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Identification and characterization of 5'-flanking region of the human riboflavin transporter 1 gene (*SLC52A1*)

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Abstract

The human *SLC52A1* gene encodes the riboflavin transporter-1 (RFVT-1), a plasma membrane protein that transports vitamin B2 (riboflavin, RF) into cells, and thus, plays a role in controlling cellular homeostasis of RF in those tissues that express the carrier protein (e.g. placenta and intestine). Currently, there is nothing known about transcriptional regulation of the *SLC52A1* gene, therefore, we aimed to clone and characterize its 5'-flanking region. Using rapid amplification of the cDNA ends (5'-RACE), we identified one transcription start site (TSS). A 579 bp segment of the 5'-flanking region of this gene was cloned which exhibited robust promoter activity upon transfection in human intestinal epithelial cells. Deletion analysis revealed the core promoter activity to be embedded in a region between -234 and -23 that lacked TATA element, was GC-rich, and harbored several putative *cis*-regulatory sites including KLFs, AP-2, EGRF and Sp-1. Mutating each of these sites led to a significant decrease in promoter activity (which was highest for the Sp-1 site), suggesting their possible involvement in regulating *SLC52A1* transcription. Focusing on the Sp-1 site, EMSA, super-shift and ChIP analysis was performed that established the interaction of the Sp-1 transcription factor with the *SLC52A1* promoter; also, co-transfection of the minimal *SLC52A1* promoter with an Sp-1 containing vector in *Drosophila* SL-2 cells led to significant promoter activation. These results are the first to reveal the identity of the minimal *SLC52A1* promoter and to establish an important role for Sp-1 in its activity.

Keywords

SLC52A1; minimal promoter; riboflavin transporter; transcription regulation

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contribution

SS, AG and HMS designed the research. SS and AG performed the experiments. SS, AG and HMS analyzed the data and wrote the paper.

1. Introduction

The water-soluble vitamin B2 (RF) in its co-enzymes forms i.e. flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), plays an important role in biochemical oxidation-reduction reactions. These reactions involve carbohydrate, lipid, and amino acid metabolism, conversion of other specific water-soluble vitamins (pyridoxine and folate) into their active forms (Cooperman and Lopez, 1984); as well as a role in proper protein folding (Tu *et al.*, 2000). Recent studies have further shown that RF has anti-oxidant and anti-inflammatory properties (Sanches *et al.*, 2014), can protect tissues against oxidative injury (Iwanaga *et al.*, 2007, Mack *et al.*, 1995, Seekamp *et al.*, 1999), and plays a role in normal immune function (Toyosawa *et al.*, 2004). Thus, it is not surprising that deficiency and sub-optimal levels of this essential micronutrient leads to clinical manifestations, including neurological disorders, anemia, and growth retardation (Cooperman and Lopez, 1984, Goldsmith *et al.*, 1975). Deficiency and sub-optimal levels of RF occurs in a variety of clinical conditions, including chronic alcoholism, diabetes mellitus, and inflammatory bowel diseases (Bonjour, 1980, Fennelly *et al.*, 1964, Fernandez-Banares *et al.*, 1989, Kodentsova *et al.*, 1993, 1994, Majumdar *et al.*, 1981, Rosenthal *et al.*, 1973); they also occur in patients with riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency (Law *et al.*, 2009, Liang *et al.*, 2009), and those with Brown-Vialetto-Van Laere and Fazio Londe syndromes (BVVL/FL syndromes) (Anand *et al.*, 2012, Bosch *et al.*, 2011).

RF is obtained from exogenous sources, via intestinal absorption, as humans and all other mammals lack the ability to synthesize the vitamin endogenously. Two sources of RF are available to the mammalian gut: dietary sources and bacterial sources (the latter is in reference to the vitamin that is generated by the normal microbiota of the large intestine) (Wrong *et al.*, 1981, Iinuma, 1955, Kasper, 1970, Said *et al.*, 2000). Previous studies have shown that absorption of free RF in the small and large intestine is via specific carrier-mediated process (Said, 2004, Said and Arianas, 1991, Said *et al.*, 1993). Recently, three specific human RFVTs have been identified: RFVT-1, RFVT-2 and RFVT-3; these transporters are encoded by the *SLC52A1*, *SLC52A2* and *SLC52A3* genes, respectively (Yamamoto *et al.*, 2009, Yao *et al.*, 2010, Yonezawa *et al.*, 2008). The hRFVT-1, -2 & -3 are 448, 445, and 469 amino acid proteins, respectively, and are predicted to have 10-11 transmembrane domains. The hRFVT-1 & -2 share 87 percent identity at amino acid levels, whereas hRFVT-3 shares 41 and 44 percent identity with hRFVT-1 & -2, respectively (Yonezawa *et al.*, 2008, Yao *et al.*, 2010, Yonezawa and Inui, 2013). Expression of the RFVTs shows tissue specific distribution, but all are expressed in the intestine, although at different levels (Yonezawa *et al.*, 2008, Subramanian *et al.*, 2011). Also, while expression of RFVT-1 was found to be mainly localized at the basolateral membrane domain of the polarized intestinal epithelial cells, that of RFVT-3 was restricted only to the apical brush border membrane domain; expression of RFVT-2 is mostly intracellular with some expression at the basolateral membrane domain of these epithelial cells (Subramanian *et al.*, 2011). Other studies have identified mutations in RFVT-3 and -2 as the cause of BVVL/FL syndromes (Green *et al.*, 2010; Bosch *et al.*, 2011, Foley *et al.*, 2014). Also, a homozygous deletion of exons 2 and 3 in the *SLC52A1* gene (RFVT-1) has been reported in a mother

whose newborn developed clinical features of multiple acyl-CoA dehydrogenase deficiency that were corrected by RF supplementation (Ho *et al.*, 2011).

Little is currently known about how the recently identified RFVTs are regulated at the transcriptional and post-transcriptional level in any cell type. We have been interested in understanding the mechanisms of RF transport and its regulation in intestine and other tissues for sometimes (see reviews, Said, 2004, Said and Ross, 2011, Said and Nexo, 2012, Said, 2011). With regards to the latter, we have for example shown that the intestinal RF uptake process is adaptively regulated by RF levels and during enterocyte differentiation (Said, 2011, Said and Nexo, 2012). In this study, we aimed to characterize the basal regulation of *SLC52A1* transcription in a model of human intestinal epithelial cells (HuTu 80). Our results identify the *SLC52A1* minimal promoter needed for basal activity, and establish an important role for the Sp-1 transcription factor in regulating activity of this promoter.

2. Materials and Methods

2.1. Materials

All molecular biology grade routine biochemical and cell culture reagents were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Tustin, CA). Primers for promoter constructs, EMSA and ChIP analysis were obtained from Sigma Genosys (Woodlands, TX). PCR kit and DNA LA polymerase were bought from Clontech (Palo Alto, CA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Anti-Sp-1 antibody and rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. 5'-RACE

Transcription start site(s) for *SLC52A1* in HuTu 80 cells was probed with the rapid amplification of the cDNA ends (RACE) utilizing 5'-RACE kit version 2.0 (Life Technologies) following the manufacturer's protocols. For primer designing, the sequence of the human *SLC52A1* mRNA was utilized from *GenBank* (accession no. NM_001104577.1). Two micrograms of total RNA from HuTu 80 cells was utilized for the initial RT-PCR. The following gene specific reverse primer was used 5'-ACCGGCTGTGCGTTTGGGT-3'. Next, the first-strand cDNAs were isolated and tailed. Then the tailed cDNAs was amplified using another gene-specific reverse primer 5'-CAAAGAGGGAGCTGATTTTCCTA-3' and the manufacturer's Abridged Anchor primer. Subsequently a nested PCR was done with the manufacturer's Abridged Universal Amplification primer and the gene-specific primer 5'-CAGGAGGCTGAGGCAGGAGATT-3'. Finally PCR products were visualized on 2% agarose gel and also subcloned into the pGEM-T Easy vector (Promega, Madison, WI) for sequencing by Laragen Sequencing Facility (Los Angeles, CA).

2.3. Cloning of the 5'-regulatory region for the *SLC52A1* gene

As an outline for designing primers to amplify *SLC52A1* gene the 5'-flanking sequence of *SLC52A1* was derived from GenBank (accession no. NG_033117.1; *Homo sapiens* solute carrier family 52, riboflavin transporter, member 1 (*SLC52A1*), RefSeqGene on

chromosome 17). PCR was performed by using two different forward primers (forward-1: 5'-CGGGGTACCATGGTGATGCTCGCCTGT-3' for 579-bp fragment, forward-2: 5'-CGGGGTACCGCCGGGGCAGGTGGATCACCT-3' for 1000-bp fragment and one reverse primer 5'-CCGCTCGAGGCGACAGAACGAGATTCG-3'. Human genomic DNA (100 ng) from Promega, Madison, WI was used for templates. PCR conditions were following an initial denaturation at 95°C for 5 min, subsequent 34 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 40 sec, extension for 1 min 35 sec at 72°C, and a final extension for 5 min at 72°C. The PCR products of 579-bp and 1000-bp length were purified from a 1.5% agarose gel, and cloned into the pGEM-T Easy vector (Promega, Madison, WI). The harvested PCR products were double digested with *KpnI* and *XhoI* (both the primers were underlined with this set of restriction sites), and subcloned into the *KpnI/XhoI* digested pGL3-Basic luciferase reporter vector (Promega) placed upstream of the luciferase gene. The DNA sequences were confirmed by sequencing (Laragen sequencing facility, Los Angeles, CA).

2.4. Construction of deleted and mutated fragments of the SLC52A1 promoter

5'-Deleted fragments of DNA were generated by PCR using specific primers (Table 1) followed by subsequent cloning of the PCR products into the *KpnI/XhoI* digested pGL3-Basic vector. Mutations were introduced using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers containing mutated nucleotides (Table 2) following the manufacturer's protocols in the consensus sites for transcriptional factors in the minimal promoter region. The sequence of all constructs (5'-deleted and mutated) was confirmed before using.

2.5. Cell culture, transfection and Luciferase assay

The human-derived duodenal epithelial HuTu 80 cells (passages 5–20, American Type Culture Collection, Manassas, VA) were maintained in EMEM growth medium with 10% fetal bovine serum, sodium bicarbonate (3.6 g/l), penicillin (100,000 U/l), and streptomycin (10 mg/l). 60% confluent HuTu 80 cells grown in a 12-well plate were co-transfected with 1 μ g of test *SLC52A1* promoter construct and 100 ng of control plasmid *Renilla* luciferase–thymidine kinase (pRL–TK) (Promega) along with Lipofectamine reagent (Life Technologies). The *Renilla*-normalized firefly luciferase activity was quantified after 48 h of transfection using the Dual Luciferase Assay system (Promega). Data are presented as fold increase over the expression of pGL3-basic set arbitrarily as one.

Drosophila SL-2 cells were seeded at approximately 5×10^5 cells per well in 12-well plates one day before transfection with Lipofectamine. Two micrograms of pGL3-Basic or *SLC52A1* promoter construct were used along with the control plasmid pP_{ac}0 and 2 μ g of the *Drosophila* Sp expression plasmid (pP_{ac}Sp1 or pP_{ac}Sp3 or pPacSp4), keeping the total amount of DNA constant at 4 μ g (Courey and Tjian, 1988, Reidling and Said, 2003). At 48-h post-transfection, cells were harvested and firefly luciferase activity was assayed with the Luciferase Assay system (Promega). The activity was normalized to the *Renilla* luciferase activity from pRL-TK in the same extract. Data represented are means \pm standard error of at least three independent experiments and given as fold expression over pGL3 basic expression.

2.6. Electrophoretic mobility shift assay (EMSA)

We prepared nuclear extract from HuTu 80 cells with the commercially available NE-PER Nuclear and Cytoplasmic extraction kit from Thermo Scientific Pierce, Rockford, IL following the manufacturer's protocol. For EMSA, a 28-bp minimal promoter region (a sequence: 5'-CCTCCGGGCAGAGCCCCGCCCACT-3'), a sequence between -60 and -33 was used. The promoter was biotinylated using a Biotin 3'-end DNA labeling kit (Thermo Scientific Pierce). LightShift Chemiluminescent EMSA kit from Thermo Scientific Pierce was used for EMSA. Briefly, for binding reactions the reaction volume was 20 μ l with 5 μ g of nuclear extract, 20 fmol of biotin end-labeled DNA, and 50 ng/ μ l poly (dI.dC), and was incubated at room temperature for 20–30 min. Analysis of oligonucleotide competition was carried out with a 50-fold molar excess of unlabeled *SLC52A1* DNA fragment. For super-shift assays pretreatment of the nuclear extract was carried out with 2 μ g anti-Sp-1 antibody or 2 μ g normal rabbit IgG. Subsequently DNA-protein complexes were separated on a 6% DNA retardation gel (Invitrogen Life Technologies) in 0.5 \times Tris borate-EDTA buffer at 100V, followed by electrophoretic transfer of binding reactions to nylon membrane and crosslinking of transferred DNA to membrane in UV. Biotinylated DNA was detected by chemiluminescence using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific Pierce).

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was performed with the ChIP assay kit (Millipore, Temecula, CA) following the provided manufacturer's protocol. HuTu 80 cells (1×10^6 cells) were incubated with 1% formaldehyde (final concentration) for 10 min at 37°C to crosslink proteins to DNA, then lysed by SDS containing lysis buffer and sonicated to shear the DNA into 200–1000 bp fragments. The Sonicated cell supernatant was initially incubated with 10 μ g of anti-Sp-1 or normal rabbit IgG antibody overnight at 4°C before precipitation of the immunocomplex with protein A agarose/salmon sperm DNA for 1 h at 4°C. Immunoprecipitates were finally eluted and incubated for 4 h at 65°C (to reverse protein/DNA crosslinks). Eluted samples were treated with proteinase K and the DNA was extracted by phenol/chloroform method followed by ethanol precipitation before using for PCR. Primers used for amplification of the *SLC52A1* promoter region from -234 to +66 were 5'-AGCTGTGTGGGCTCTGGGC-3' (forward) and 5'-ATGGACCCCGGGACAAGG-3' (reverse). PCR conditions was as follows- denaturation at 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 35 sec.

2.8. Data presentation and Statistical analysis

Data are presented as mean \pm SE of at least three independent experiments. Statistical significance of each data was evaluated by Student's *t*-test using Microsoft Excel software; $p < 0.05$ and $p < 0.01$ were considered as significant (*) and highly significant (**), respectively.

3. Results

3.1. Determination of the TSS of the human *SLC52A1* gene in intestinal epithelial HuTu 80 cells using 5'-RACE

In this study, we used 5'-RACE techniques to identify the TSS of *SLC52A1* in intestinal epithelial HuTu 80 cells as the first step in cloning of the putative promoter. RefSeqGene information for the *SLC52A1* gene deposited in GenBank (accession number, NG_033117.1) was used for primer design. A number of clones for the single prominent PCR product of ~300 bp were obtained (Fig. 1A) and sequenced. The sequencing results from several randomly selected clones revealed the existence of one distinct TSS for *SLC52A1* in HuTu 80 cells represented as cytosine ('C'), located 2 bp before exon 1 and 828 bp upstream of the ATG codon of the *SLC52A1* (Fig. 1B). We denoted the TSS as + 1 (Fig. 1B).

3.2. Cloning of the 5'-regulatory region of the human *SLC52A1* gene and determination of promoter activity

We cloned the 579-bp genomic fragment containing the 5'-regulatory region of the human *SLC52A1* gene from human genomic DNA and verified its identity by sequencing. The cloned fragment starts within exon 1 of the *SLC52A1* gene at position +150 (using TSS as +1) and extends to -428 upstream of the TSS. Transient transfection of human intestinal epithelial HuTu 80 cells with the cloned -428/+150 genomic fragment using the *Firefly* luciferase reporter construct resulted in a significant ($P < 0.01$) increase in promoter activity, which was over 190-fold above the activity observed with the pGL3-basic vector (Fig. 2A). In contrast, a larger 1000 bp genomic fragment (-849/+150) of the *SLC52A1* 5'-regulatory region cloned into pGL3-basic plasmid and transfected in HuTu 80 cells did not show any promoter (luciferase) activity (Fig. 2A). The latter observation suggests possible existence of strong suppressor element between - 849 nt and -429 nt of the *SLC52A1* 5'-regulatory region.

3.3. Determination of the minimal *SLC52A1* promoter

We performed 5'-deletion analysis to determine the minimal region required for basal activity of the *SLC52A1* promoter. A series of 5'-deletion reporter plasmid constructs containing specific lengths of the *SLC52A1* (ranging from -428 to +2) were generated by PCR amplification (and verified by sequencing) and cloned upstream of the *Firefly* luciferase gene. Promoter activity of each of the deletion constructs was assessed by measuring luciferase activities in transiently transfected HuTu 80 cells. The results showed luciferase activity of the shorter 384 bp promoter fragment (-234/+150) had ~50% less activity than the full-length 579 bp (-428/+150) promoter (Fig 2B). A further deletion of 88 bp (-234/-146) from 5'-end of the 384 bp fragment resulted in a drastic reduction in promoter activity, whereas deletion of an additional 123 bp (-146/-23) completely abolished the luciferase activity (Fig. 2B). These results suggest that the minimal promoter sequence of the *SLC52A1* gene has encoded in the sequence between -234 and -23.

3.4. Identification of putative cis-regulatory elements in the SLC52A1 minimal promoter

To obtain information on putative *cis*-regulatory binding sites in the minimal promoter region of the *SLC52A1* gene, we subjected the DNA fragment (–234/–23) to *MatInspector* analysis (www.genomatix.de). The analysis revealed an absence of the typical TATA motif and the existence of multiple putative *cis*-regulatory elements including Stimulating protein 1 (Sp-1)/GC-box, Activator protein 2 (AP2), Krueppel like transcription factors (KLFs) and EGR/nerve growth factor induced protein C & related factors (EGRF) (Fig. 3A). To assess the involvement of these putative *cis*-regulatory sites in regulating the basal activity of the *SLC52A1* promoter, we introduced specific point mutations in the core sequences of these sites and examined the effect of these mutations on promoter activity. As a negative control, the effect of mutating a random site (–71/–72) in the minimal promoter was examined. In this context we compared the minimal promoter (–234/+150) activity with mutant promoters after transfection into HuTu 80 cells. The results showed that mutating all the *cis*-elements identified by *MatInspector*, but not the randomly mutated sites, led to a significant ($P < 0.01$) reduction in promoter activity compared to the minimal promoter (Fig. 3B). The reduction, however, was most pronounced in the Sp-1 site located at –47/–41 position, where an approximate 72% reduction in promoter activity was observed. For this reason we focused on this site and its interacting transcription factor in subsequent experiments.

3.5. Conformation of binding of Sp-1 in the SLC52A1 minimal promoter

To further establish the importance of the Sp-1 *cis*-element in *SLC52A1* promoter activity, we first examined the interaction of a 28-bp region (–60/–33) of the minimal promoter containing this site and nuclear extract from HuTu 80 cells, i.e., performed EMSA. The results showed the formation of two major DNA-protein complexes (two major bands, *bands 1 and 2*) with decreased gel mobility (Fig. 4A, lane 2). One of these two major DNA-protein complexes was specific and was competed away with a 50-fold molar excess of the unlabeled DNA probe (Fig. 4A, lane 3). To establish the involvement of the Sp-1 transcription factor in the formation of the protein - DNA complex that was detected in the EMSA, we then performed super-shift analysis in which we pre-incubated the HuTu 80 cells nuclear extract with specific anti-Sp-1 antibodies. The result showed clear disappearance of the specific protein-DNA complex (Fig. 4B, lane 4), indicating that the interacting protein is Sp-1. The nonspecific IgG antibodies (served as negative control) had no effect on mobility of the protein-DNA complex (Fig. 4B, lane 3). These results suggest that Sp-1 can specifically bind to its site in the *SLC52A1* promoter fragment.

To further confirm the existence of direct binding of Sp-1 to the *SLC52A1* promoter and to do so *in vivo*, we carried out ChIP assay using cross-linked chromatin prepared from HuTu 80 cells. Chromatin was sheared by sonication into fragments of 200-1000 bp followed by immunoprecipitation using anti-Sp-1 antibodies or nonspecific rabbit IgG (as negative control). The purified DNA from the immune-precipitated chromatin was used as template for PCR amplification, using primers that specifically amplify a 300 bp region (–234/+66) harboring the Sp-1 site. As shown in Fig. 4C, the amplification of the region –234 to +66 was detected in immunoprecipitated obtained with anti-Sp-1 antibody, but not with the nonspecific rabbit IgG. These data further demonstrate that anti-Sp-1 antibody is able to

specifically precipitate the minimal *SLC52A1* promoter region, and provide a direct conformation of Sp-1 association with the minimal *SLC52A1* promoter *in vivo*.

3.6. Role of Sp-1 in the activation of the *SLC52A1* promoter: studies with *Drosophila* SL-2 cells

To further confirm the role of Sp-1 in the activation of the *SLC52A1* promoter described earlier, we used *Drosophila* SL-2 cells since these cells lacks endogenous Sp factors (Courey and Tjian, 1988, Reidling and Said, 2003). For this, we co-transfected the minimal promoter construct (–234/+149) of *SLC52A1* with *Drosophila* expression vectors pP_{ac}Sp0, and pP_{ac}Sp1 into *Drosophila* SL-2 cells and also tested the effect on promoter activity of pP_{ac}Sp3 and pP_{ac}Sp4. The level of reporter gene expression detected for the construct in the absence of co-expression of exogenous Sp protein was assigned the arbitrary value of 1. The results showed a significant ($P < 0.01$; 8-fold) induction in promoter activity upon co-expression with pP_{ac}Sp1 but not with pP_{ac}Sp3 or pP_{ac}Sp4 (Fig. 5). These findings confirm the important role and specificity of Sp-1 in activating the *SLC52A1* minimal promoter.

4. Discussion

Our laboratory has been interested in delineating the mechanisms and regulation of RF transport in intestinal and other epithelial cells (Said, 2004, Said and Arianas, 1991, Said *et al.*, 1993, Said and Ross, 2011, Said and Nexo, 2012, Said, 2011), but it has been difficult to characterize the regulatory aspects at the molecular level due to lack of knowledge of the identity of RF transporters. With the recent cloning and molecular identification of three specific RFVTs, this issue became feasible. Taking advantage of this advancement, we report here on the first characterization of the transcriptional regulation of the *SLC52A1* gene (which encodes RFVT-1). First we identified the TSS of the *SLC52A1* gene in human-derived intestinal HuTu 80 cells using 5'-RACE approach and found one TSS, located outside of predicted exon 1. Based on the location of TSS, we subsequently amplified and cloned a 579 bp and a 1000 bp genomic DNA fragments and tested their promoter activity after transfecting them into HuTu 80 cells. The 579 bp (–428/+150) fragment had a robust promoter activity in these cells while the 1000 bp (–849/+150) fragment had virtually no promoter activity. These findings suggest the presence of a possible suppressor in the upstream region (–849 to –429) of the *SLC52A1* promoter; further studies, however, are needed to test this possibility. Our results showed the minimal *SLC52A1* promoter region to be between –234 nt and –23 nt using a 5'-deletion approach. This region retained approximately 50% of the activity of the 579 bp promoter fragment. The minimal region was found to be TATA-less, GC-rich, and contained a number of putative *cis*-regulatory elements that include EGRF, AP-2, KLFs and Sp-1. The Role of these putative *cis*-elements in regulating the activity of the *SLC52A1* minimal promoter was examined by mutating these sites and testing the effect of such mutations on promoter activity. Mutating all the putative *cis*-elements led to a significant reduction in promoter activity, with the degree of reduction being highest for the Sp-1 binding site. For that reason we focused our subsequent investigations on this site and on the nuclear factor that interacts with it.

To establish the interaction of Sp-1 with the *SLC52A1* promoter, we first performed EMSA using a 28 bp region of the minimal promoter that contain this *cis*-regulatory site and observed two major bands representing DNA-protein complexes. One of these bands was found to be specific and was competed away by an excess of the unlabeled probe. We then performed super-shift analysis using anti Sp-1 antibody with the results showing a complete disappearance of the specific DNA-protein complex. Similar disappearance of the specific DNA-protein complex has been observed with other genes (Han *et al.*, 2001; Zatyka *et al.*, 2002; Omotehara *et al.*, 2002; Chen *et al.*, 2007), which could be due to precipitation of the corresponding protein-DNA complex by Sp-1 antibodies, or due to interaction of the Sp-1 antibodies with the nuclear Sp-1 protein leading to inability of the latter to bind its specific site in the DNA. This suggests that the Sp-1 nuclear factor (which is ubiquitously expressed in mammalian cells) does indeed bind to the *SLC52A1* promoter. The latter was further confirmed by the ChIP assay, which showed anti-Sp-1 antibody specifically precipitates the *SLC52A1* promoter whereas nonspecific rabbit IgG antibody was not able to do so. Based on the above observations, it is concluded that the Sp-1 transcriptional factor does bind to the *SLC52A1* promoter.

To further establish a role for Sp-1 in the activity of the *SLC52A1* promoter, we also used the *Drosophila* SL-2 cellular system. These cells lack Sp nuclear factors and have been used as a model to verify role of Sp factors in activity of promoters of different genes (Courey and Tjian, 1988, Reidling and Said, 2003, Wang and Bannon, 2005, Wan *et al.*, 2005). For this we co-transfected the minimal *SLC52A1* promoter with a human Sp-1 containing vector and observed a significant induction to promoter activity. It is interesting that transfecting these cells with the transcription factors Sp-3 and Sp-4 (which can also interact with Sp *cis*-elements in gene promoters, Han *et al.*, 2001, Wan *et al.*, 2005, Nabokina and Said, 2004) did not affect the activity of the *SLC52A1* minimal promoter demonstrating the specificity of the Sp-1 effect.

In conclusion, this study describes the first characterization of the *SLC52A1* promoter and reports on the identification of the minimal promoter and key transcription factors involved in its activity. Results of these investigations should assist us in understanding how expression of the *SLC52A1* gene is affected in conditions like RF deficiency or over-supplementation, and how exposure to environmental/external factors like chronic alcohol use could affect this expression. The findings should serve as base for future investigations into the molecular regulation of this important human gene in health and disease.

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Abbreviations

RFVT-1	riboflavin transporter-1
RF	riboflavin
5'-RACE	5'-rapid amplification of the cDNA ends

TSS	Transcription start site
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
EMSA	Electrophoretic mobility shift assay
ChIP	Chromatin immunoprecipitation

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Highlights

- *SLC52A1* minimal promoter was identified
- Roles of putative *cis*-regulatory elements were established by point mutations; Sp-1 site appears to play a predominant role
- Binding of Sp-1 to the minimal promoter was established by EMSA and ChIP assays
- Expression of Sp-1 in *Drosophila* SL-2 cells activated *SLC52A1* promoter
- Sp-1 plays a key role in the transcriptional regulation of the *SLC52A1* gene

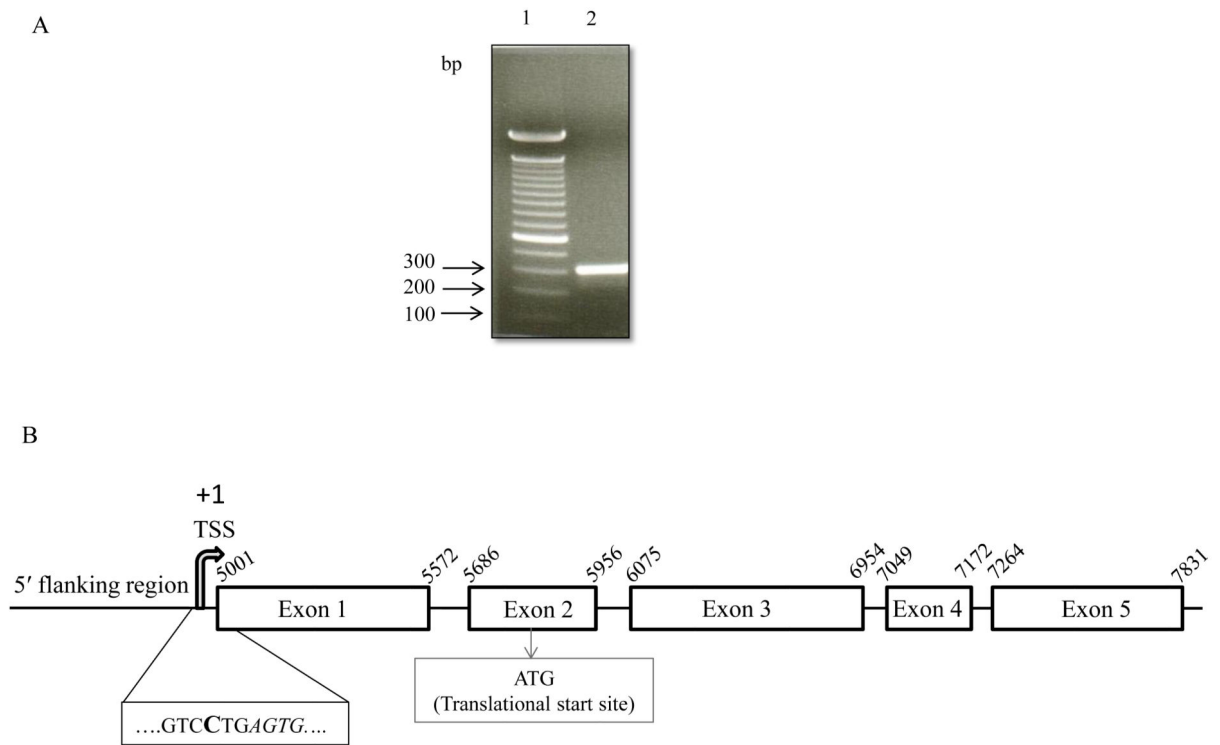


Fig. 1. Identification of the transcriptional start site of the *SLC52A1* gene

A. Results of 5'-RACE experiments on total RNA from HuTu 80 cells; DNA sequences were detected by gel electrophoresis using 2% agarose. Lane 1, 1Kb marker; Lane 2, A single DNA band of 300 bp was detected. **B.** Schematic diagram of *SLC52A1* gene depicting the positions of the TSS. The TSS and the first four nucleotide of exon 1 are shown in bold type and italic, respectively. The start codon is shown in the box that is located inside exon 2.

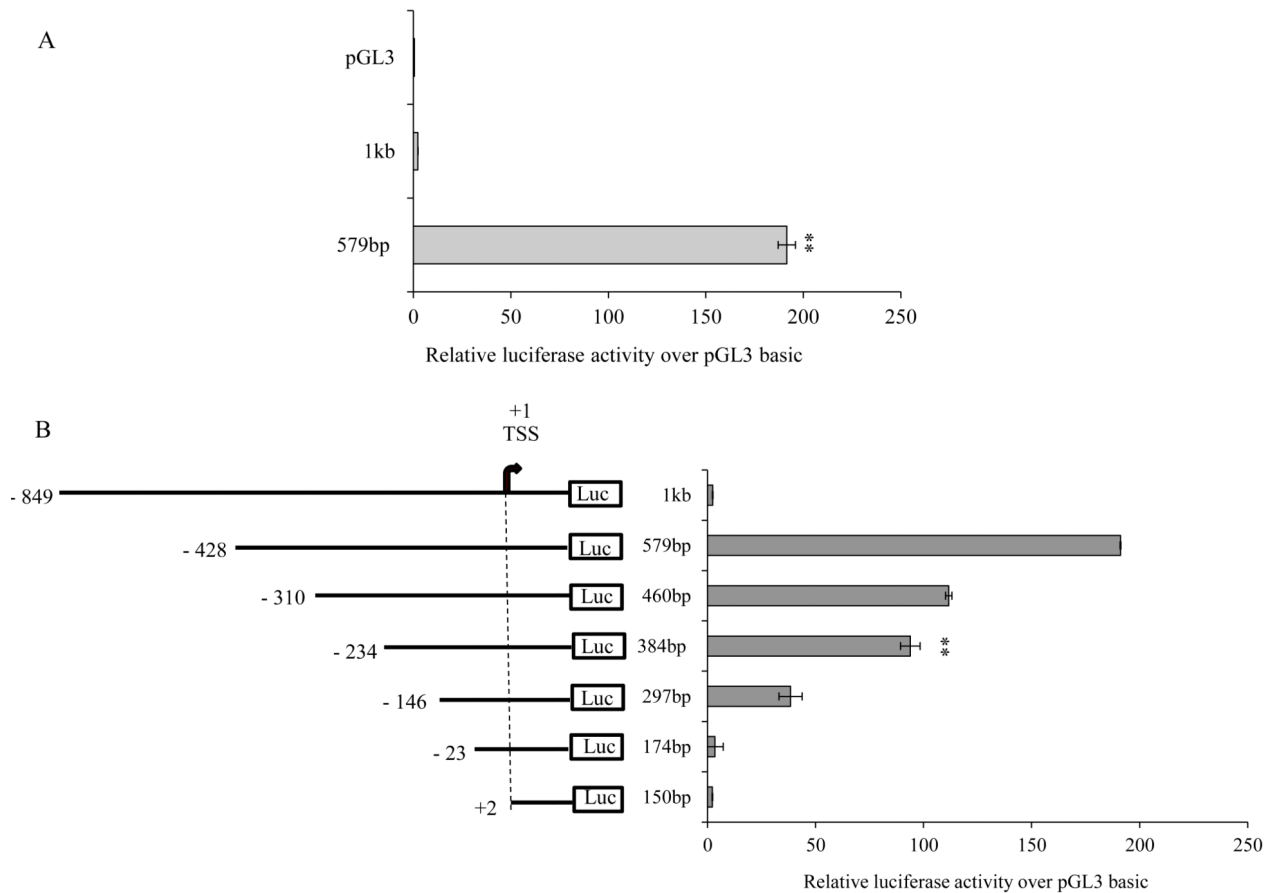


Fig. 2. *SLC52A1* promoter activity in HuTu 80 cells and deletion analysis of the *SLC52A1* promoter

A. Promoter activity of 1000bp and 579bp promoter fragments of *SLC52A1* in HuTu 80 cells. **B.** Schematic diagrams of the 5'-deleted promoter constructs are shown on the left. 5'-deletions were introduced into the 1000 bp promoter fragment as described in MATERIALS AND METHODS section. All construct are derivatives of the pGL3-basic vector. Promoter constructs in pGL3-basic vector were transiently expressed in HuTu 80 cells for luciferase assay. Firefly luciferase activity was normalized relative to *Renilla* luciferase activity and results were expressed as fold over pGL3-basic expression; data are presented as mean \pm SE of at least 3 independent experiments.

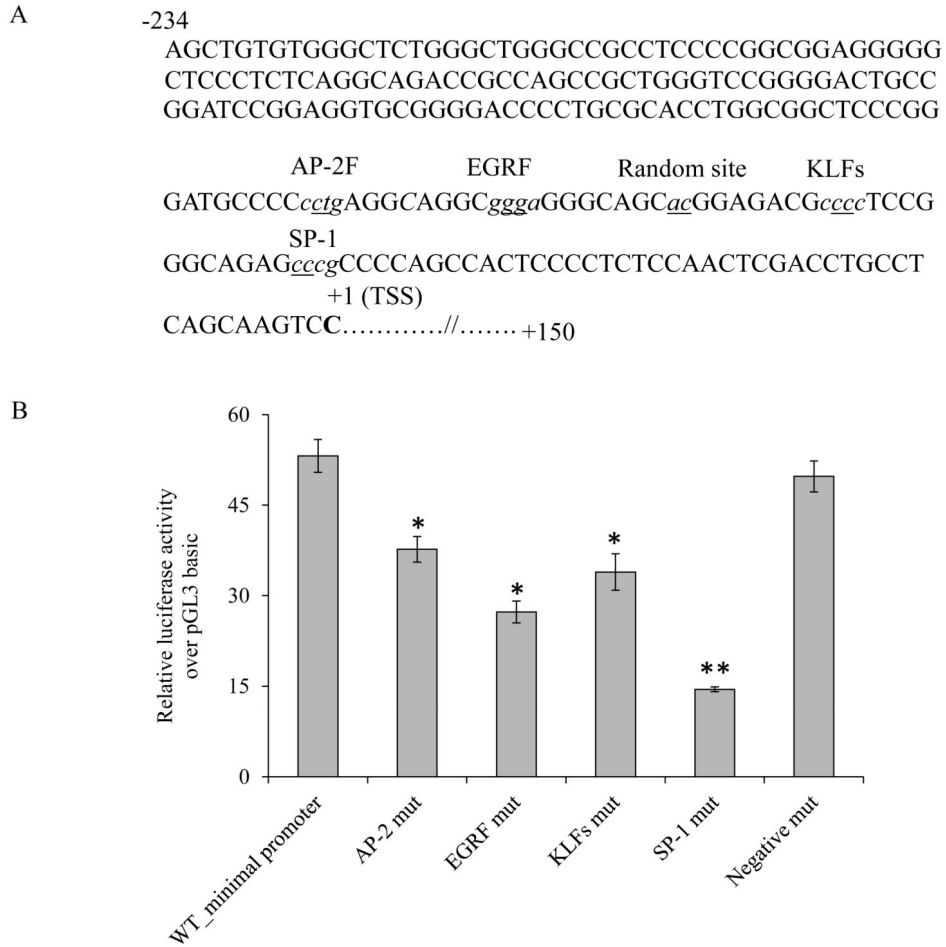


Fig. 3. Schematic of the *SLC52A1* minimal promoter region and mutational analysis of *cis*-regulatory elements

A. Schematic depicting the minimal promoter sequence (–234 to +150) with annotated putative *cis*-elements. *Cis*-elements on the complementary strand are shown in italicized letters. The nucleotides that were altered for mutational analysis are underlined. TSS is bold type and numbered as +1. **B:** The mutated minimal promoter constructs in pGL3-Basic were transiently expressed into HuTu 80 cells for luciferase assays. Firefly luciferase activity was normalized relative to *Renilla* luciferase activity and results were expressed as fold over pGL3-basic expression; data are presented as mean ± SE of at least 3 independent experiments.

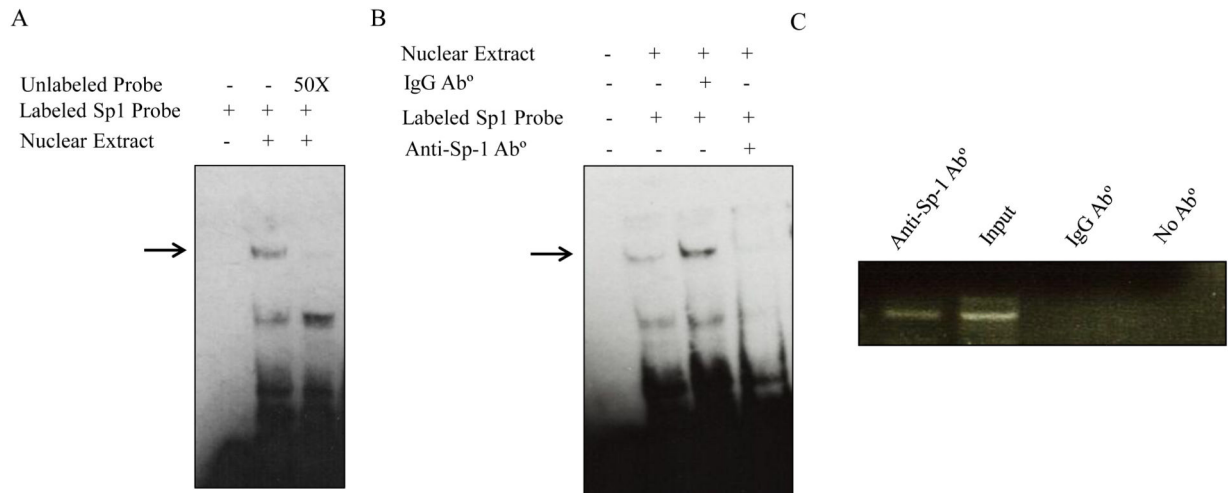


Fig. 4. Binding of Sp-1 transcription factor to the *SLC52A1* promoter *in vitro* and *in vivo*
A. DNA/protein profile from EMSA using the *SLC52A1* minimal promoter region. Gel shift assays were conducted with HuTu 80 cells nuclear extract as described in MATERIALS AND METHODS section. The labeled fragment of the *SLC52A1* promoter region (-60/-33) was used. DNA/protein complexes were resolved on 6% retardation acrylamide gel. Competition experiments were performed using a 50 fold molar excess of unlabeled promoter fragment. The specific complex is indicated with an arrow. **B.** Presence of Sp1 in DNA-protein complexes. Nuclear extracts were pre-incubated with anti-Sp-1 antibody or nonspecific IgG before the addition of the biotin labeled Sp-1 probe. Data are representative of three separate experiments. **C.** Chromatin immunoprecipitation analysis. Formaldehyde cross-linked chromatin from HuTu 80 cells was incubated with anti-Sp-1 antibody. As a negative control, the chromatin was incubated without antibodies or with nonspecific IgG antibody. Total input DNA at a 1:10 dilution was used as a positive control for the PCR reaction. Immunoprecipitated DNA was analyzed by PCR with primers specific for the *SLC52A1* promoter.

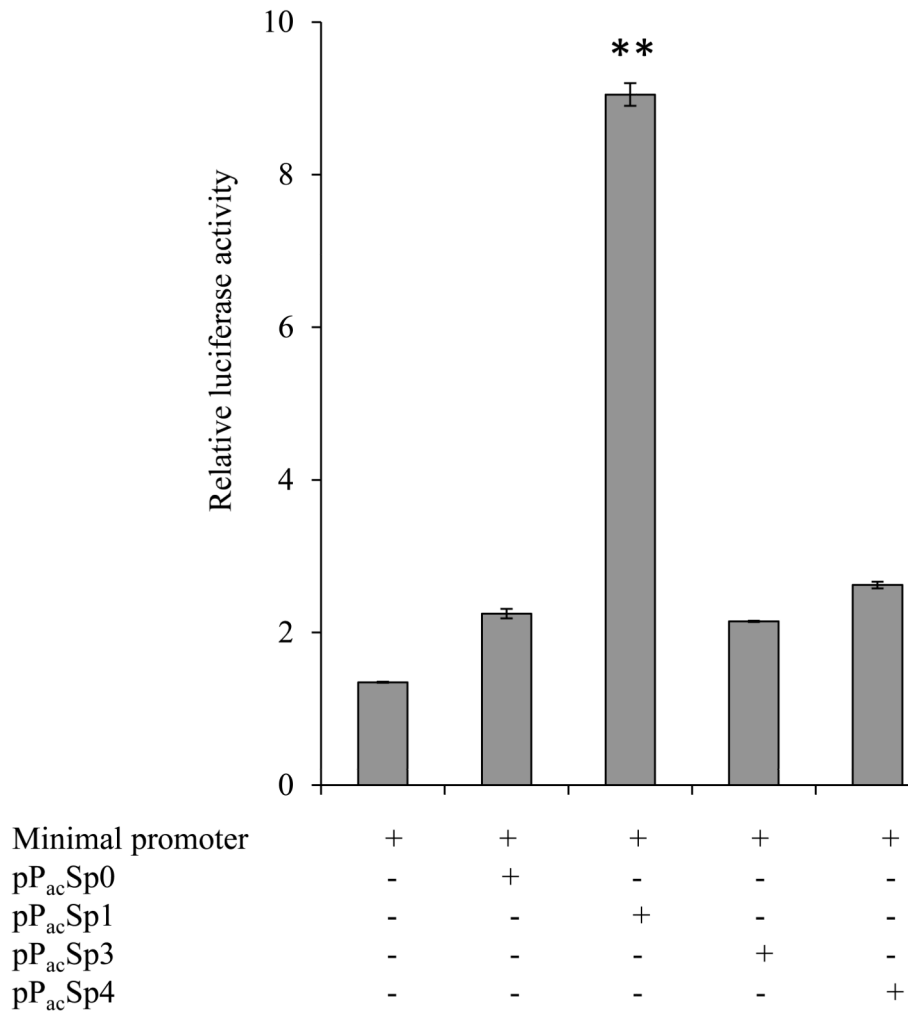


Fig. 5. Co-transfection assays using both pP_{ac}Sp1 or pP_{ac}Sp3 or pP_{ac}Sp4 vectors and *SLC52A1* minimal promoter luciferase construct in *Drosophila* SL-2 cells

SL-2 cells were co-transfected with 3 μ g of the minimal promoter construct in pGL3-basic and 2 μ g of either control plasmid pPac0 or *Drosophila* Sp expression plasmids (pP_{ac}Sp1 or pP_{ac}Sp3 or pP_{ac}Sp4). At 48 h post-transfection, cells were harvested and firefly luciferase activity was assayed as described in MATERIALS AND METHODS. Firefly luciferase activity was normalized relative to *Renilla* luciferase activity and results were expressed as fold over pGL3-basic expression; data are presented as mean \pm SE of at least 3 independent experiments.

TABLE-1
Primer sequences used in PCR for deletion of 5'-regulatory region of *SLC52A1* promoter

Sequence (5'-3')	Position
CGGGGTACCGCTCTAGCCTGGGTGAC	-310 to -292
CGGGGTACCAGCTGTGTGGGCTCTGG	-234 to -216
CGGGGTACCGGATCCGGAGGTGCGG	-146 to -131
CGGGGTACCAACTCGACCTGCCTCAGC	-23 to -7
CGGGGTACCTGAGTGTGCGAAAAGCGC	+2 to +20
<u>CCGCTCGAGGCGACAGAACGAGATTCG</u>	+134 to 150

KpnI and *XhoI* restriction sites are shown by bold and underline, respectively. The relative position of primers is based on numbering the start site of transcription of *SLC52A1* as position+1.

TABLE-2
Sequence of primers used in PCR for generating mutant *SLC52A1* minimal promoter

<i>cis</i> -elements	Forward primer (5'-3')	Reverse primer (5'-3')
AP2	CGGGATGCCCC <u>AG</u> GAGGCAGGCGGG	CCCGCCTGCCTC <u>CT</u> TGGGGGCATCCCG
EGR	CCCTGAGGCAGGC <u>AA</u> GAGGGCAGCACGG	CCGTGCTGCCCTC <u>TT</u> GCCTGCCTCAGGG
KLFs	CAGCACGGAGACG <u>AA</u> CCTCCGGGCAGAG	CTTGCCCGGAGG <u>TT</u> CGTCTCCGTGCTG
Sp-1	CCTCCGGGCAGAG <u>AA</u> CGCCCAGCCACT	AGTGGCTGGGGC <u>TT</u> TCTCTGCCCGGAGG

Location of point mutations are shown by bold and underlined text