]. The CpG islands were identified by using CpGplot of the European Molecular Biology Open Software Suite (EMBOSS 5.0.0) (http://www.ebi.ac.uk/emboss/cpgplot).

2.7. Statistical analysis

Experiments were repeated at least three times, with two replicates per sample for each experiment. Luciferase activity was normalized by β-galactosidase activity. Results are presented as means ± standard deviation (SD). Statistical significance was determined using the Student's *t*-test. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Identification of the upstream promoter region of human SLC5A8 gene

To better understand the mechanisms involved in *SLC5A8* gene expression, we cloned and analyzed its promoter region containing the 5′-regulatory region and a part of exon 1. A Blast search using *SLC5A8* (GeneID: 160728) as query performed online (basic local alignment search tool: BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed the 5′-upstream sequence of the *SLC5A8* gene.

A genomic DNA fragment that spans from −1645 to +396 relative to the transcription start site of the *SLC5A8* gene was amplified by PCR (with primers as listed in Table 1). The fragment was then cloned into the pGL2 basic vector and sequenced.

3.2. Analysis of minimal SLC5A8 promoter region

To find cell lines suitable for *SLC5A8* promoter analysis, pGL2 − 1645/+396 or pGL2 basic vector were cotransfected with pCMV-β-galactosidase plasmid into eight different cell lines (specified in section 2.3). Transient transfection showed that the highest *SLC5A8* promoter activity was found in HEK 293 T cells (Fig. 1). Other cell lines, especially in colorectal cancer cell RKO and colon adenocarcinoma SW480, showed lower promoter activity, but they showed the same trend. The HEK 293 T cells were therefore selected for further analysis of the regulation of *SLC5A8* expression.

To identify the proximal promoter region of the *SLC5A8* gene, we generated six promoter deletion constructs: pGL2 − 1645/+396, pGL2 − 1645/−567, pGL2 − 567/+396, pGL2 − 294/+396, pGL2 − 36/+396, and pGL2 − 294/+1 (Fig. 2). The constructs were transfected individually with Gene Comparison reagent into HEK 293 T cells. The luciferase activity driven by *SLC5A8* promoter constructs was measured 48 hours after transfection and normalized to the pCMV-β-galactosidase activity.

Compared to the basal luciferase activity of pGL2 basic, pGL2 − 1645/+396 had a high promoter activity, ~30-fold higher than the promoterless pGL2 basic vector (Fig. 2). The highest transcriptional activity was observed with pGL2 − 567/+396, and luciferase activity by pGL2 − 294/+396 and pGL2 − 294/+1 exhibited a high activity similar to pGL2 − 567/+396, whereas deletion of region from −294 to −36 dramatically decreased *SLC5A8* transcriptional activity, indicating that this region probably contained positive regulatory elements essential for the activation of *SLC5A8* gene expression.

Taken together, these results indicate that a 295-bp fragment is required for the basal transcriptional activity of the human *SLC5A8* gene.

3.3. Bioinformatics analysis of SLC5A8 promoter region

Several bioinformatics approaches were used to identify potential cis-acting elements required for expression of the *SLC5A8* gene. A potential TATA box was identified by MatInspector at −60 upstream of the putative transcriptional start site. Two CpG islands, spanning positions

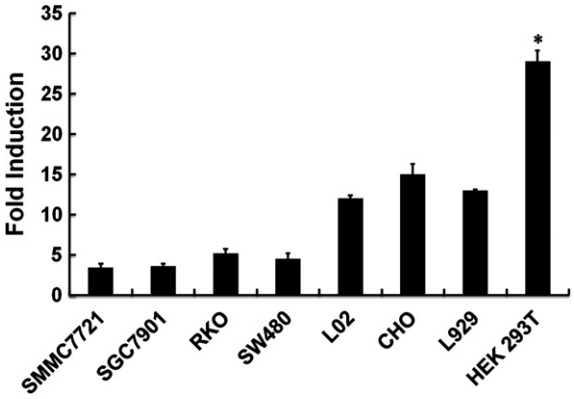

Fig. 1. Determination of *SLC5A8* promoter by luciferase assay. pGL2 – 1645/+396 and pGL2 basic vector were transiently transfected into indicated eight different cell lines, together with pCMV-β-galactosidase plasmid: SMMC7721, human hepatic carcinoma; SGC7901, human gastric carcinoma; RKO, human colorectal carcinoma; SW480, human colon adenocarcinoma; L02, human liver; CHO, Chinese hamster ovary; L929, mouse fibroblast; HEK 293 T, human embryonic kidney. Luciferase activity was measured 48 hours after transfections. Relative pGL2 basic vector activity (set to 1) of a representative experiment is shown. Data are means ± SD of three independent experiments. * $P < 0.01$ vs. control (pGL2 basic).

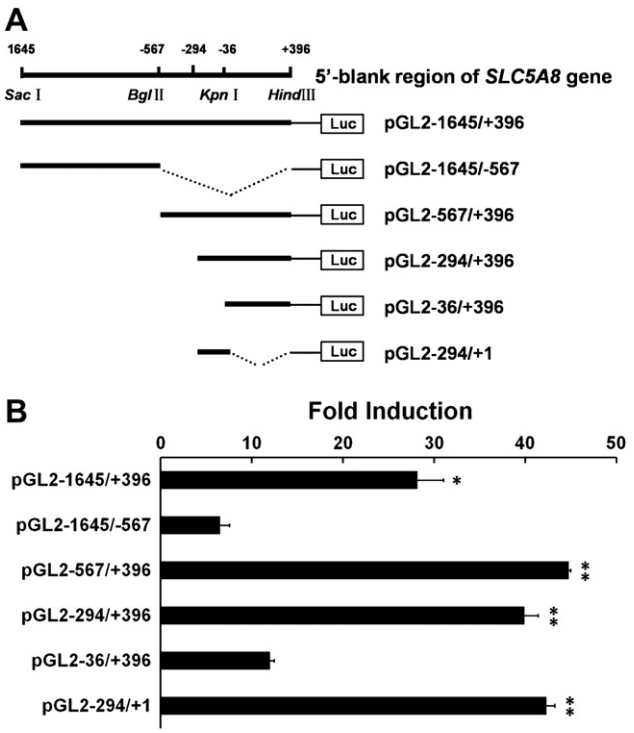

Fig. 2. Analysis of the proximal promoter region of *SLC5A8* gene. (A) Schematic illustration of 5′-deletion and 3′-deletion constructs of the *SLC5A8* promoter. (B) Analysis of the promoter activity of *SLC5A8* gene. The six *SLC5A8* promoter constructs shown or pGL2 basic vector and pCMV-β-galactosidase plasmid were cotransfected into HEK 293 T human embryonic kidney cells. Luciferase activities were measured 48 hours after transfections and normalized by β-galactosidase activities. Relative pGL2 basic vector activity (set to 1) of a representative experiment is shown. Data are the mean ± SD of three independent experiments. * $P < 0.05$ vs. control (pGL2 basic) and ** $P < 0.01$ vs. control (pGL2 basic).

−166 to −20, and +17 to +344, were detected by using the EMBOSS CpGplot program (Fig. 3A). A comprehensive computational analysis of the human *SLC5A8* 5′-flanking region from −317 to +396 revealed the presence of several GC-rich boxes, potential binding sites for Sp1 transcription factors, and several CCAAT boxes, potential binding sites for C/EBP transcription factors, as well as several putative binding sites for transcription factors such as activator protein (AP1), cAMP-responsive element-binding (CREB), p53, and E2F (Fig. 3B).

3.4. Identification of the regulatory regions of SLC5A8 promoter

Expression of *SLC5A8* is known to be under the control of the transcription factor CCAAT/enhancer binding protein [12]. So, to define the important regulatory regions in the *SLC5A8* promoter, we first analyzed the putative C/EBP transcription factor binding sites in *SLC5A8* promoter. Four 5′-deletion constructs based on the deletion of putative binding sites for C/EBP including pGL2 – 294/+1, pGL2 – 284/+1, pGL2 – 188/+1, and pGL2 – 165/+1 were transfected individually into HEK 293 T cells, and the relative activity of luciferase was measured 48 hours later. The construct pGL2 – 284/+1 showed a lower promoter activity (decreased 30%), compared with pGL2 – 294/+1, and pGL2 – 165/+1 displayed a much lower promoter activity (decreased 33%), compared with pGL2 – 188/+1 (Fig. 4). These results indicate that the fragment spanning positions −294 to −284 and −188 to −165 are all important for the basal transcription activity of the human *SLC5A8* promoter, which suggests that C/EBP transcription factor is essential for the basal transcription activity of the human *SLC5A8* promoter, and the results also showed that the fragment spanning positions −284 to −188 should contain some important positive elements.

Besides C/EBP binding sites, there are many putative Sp1 binding sites, according to the comprehensive computational analysis, so we analyzed two fragments, spanning positions −284 to −273 and −165 to −155, that contained the putative Sp1 binding site. Two 5′-deletion constructs, pGL2 – 273/+1 and pGL2 – 155/+1, were created based on the deletion of putative Sp1 binding sites compared with pGL2 – 294/+1 and pGL2 – 165/+1, respectively. Four constructs were transfected into HEK 293 T cells individually, and the relative activity of luciferase was measured 48 hours later.

The construct pGL2 – 273/+1 showed a lower promoter activity (decreased 26%), compared with pGL2 – 284/+1, and pGL2 – 155/+1 showed little change (decreased 18%) on promoter activity, compared with pGL2 – 165/+1 (Fig. 5). These results indicate that the fragment spanning positions −284 to −273 is important for the basal transcription activity of the human *SLC5A8* promoter,

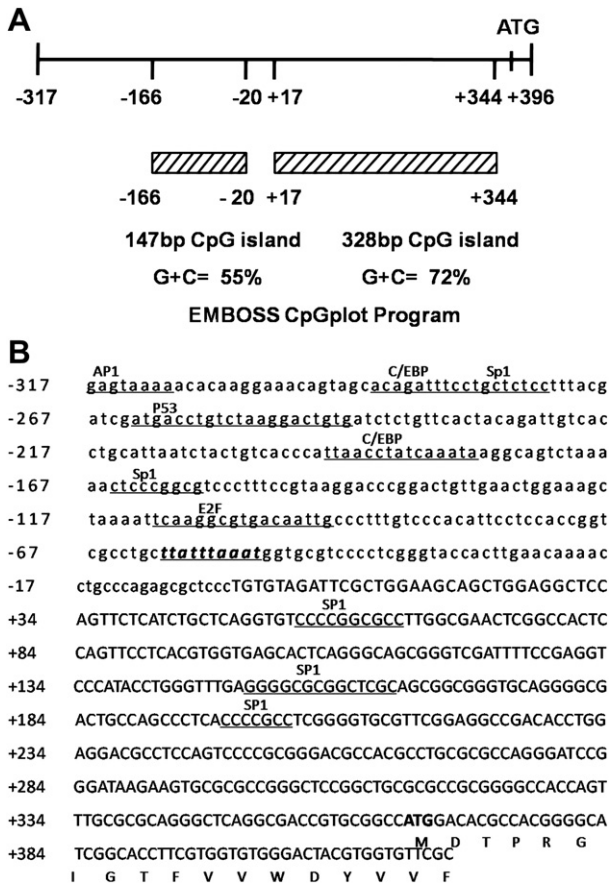

Fig. 3. Analysis of promoter region and cis-acting element of the *SLC5A8* gene by bioinformatics analysis. (A) Schematic representation of the putative *SLC5A8* promoter and CpG islands. The CpG islands are shown as a hatched box. The transcriptional initiated site is position +1, and the rest of the sequence is numbered relative to it. (B) Analysis of the putative cis-acting element of the *SLC5A8* gene (−317/+396) by bioinformatics analysis. The 5′-flanking region is in lower case letters; exon 1 is in uppercase letters. The amino acid sequence starting from the ATG codon is shown below the sequence. The putative TATA box is shown italic, bold, and underlined. The A of the initiation codon is numbered +1, and the rest of the sequence is numbered relative to it. The consensus sequence for the putative binding sites of transcription factors are underlined, the names are indicated above the sequence. Nucleotide numbers are indicated on the left side of the sequence.

which suggests that Sp1 transcription factor is essential for the basal transcription activity of the human *SLC5A8* promoter.

3.5. The effect of C/EBPβ and Sp1 on SLC5A8 promoter activity and protein expression

The deletion constructs of C/EBP and Sp1 potential binding site decreased the luciferase activity, which suggests that transcription factors C/EBPβ and Sp1 contribute to the regulation of the *SLC5A8* transcription. To test this, we cotransfected the *SLC5A8* promoter reporter gene constructs and the expression plasmid for transcription factor C/EBPβ or Sp1 into HEK 293 T cells. Overexpression of C/EBPβ increased *SLC5A8* promoter-driven luciferase activity by about onefold, whereas cotransfection of C/EBPβ with pGL2 − 284/+1 and pGL2 − 165/+1 had a lesser effect on *SLC5A8* promoter activity, compared with pGL2 − 294/+1 and pGL2 − 188/+1 (Fig. 6A). On the other hand, the protein expression can be upregulated by C/EBPβ (Fig. 6C). These data suggest that transcription factor C/EBPβ can upregulate the activity of the *SLC5A8* promoter and protein expression.

We examined whether Sp1 can activate the *SLC5A8* promoter in cotransfection experiments in HEK 293 T cells. Because endogenous Sp1 expression levels are low in HEK 293 T cells, this cell line can be used for cotransfection of Sp1 expression vector with promoter reporter gene constructs [26]. The basal *SLC5A8* promoter activities after cotransfection with an Sp1-expressing plasmid were greatly increased, compared with the empty vector pcDNA3 control (twofold increase), whereas cotransfection of Sp1 with pGL2 − 273/+1, pGL2 − 165/+1, or pGL2 − 155/+1 presented relative decreasing effects on *SLC5A8* promoter activity (onefold increase) (Fig. 6B). On the other hand, protein expression can be greatly upregulated by Sp1 (Fig. 6C). These data suggest that Sp1 transcription factors can upregulate the activity of the *SLC5A8* promoter, and also predict that some other putative Sp1 binding sites spanning position −155 to +1 could play an important role in regulating *SLC5A8* transcription.

4. Discussion

SLC5A8 has been proposed as a tumor-suppressor gene in colon cancer [15]. When reexpressing *SLC5A8* in colon cancer cell lines in which it had been silenced, *SLC5A8* decreases colony formation by at least 75%. The mechanism of the tumor-suppressive role of *SLC5A8* was proposed by the Ganapathy research group [12,21,22]. The potential function of *SLC5A8* in normal tissues is likely to facilitate the active absorption of short-chain fatty acids. The histone deacetylase inhibitors butyrate and pyruvate have been shown to cause growth arrest and apoptosis in a variety of tumors [19]. The capacity to transport butyrate and pyruvate into colonic epithelial cells may explain the potential tumor-suppressive role of *SLC5A8* in tumors [6,21,27].

Investigation of the transcriptional regulation mechanism of *SLC5A8* gene may lead to findings useful for cancer therapy. Previous studies, however, focused mainly on DNA methylation [7,10,15], and little is known about the other transcription mechanisms controlling *SLC5A8* gene expression. To delineate the regulatory mechanisms of *SLC5A8* gene expression, we reported the cloning and functional characterization of the *SLC5A8* promoter.

The 2042-bp fragment, which contains the 5′-blank region and part of exon 1 of the *SLC5A8* gene, was

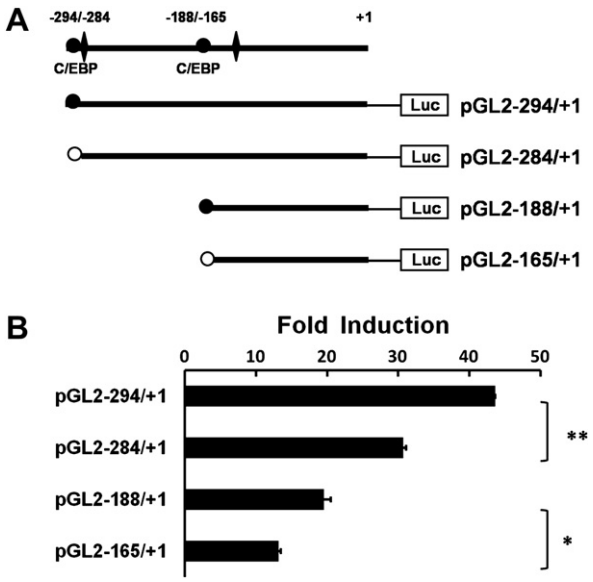

Fig. 4. Identification of the putative C/EBP binding sites in *SLC5A8* promoter. (A) Schematic representation of 5′-deletion constructs of the *SLC5A8* promoter. The putative C/EBP binding sites in the region from −294 to −284 and −188 to −165 of the *SLC5A8* gene are shown. (B) Analysis of the deletion mutant construct activities of *SLC5A8* gene promoter. The four *SLC5A8* promoter constructs shown or pGL2 basic vector and pCMV-β-galactosidase plasmid were cotransfected into HEK-293 T cells, and the luciferase activities were measured 48 hours after transfections and normalized by β-galactosidase activities. Data are means ± SD ($n = 3$). * $P < 0.05$ vs. control (pGL2 − 188/+1) and ** $P < 0.01$ vs. control (pGL2 − 294/+1) by *t*-test.

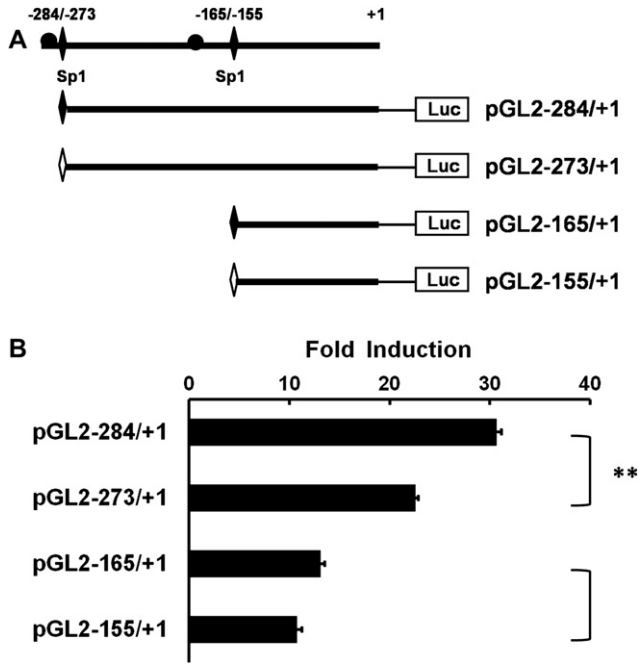

Fig. 5. Identification of the putative Sp1 binding sites in *SLC5A8* promoter. (A) Schematic representation of and 5′-deletion deletion constructs of the *SLC5A8* promoter. The putative Sp1 binding sites in the region from −284 to −273 and −165 to −155 of the *SLC5A8* promoter were shown. (B) Analysis of the deletion mutant construct activities of *SLC5A8* gene promoter. The four *SLC5A8* promoter constructs shown or pGL2 basic vector and pCMV-β-galactosidase plasmid were cotransfected into HEK 293 T cells and the luciferase activities were measured 48 hours after transfection and normalized by β-galactosidase activities. Data are means ± SD ($n = 3$). ** $P < 0.05$ vs. control (pGL2 − 284/+1) by *t*-test.

originally obtained from human genomic DNA by PCR. Reporter gene activity of each *SLC5A8* promoter–luciferase construct was measured by transient transfection assays and compared with pGL2 basic vector plasmid. The luciferase reporters driven by the promoter constructs of *SLC5A8* showed a higher activity in HEK 293 T cells, but lower in other tumor cell lines, especially in RKO colorectal cancer cells and SW480 colon adenocarcinoma cells (Fig. 1). Our transient transfection results suggest that the analyzed upstream sequences of the *SLC5A8* gene may contain some important—but not sufficient—sequence information to confer *SLC5A8* gene expression.

To define the proximal regulatory region in the *SLC5A8* promoter, we generated a series of deletion constructs containing the progressive 5′-deletion and the 3′-deletion, then transfected into HEK 293 T cells. The luciferase activity results indicated that a 295-bp region is necessary and sufficient for the transcriptional activity of the *SLC5A8* gene (Fig. 2). Because deletion analysis had showed that the cis-regulatory elements required for the promoter activity of the human *SLC5A8* gene were mainly located in the region from −294 to +1, we focused on this region and investigated which sequences within this region contribute to the activation of *SLC5A8*.

Transcriptional regulation is the result of the balance between chromatin remodeling and RNA polymerase activity on accessible regulatory regions [28]. Processes that regulate gene transcription are directly under the influence of the genome organization. Even though our results have identified a minimal promoter regulation mostly relying on basal transcription factors, *SLC5A8* transcription could require more complex pathways, involving cell cycle progression or cell differentiation.

Consensus recognition sequences for several transcription factors known to be involved in the regulation of gene expression were determined in this region by the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html). Some of them are ubiquitous transcription factors, such as AP1, C/EBP, CREB, p53, and E2F (Fig. 3).

SLC5A8 is downregulated in the kidney of C/EBPδ null mice, which suggested that C/EBPδ controls the expression of *SLC5A8* in the kidney; this was confirmed by luciferase reporter assay, which showed that expression of C/EBPδ induced the *SLC5A8* activity [12]. We therefore studied the effects of three C/EBP transcription factors binding sites in the *SLC5A8* promoter. The 5′-deletion constructs were used to determine the function of the C/EBP binding sites. The results showed that C/EBP binding sites from −294 to −284 and −188 to −165 are essential for the basal transcription activity of the human *SLC5A8* promoter. Given that all members of the C/EBP family of transcription factors have highly similar or identical DNA sequence

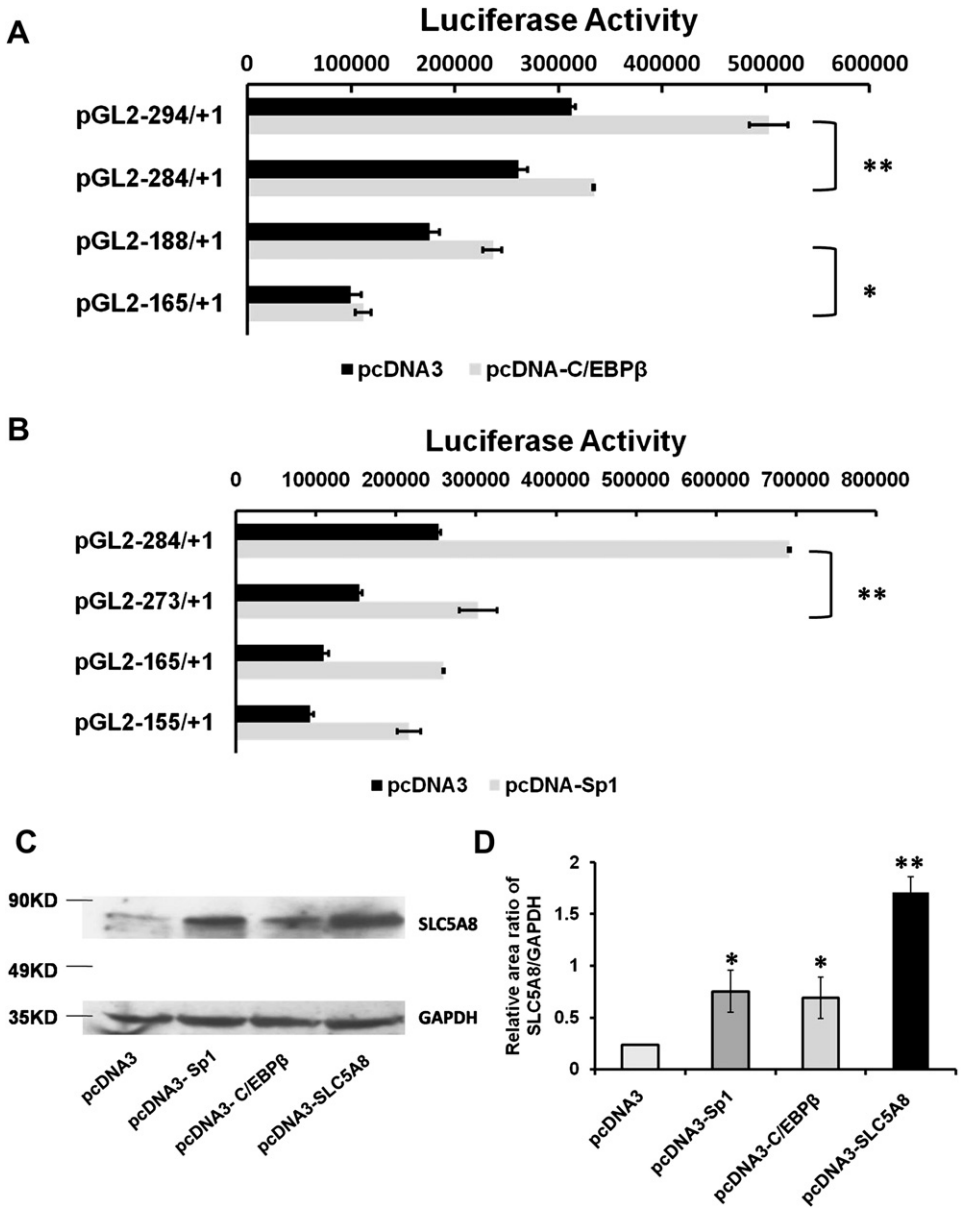

Fig. 6. Effect of transcription factors Sp1 and C/EBPβ on *SLC5A8* promoter activity and protein expression. (A,B) *SLC5A8* promoter constructs or deletion constructs were cotransfected with eukaryote expression vector of C/EBPβ or pcDNA3 vector (A) or Sp1 or pcDNA3 vector (B). Luciferase activity was measured 48 hours after transfection. All of the constructs were cotransfected with the pCMV-β-galactosidase plasmid for normalizing transfection efficiency. Data are means ± SD ($n = 3$). * $P < 0.05$ vs. control by *t*-test. ** $P < 0.01$ vs. control by *t*-test. (C) Overexpression of C/EBPβ and Sp1 led to increased expression of the endogenous *SLC5A8*. HEK 293 T cells were transiently transfected with pcDNA3, pcDNA3-Sp1, pcDNA-C/EBPβ, or pcDNA-SLC5A8 expression plasmid. Overexpression of pcDNA-SLC5A8 was considered as positive control. At 48 hours after transfection, cell lysates were prepared for Western blot analysis. Western blots showed levels of SLC5A8 protein in the cells transfected with specific plasmids. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. (D) The protein levels were quantified by Image J software.

specificities, and cotransfection of C/EBPβ also upregulated the activities of the *SLC5A8* promoter (Fig. 4). Therefore, it is concluded that C/EBP family transcription factors control *SLC5A8* expression.

DNA methylation on CpG islands constitutes an epigenetic mark generally correlated with transcriptionally silent condensed chromatin [29]. CpG islands are stretches of unmethylated DNA with a higher frequency of CpG dinucleotides, relative to the entire genome. CpG islands are known to preferentially occur at the transcriptional start of genes; almost 50% of all genes and almost all housekeeping genes have CpG islands at the 5′-end of the transcript [30–32]. The presence of CpG islands suggested the possibility that the gene might be regulated through changes in methylation status, especially the methylation of GC boxes (Sp1 sites), which has been showed to cause gene silencing [33,34].

Regarding the GC-rich region in the *SLC5A8* promoter, the CpG islands can be methylated and regulate transcription activity dependent on the methylation level. A dense

CpG island (G + C = 70%) was identified located in *SLC5A8* exon 1 and surrounding the 3D41 *Not*I site. This region covered 573 bp and included 62 CpG dinucleotides [15]. The region immediately 5′ of exon 1 showed only a 46% G + C content. In the present study, the region from −567 to +396 showed a higher luciferase activity, whereas the region from −36 to +396, which contained the part of exon 1 and had a high G + C content, showed the lower activity. The GC-rich regions are the putative binding site for the Sp family of transcription factors [35]. Sp1, a member of the Sp transcription factor family that is extensively expressed in mammalian cells, can bind with similar affinities for GC boxes, GC similar sequence, and acts through GC boxes to regulate gene expression of many housekeeping, tissue-specific, viral, and inducible genes [36,37]. In addition to its role in transcription activation, Sp1 appears to play a critical role for the maintenance of the hypomethylation status of CpG islands [38].

Therefore, we next analyzed the Sp1 sites within the region from −294 to +1, which showed a higher promoter activity. We deleted the fragments from −284 to −273 and −165 to −155 in *SLC5A8* gene promoter, fragments that contain the putative Sp1 transcription factor binding sites; the result showed that the region from −284 to −273 is important for the *SLC5A8* gene basal transcriptional activity (Fig. 5). Meanwhile, cotransfection of Sp1 showed that Sp1 transcription factors act as transcriptional activators for *SLC5A8* promoter activities (Fig. 6). Methylation of the CpG island directly inhibits the binding of Sp1 [39]. Therefore, it is predicted that methylation of this site could interfere with the binding of Sp1 to DNA and associate with the silencing of *SLC5A8*, thus leading to tumorigenesis.

Generally, one or more transcription factors recruit specific proteins to cooperatively mediate the regulation of a target gene. There may also be competitive binding, in which multiple transcription factors simultaneously interact with the promoter region of a target [40]. The signal-dependent transcription regulation on *SLC5A8* gene could be more complex, so further analysis and detailed elucidation of *SLC5A8* gene transcription regulation are needed.

5. Conclusion

Here we have reported identification, cloning, and characterization of the promoter of human *SLC5A8* gene and its transcription regulatory elements in HEK 293 T cells. Transient transfection results suggested that the upstream sequences of the *SLC5A8* gene possess promoter activity. Deletion analysis showed that the region between −294 and the putative transcriptional initiation site (+1) is indispensable for the promoter activity. Detailed sequence analysis of the *SLC5A8* 5′-flanking region revealed the presence of several GC-rich boxes, and CAAT boxes. Further analysis revealed that two C/EBP sites (−294/−284, −188/−165) and one Sp1 site (−284/−273) are important for the basal transcription activity of the human *SLC5A8* promoter. Overexpression of C/EBPβ and Sp1 upregulated the human *SLC5A8* promoter activities, whereas the deletion of these sites markedly blocked its activities. Both transcription factors can upregulate the protein expression of *SLC5A8*. These results suggest some possible mechanisms that transcriptional factors Sp1 and C/EBP are associated with *SLC5A8* promoter regions and regulate *SLC5A8* promoter activity.

The present results contribute to understanding of the molecular mechanism of the *SLC5A8* expression and its role in transcription regulation, and such understanding can contribute to developing reagents for therapeutic upregulation of *SLC5A8* in human cancers. Continued investigation of the molecular mechanisms may eventually lead to novel strategies for increasing *SLC5A8* expression in various cancers.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (grant no. 30873409), the Program for New Century Excellent Talents in University (grant no. NCET-06-0320), and the Cultivation Fund of the Scientific and Technical Innovation Project of Northeast Normal University (grant no. NENU-STB07008).

References

[1] Miyauchi S, Gopal E, Fei YJ, Ganapathy V. Functional identification of SLC5A8, a tumor suppressor downregulated in colon cancer, as a Na^+-coupled transporter for short-chain fatty acids. J Biol Chem 2004;279:13293–6.

[2] Coady MJ, Chang MH, Charron FM, Plata C, Wallendorff B, Sah JF, Markowitz SD, Romero MF, Lapointe JY. The human tumour suppressor gene *SLC5A8* expresses a Na^+-monocarboxylate cotransporter. J Physiol 2004;557:719–31.

[3] Gopal E, Fei YJ, Miyauchi S, Zhuang L, Prasad PD, Ganapathy V. Sodium-coupled and electrogenic transport of B-complex vitamin nicotinic acid by slc5a8, a member of the Na/glucose co-transporter gene family. Biochem J 2005;388:309–16.

[4] Martin PM, Gopal E, Ananth S, Zhuang L, Itagaki S, Prasad BM, Smith SB, Prasad PD, Ganapathy V. Identity of SMCT1 (SLC5A8) as a neuron-specific Na+-coupled transporter for active uptake of l-lactate and ketone bodies in the brain. J Neurochem 2006;98:279–88.

[5] Rodriguez AM, Perron B, Lacroix L, Caillou B, Leblanc G, Schlumberger M, Bidart JM, Pourcher T. Identification and characterization of a putative human iodide transporter located at the apical membrane of thyrocytes. J Clin Endocrinol Metab 2002;87:3500–3.

[6] Gupta N, Martin PM, Prasad PD, Ganapathy V. SLC5A8 (SMCT1)-mediated transport of butyrate forms the basis for the tumor suppressive function of the transporter. Life Sci 2006;78:2419–25.

[7] Ueno M, Toyota M, Akino K, Suzuki H, Kusano M, Satoh A, Mita H, Sasaki Y, Nojima M, Yanagihara K, Hinoda Y, Tokino T, Imai K. Aberrant methylation and histone deacetylation associated with silencing of *SLC5A8* in gastric cancer. Tumour Biol 2004;25:134–40.

[8] Dong SM, Lee EJ, Jeon ES, Park CK, Kim KM. Progressive methylation during the serrated neoplasia pathway of the colorectum. Mod Pathol 2005;18:170–8.

[9] Hong C, Maunakea A, Jun P, Bollen AW, Hodgson JG, Goldenberg DD. Shared epigenetic mechanisms in human and mouse gliomas inactivate expression of the growth suppressor *SLC5A8*. Cancer Res 2005;65:3617–23.

[10] Porra V, Ferraro-Peyret C, Durand C, Selmi-Ruby S, Giroud H, Berger-Dutrieux N, Decaussin M, Peix JL, Bournaud C, Orgiazzi J, Borson-Chazot F, Dante R, Rousset B. Silencing of the tumor suppressor gene *SLC5A8* is associated with *BRAF* mutations in classical papillary thyroid carcinomas. J Clin Endocrinol Metab 2005;90:3028–35.

[11] Hu S, Liu D, Tufano RP, Carson KA, Rosenbaum E, Cohen Y, Holt EH, Kiseljak-Vassiliades K, Rhoden KJ, Tolaney S, Condouris S, Tallini G, Westra WH, Umbricht CB, Zeiger MA, Califano JA, Vasko V, Xing M. Association of aberrant methylation of tumor suppressor genes with tumor aggressiveness and *BRAF* mutation in papillary thyroid cancer. Int J Cancer 2006;119:2322–9.

[12] Thangaraju M, Ananth S, Martin PM, Roon P, Smith SB, Sterneck E, Prasad PD, Ganapathy V. c/ebpδ Null mouse as a model for the double knock-out of slc5a8 and slc5a12 in kidney. J Biol Chem 2006;281:26769–73.

[13] Park JY, Zheng W, Kim D, Cheng JQ, Kumar N, Ahmad N, Pow-Sang J. Candidate tumor suppressor gene *SLC5A8* is frequently down-regulated by promoter hypermethylation in prostate tumor. Cancer Detect Prev 2007;31:359–65.

[14] Park JY, Helm JF, Zheng W, Ly QP, Hodul PJ, Centeno BA, Malafa MP. Silencing of the candidate tumor suppressor gene solute carrier family 5 member 8 (*SLC5A8*) in human pancreatic cancer. Pancreas 2008;36:e32–9.

[15] Li H, Myeroff L, Smiraglia D, Romero MF, Pretlow TP, Kasturi L, Lutterbaugh J, Rerko RM, Casey G, Issa JP, Willis J, Willson JK, Plass C, Markowitz SD. *SLC5A8*, a sodium transporter, is a tumor suppressor gene silenced by methylation in human colon aberrant crypt foci and cancers. Proc Natl Acad Sci U S A 2003;100:8412–7.

[16] Paroder V, Spencer SR, Paroder M, Arango D, Schwartz S Jr, Mariadason JM, Augenlicht LH, Eskandari S, Carrasco N. Na^+/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: molecular characterization of SMCT. Proc Natl Acad Sci U S A 2006;103:7270–5.

[17] Whitman SP, Hackanson B, Liyanarachchi S, Liu S, Rush LJ, Maharry K, Margeson D, Davuluri R, Wen J, Witte T, Yu L, Liu C, Bloomfield CD, Marcucci G, Plass C, Caligiuri MA. DNA hypermethylation and epigenetic silencing of the tumor suppressor gene, *SLC5A8*, in acute myeloid leukemia with the *MLL* partial tandem duplication. Blood 2008;112:2013–6.

[18] Bennett KL, Karpenko M, Lin MT, Claus R, Arab K, Dyckhoff G, Plinkert P, Herpel E, Smiraglia D, Plass C. Frequently methylated tumor suppressor genes in head and neck squamous cell carcinoma. Cancer Res 2008;68:4494–9.

[19] Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 2001;1:194–202.

[20] Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005;45:495–528.

[21] Ganapathy V, Gopal E, Miyauchi S, Prasad PD. Biologic functions of *SLC5A8*, a candidate tumor suppressor. Biochem Soc Trans 2005;33:237–40.

[22] Ganapathy V, Thangaraju M, Gopal E, Martin PM, Itagaki S, Miyauchi S, Prasad PD. Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. AAPS J 2008;10:193–9.

[23] Kel AE, Gössling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, Wingender E. MATCH: a tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res 2003;31:3576–9.

[24] Marinescu VD, Kohane IS, Riva A. MAPPER: a search engine for the computational identification of putative transcription factor binding sites in multiple genomes. BMC Bioinformatics 2005;6:79.

[25] Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 2005;21:2933–42.

[26] Banks EB, Crish JF, Eckert RL. Transcription factor Sp1 activates involucrin promoter activity in non-epithelial cell types. Biochem J 1999;337:507–12.

[27] Thangaraju M, Cresci G, Itagaki S, Mellinger J, Browning DD, Berger FG, Prasad PD, Ganapathy V. Sodium-coupled transport of the short chain fatty acid butyrate by SLC5A8 and its relevance to colon cancer. J Gastrointest Surg 2008;12:1773–81.

[28] Stirzaker C, Song JZ, Davidson B, Clark SJ. Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. Cancer Res 2004;64:3871–7.

[29] Métivier R, Gallais R, Tiffoche C, Le Péron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G. Cyclical DNA methylation of a transcriptionally active promoter. Nature 2003;452:45–50.

[30] Gardiner-Garden M, Frommer M. CpG islands in vertebrate genome. J Mol Biol 1987;196:261–82.

[31] Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. Genomics 1992;13:1095–107.

[32] Antequera F, Bird A. Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci U S A 1993;90:11995–9.

[33] Uhl J, Klan N, Rose M, Entian KD, Werz O, Steinhilber D. The 5-lipoxygenase promoter is regulated by DNA methylation. J Biol Chem 2002;277:4374–9.

[34] Murumägi A, Vähämurto P, Peterson P. Characterization of regulatory elements and methylation pattern of the autoimmune regulator (AIRE) promoter. J Biol Chem 2003;278:19784–90.

[35] Kadonaga JT, Carner KR, Masiarz FR, Tjian R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 1987;51:1079–90.

[36] Bouwman P, Philipsen S. Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol 2002;195:27–38.

[37] Li L, He S, Sun JM, Davie JR. Gene regulation by Sp1 and Sp3. Biochem Cell Biol 2004;82:460–71.

[38] Macleod D, Charlton J, Mullins J, Bird AP. Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. Genes Dev 1994;8:2282–92.

[39] Clark SJ, Harrison J, Molloy PL. Sp1 binding is inhibited by ^{m}Cp^{m}CpG methylation. Gene 1997;195:67–71.

[40] Wang RS, Zhang XS, Chen L. Inferring transcriptional interactions and regulator activities from experimental data. Mol Cells 2007;24:307–15.