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Molecular analysis of the human *SLC13A4* sulfate transporter gene promoter

J. Jefferis [a], J. Rakoczy [a,b,c], D.G. Simmons [b], P.A. Dawson [a,c,*]

[a] Mater Medical Research Institute, South Brisbane, Queensland, Australia
[b] School of Biomedical Sciences, University of Queensland, St. Lucia, Queensland, Australia
[c] Translational Research Institute, Woolloongabba, Queensland, Australia

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ABSTRACT

The human solute linked carrier (SLC) 13A4 gene is primarily expressed in the placenta where it is proposed to mediate the transport of nutrient sulfate from mother to fetus. The molecular mechanisms involved in the regulation of *SLC13A4* expression remain unknown. To investigate the regulation of *SLC13A4* gene expression, we analysed the transcriptional activity of the human *SLC13A4* 5′-flanking region in the JEG-3 placental cell line using luciferase reporter assays. Basal transcriptional activity was identified in the region −57 to −192 nucleotides upstream of the *SLC13A4* transcription initiation site. Mutational analysis of the minimal promoter region identified Nuclear factor Y (NFY), Specificity protein 1 (SP1) and Krüppel like factor 7 (KLF7) motifs which conferred positive transcriptional activity, as well as Zinc finger protein of the cerebellum 2 (ZIC2) and helix–loop–helix protein 1 (HEN1) motifs that repressed transcription. The conserved NFY, SP1, KLF7, ZIC2 and HEN1 motifs in the *SLC13A4* promoter of placental species but not in non-placental species, suggests a potential role for these putative transcriptional factor binding motifs in the physiological control of *SLC13A4* mRNA expression.

1. Introduction

Sulfate SO_4^{2-} is an essential nutrient for fetal growth and development [1]. The developing fetus has negligible capacity to generate its own sulfate and therefore is completely reliant on sulfate from the maternal circulation [2,3]. During pregnancy in humans and rodents, circulating maternal sulfate levels increase by ≈two-fold to meet the gestational requirements of the developing fetus [4–6]. Maternal hyposulfataemia has been linked to fetal sulfate deficiency and late gestational miscarriage in mice [6,7], demonstrating the importance for maintaining a sufficiently high sulfate supply to the developing fetus.

Supply of sulfate from maternal circulation to the fetus is mediated by sulfate transporter proteins that are expressed on the plasma membrane of placental trophoblast cells [1]. The mammalian genome contains ten sulfate transporter genes that have different tissue and cell membrane expression patterns [8]. Recent studies have shown abundant *SLC13A4* sulfate transporter mRNA expression in the syncytiotrophoblast cells (site of maternal–fetal nutrient exchange) of human and mouse placentae [9,10]. During mouse gestation, placental *SLC13A4* mRNA and protein levels increase by ⩾four-fold in late gestation when fetal sulfate requirements are high [9]. These findings indicate that *SLC13A4* most likely plays an important role in mediating the placental transport of sulfate from mother to fetus.

Human, mouse and rat *SLC13A4* mRNA is most abundant in the placenta, with lower levels found in the choroid plexus [11–13], suggesting a physiological role for *SLC13A4* in both the placenta and brain. Lower levels of *SLC13A4* mRNA have been detected by PCR in other tissues, including testes and liver but the physiological contribution of *SLC13A4* to sulfate transport in those tissues is not known [11–13]. Nonetheless, abundant *SLC13A4* mRNA levels in the placentae of numerous species, indicates an important role of *SLC13A4* in mediating sulfate transport through the placenta. In addition, human *SLC13A4* amino acid sequence shares high identity (⩾88%) with mouse and rat [11,12], indicating a possible conserved role of *SLC13A4* in mammalian physiology.

The human *SLC13A4* protein shares 40% amino acid identity with the human *SLC13A1* sulfate transporter, which is primarily expressed in the ileum and kidney where it maintains circulating sulfate levels [8]. Both *SLC13A1* and *SLC13A4* are localised to chromosome 7q31-33 [8], suggesting a gene duplication event through evolution. Numerous metabolic and physiological factors are known to regulate renal *SLC13A1* gene expression, including vitamin D and T3 responsive elements in the *SLC13A1* gene promoter [14,15]. Unlike the *SLC13A1* gene, there is currently no information regarding the molecular factors that regulate *SLC13A4* gene expression. The 5′-flanking regions of human, mouse and rat

* Corresponding author. Address: Mater Research, Translational Research Institute, Woolloongabba, Queensland 4102, Australia.
E-mail address: paul.dawson@mmri.mater.org.au (P.A. Dawson).

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SLC13A4 contain numerous putative transcription factor binding motifs [11–13] but the role of these DNA sequences in regulating transcription have yet to be investigated.

In this study, we identified the minimal promoter sequence of the human *SLC13A4* 5′-flanking region that confers transcriptional activity. Phylogenetic alignment of the human minimal promoter sequence with multiple species, including both placental and non-placental animals, was performed to identify conserved putative transcription factor binding motifs. Finally, site-directed mutagenesis identified motifs that either up- or down-regulated transcriptional activity within the *SLC13A4* promoter. This is the first study to identify DNA sequences that potentially regulate the transcriptional activity of *SLC13A4* in numerous placental species.

2. Materials and methods

2.1. Bioinformatics

The NCBI database (http://www.ncbi.nlm.nih.gov) was queried for homologues of human *SLC13A4*. A phylogenetic tree of all species with *SLC13A4* homologues was generated online using the Interactive Tree of Life program (iTOL; http://itol.embl.de/) [16]. The 5′-flanking region of these genes from the predicted transcriptional start site to approximately −3500 bases upstream, were selected for sequence analysis as previously done for the related *SLC13A1* sulfate transporter gene [17]. Putative transcription factor binding motifs were identified using MatInspector [18] and then a multiple species alignment of those motifs was generated using the DiAlign TF program (http://www.genomatix.de). *In silico* methylation analysis of the human, rat and mouse *SLC13A4* 5′-flanking regions as well as *CYP24A1*, *Cyp24a1* and *SERPINB5* (control genes known to have methylated promoters) [19,20], was performed using CpG island searcher (http://cpgislands.usc.edu/) using recommended parameters for %GC content (55%), ratio of observed to expected GC islands (0.65), 200 bp CpG island lengths and 100 bp distances between CpG islands [21].

2.2. Cell culture

The JEG-3 placental cell line was cultured in DMEM medium containing L-glutamine and glucose (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin–streptomycin and 0.1% plasmocin (InvivoGen). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Promoter plasmid constructs

Fragments of the human *SLC13A4* 5′-flanking region, with a common 3′-end at +690 (+1 is the transcription start site) and 5′-ends of −3004, −1054, −488, −380, −192, −57 and −5 were amplified by PCR using 200 nM forward and reverse primers (Table 1) and 1.25 U LA TAQ polymerase (TakaRa). The thermal cycling protocol was: 95 °C for 1 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min. Each fragment was cloned upstream of a luciferase reporter gene in the multiple cloning site of the pMetLuc2 expression vector (Clontech) using In-Fusion reagent as described by the manufacturer (Clontech). Sequence variants were introduced into the cloned −380 *SLC13A4* 5′-flanking region by PCR using 200 nM forward and reverse primers (Table 1) and thermal cycling parameters: 95 °C for 1 min; 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min. These PCR products were then treated with In-Fusion regent (Clontech) to generate circularized vectors, as previously described [22]. Nucleotide sequences of all cloned *SLC13A4* sequences were verified by DNA sequence analysis using 1 μg plasmid DNA, 9.6 pmol forward and reverse primers (Table 1), ABI Prism BigDye

Table 1
Primers used for PCR and DNA sequencing.

Primer	#Direction	Sequence (5′–3′)	##Primer location
**Primers used for PCR-amplifying SLC13A4 5′-flanking regions*			
P1	F	CATGGTGGCGACCGGTCGCGCCTCTGTCCTCTCCAG	+690
P2	R	CCCGGGATCCACCGGTCTCCACTCCCTTGACAATGG	−3004
P3	R	CCCGGGATCCACCGGTTTAGCCTGGGGATCATTTCC	−1054
P4	R	CCCGGGATCCACCGGTCGATCTTTGCCAGCAGGGAG	−488
P5	R	CCCGGGATCCACCGGTCCAAGGTCTAGATCAGCAGG	−380
P6	R	CCCGGGATCCACCGGTCCGCTGTGACCAATCAAGAG	−192
P7	F	CCCGGGATCCACCGGTGGCCTCCAGCAAGTTTTGTC	−57
P8	R	CCCGGGATCCACCGGTCTTTTCTGCTTTGCAGGCCCAG	−5
***Primers used for site directed mutagenesis*			
P9	F	CCATTGGTGGATGAAACCTAAAGGTGGGCCTCCAG	−67
P10	R	TTCATCCACCAATGGGAAGCCGGGACAGAGACAG	
P11	F	GATTGGCTTTGAGAAGTTCGCGGAGCTACTGTCTC	−113
P12	R	TTCTCAAAGCCAATCAGCAAATTGAAAAGAATGAG	
P13	F	TCTTTTCAATTTGCTGTTTGGCTTTGAGAAGTGGG	−129
P14	R	AGCAAATTGAAAAGAATGAGTCAATTTCTCTCCCC	
P15	F	GCGGGGAGAGAAATTGACCCATTCTTTTCAATTTG	−149
P16	R	AATTTCTCTCCCCGCGGCCGCTCTTGATTGGTCAC	
P19	F	GGAATCAGCAGGCCCCCGTTTTGACCAATCAAGAG	−189
P20	R	GGGCCTGCTGATTCCGCAGAACCTAGGCAGCCTG	
****Primers used for DNA sequencing*			
P21	F	CTGTGGATAACCGTATTAC	
P22	R	CAGATGTCGATGTTGGGG	

#F, forward primer; R, reverse primer. ##Number denotes the position of the first 5′-nucleotide in the 5′-flanking region of *SLC13A4*. *Underlined sequence homologous to the pMetLuc2 vector to allow recombination cloning. **Underlined nucleotides are mutated in the core sequences of HNF1-MYT1, SP1-KLF7, NFY, AP1 and ZIC2-HEN1 sites. ***Primers located in the pMetLuc2 vector.

Terminator Sequence chemistry and an AB3730x/96-capillary sequencer (Applied Biosystems).

2.4. Transient transfections and luciferase assay

JEG-3 cells were transiently co-transfected using Lipofectamine$_{2000}$ (Invitrogen) with pMetLuc2 containing individual cloned *SLC13A4* fragments or pMetLuc2 that lacks cloned fragments (negative control), and pSEAP2 alkaline phosphatase expression vector used to normalise luciferase activity. Cells (80% confluent in 48 well plates) were incubated with 2 µl Lipofectamine$_{2000}$ and plasmids (0.4 µg each pMetLuc2 and pSEAP2) in DMEM containing 10% fetal calf serum (total volume 50 µl) for 7 h, and then the media was replaced with 600 µl OptiMEM® (Invitrogen) containing 10% fetal calf serum for an additional 70 h. Luciferase and alkaline phosphatase activities in the cell culture media were assayed using protocols and reagents (Ready-To-Glow™ Dual secreted reporter assay) purchased from Clontech, and measured using a PolarStar Omega plate reader (BMG Labtech).

2.5. Statistical analysis

The statistical significance of the differences of luciferase activities between each clone and control vector was evaluated using a one-way ANOVA, followed by a Dunnett multiple comparisons test, with $P < 0.05$ considered significant.

3. Results and discussion

3.1. Analysis of SLC13A4 protein sequences and 5′-flanking regions of multiple species

From the NCBI database, we retrieved the *SLC13A4* gene sequences of 20 species, including human. Alignment of human *SLC13A4* amino acid sequences with the 19 homologues showed the highest homology (88%–99% identity) to hemochorial species, intermediate homology (64%–93% identity) with endotheliochorial and epitheliochorial species, and lowest homology (49%–79% identity) with non-placental species (Fig. 1A). These findings show that the human *SLC13A4* protein sequence is more highly conserved with most other placental species, particularly hemochorial species, when compared to non-placental species. In addition, the relatively high similarity (88% identity) between mouse and human *SLC13A4* amino acid sequences [11], together with recent data showing both human and mouse *SLC13A4* mRNA expression in placental syncytiotrophoblasts [9,10], indicates that mouse *SLC13A4* is likely to be a suitable model for studying the physiological roles of *SLC13A4* in the human placenta.

Within the first 3500 nucleotides upstream of the predicted transcription initiation start site of all 20 species analysed in this study, we identified 9 putative transcriptional binding motifs that were conserved in most of the placental species (Fig. 1B). These conserved motifs in the human *SLC13A4* 5′-flanking region are: High mobility group A (HMGA, 5′-TTGTGGCAATCAATGACTGAAAA-3′) at position −2805 to −2783; Homeobox D10 (HOXD10, 5′-TTTTTTTTATTGTGGTA-3′) at position −1841 to −1825; overlapping Zinc finger protein of the cerebellum 2 (ZIC2, 5′-GCCCCC GCTGTGACC-3′) at position −196 to −182 and Helix–loop–helix transcription factor (HEN1, 5′-CAGGCCCCCGCTGTGACCAAT-3′) at position −199 to −179; Nuclear factor Y (NFY, 5′-TTGCTGATT GGCTTT-3′) at position −135 to −121; overlapping Specificity protein 1 (SP1, 5′-GAAGTGGGCGGAGCTAC-3′) at position −118 to −102 and Krüppel like factor 7 (KLF7, 5′-AGAAGTGGGCGGAG CTA-3′) at position −119 to −103; and overlapping Hepatic nuclear factor 1 (HNF1, 5′-GGATGAAAGTTAAAGGT-3′) at position −75 to −59 and Myelin transcription factor 1 (MYT1, 5′-TGAAAGT-TAAAGG-3′) at position −72 to −60. In addition, human *SLC13A4* contains a consensus sequence for Activating protein 1 (AP1, 5′-AATTGACTCATTC-3′) at position −156 to −144, which is not conserved with other *SLC13A4* homologues but is found in a similar position in the 5′-flanking region of the related human *SLC13A1* orthologue [17]. Furthermore, all 20 *SLC13A4* homologues lack

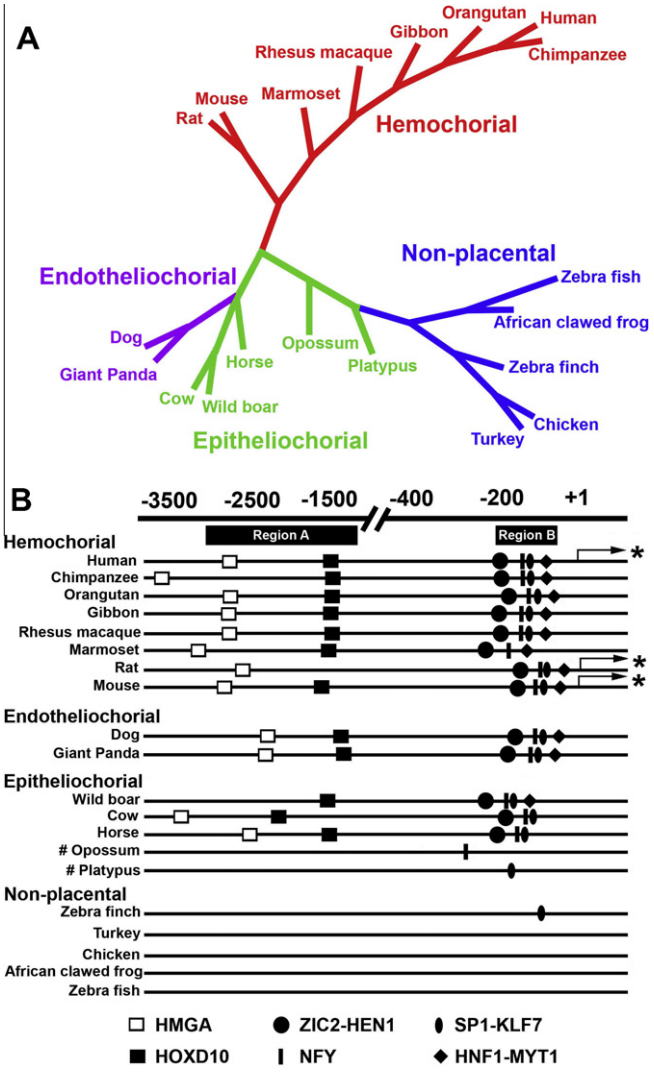

Fig. 1. Phylogenetic analysis of *SLC13A4* protein and the *SLC13A4* 5′-flanking region. (A) Phylogenetic tree of vertebrates that carry *SLC13A4*. Distances represent evolutionary divergence of species. Colors represent the placental types; hemochorial (red), endotheliochorial (purple) epitheliochorial (green); and non-placental species (blue). Image adapted from tree generated online using the Interactive Tree of Life (ITOL) [16]. (B) Multiple species alignment of conserved putative transcription factors in the *SLC13A4* 5′-flanking regions. Thirteen placental species shared six highly conserved domains with human *SLC13A4*: HMGA, HOXD10, ZIC2/HEN, NFY, SP1/KLF7 and HNF1/MYT1. Regions A and B represent segments of human *SLC13A4* 5′-flanking region that confer negative and positive transcriptional regulation (see Fig. 2A). *The transcriptional start sites have been identified in human [13], mouse [11] and rat [12], as indicated by the arrow. The species are divided into placental subtypes; hemochorial, endotheliochorial, epitheliochorial and non-placental. #The marsupial species (opossum and platypus) have a transient placenta early in development, which is functionally distinct to that of the other placental mammals [33], and share few conserved putative motifs with other placental species. Of the 5 non-placental species, only the zebra finch shares conserved putative transcription factors with the placental mammals. Scale bar at top is relative to the +1 of the transcriptional start site in human *SLC13A4*.(For interpretation of the references to color in this Fig. legend, the reader is referred to the web version of this article.)

any canonical TATA-box, suggesting that *SLC13A4* mRNA expression is likely regulated by multiple transcription factors.

To investigate the potential epigenetic regulation of *SLC13A4* mRNA expression, we performed a bioinformatic analysis of the 5′-flanking regions of human, rat and mouse *SLC13A4*, as well as human *CYP24A1*, mouse *Cyp24a1* and human *SERPINB5* (positive controls), previously shown to have promoters regulated by CpG methylation [19,20]. Methylation was predicted for *CYP24A1*, *Cyp24a1* and *SERPINB5*, but the absence of CpG islands in all three *SLC13A4* homologues (Table 2), suggests that *SLC13A4* mRNA expression is unlikely to be regulated by methylation.

3.2. Minimal promoter sequence of human SLC13A4

To localise the DNA sequences of *SLC13A4* which are important for basal promoter activity, a series of reporter constructs with 5′-flanking regions of *SLC13A4* were cloned upstream of the luciferase gene, transfected into JEG-3 cells and then assayed for luciferase activity (Fig. 2A). The highest luciferase expression was obtained with fragments with the common 3′-end +690 and upstream 5′-ends at −192, −380, −488 and −1054, when compared to constructs with 5′-ends at −3004, −57 and −5. This finding suggests the possible presence of upstream negative regulatory motifs between −1054 and −3004 (region A, Fig. 1B), as well as downstream positive regulatory elements between −192 and −57 (region B, Fig. 1B).

Within region A, binding motifs for HMGA and HOXD10 were conserved in most placental species. Previous studies have shown that HMGA and HOXD10 can act a transcriptional repressors [23,24], which is consistent with our data showing the lowest luciferase activity in fragments containing the HMGA and HOXD10 binding sequences (Fig. 2A). However, the evaluation of these 2 sites to *SLC13A4* transcription will require additional studies.

Region B contains ZIC2, HEN1, NFY, SP1, KLF7, HNF1 and MYT1 motifs conserved in most placental species (Fig. 1B). Both ZIC2 and HEN1 are primarily expressed in the central nervous system [25,26], suggesting a potential role for the putative ZIC2 and HEN1 motifs in regulating *SLC13A4* expression in the brain. NFY, SP1 and KLF7 transcriptional factors are expressed in the placenta where they play a role in regulating placental development [27,28]. SP1 is a ubiquitous transcription factor that acts in concert with other transcription factors, including NFY [29], and is expressed in mouse syncytiotrophoblasts at the time of syncytialisation, when *SLC13A4* mRNA levels are up-regulated [9]. In addition, KLF7 is expressed towards the fetal side of the placenta during the third trimester of pregnancy [30], which correlates with the spatial and temporal expression of *SLC13A4* mRNA in mouse and human gestation [9,10]. Whilst HNF1 and MYT1 transcription factors have not been detected in the placenta [31,32], the conserved sequence and location of these putative transcription factor binding motifs in *SLC13A4* of numerous placental species will require further investigation.

Table 2
Methylation analysis of the human, rat and mouse *SLC13A4* 5′-flanking regions.

Gene 5′-flanking region	CpG islands	#Exp_{CpG}/Obs_{CpG}	Predicted methylation
Human *SLC13A4*	0	0.65	No
Rat *Slc13a4*	0	0.65	No
Mouse *Slc13a4*	0	0.65	No
*Human *CYP24A1*	2	0.68	Yes
*Mouse *Cyp24a1*	1	0.68	Yes
**Human *SERPINB5*	1	0.72	Yes

#The ratio of expected CpG to observed CpG (Exp_{CpG}/Obs_{CpG}) investigates whether the number of CpG repeats is more so than expected by chance, and a CpG island is defined when Exp_{CpG}/Obs_{CpG} >0.65. To test the validity of the software analysis three positive controls, which have methylation patterns confirmed through bisulfite sequencing, were included. *CYP24A1 (vitamin D 24-hydroxylase) is methylated in human and mouse placenta [20] and **Human *SERPINB5* (encodes the Maspin protein) which is methylated in maternal blood [19].

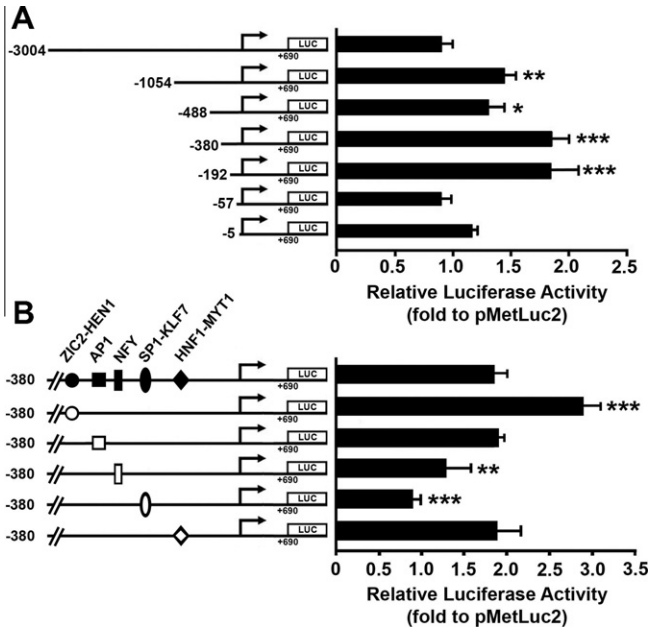

Fig. 2. Transcriptional activities of the *SLC13A4* 5′-flanking region. (A) Transcriptional activity of the 5′-truncated *SLC13A4* 5′-flanking region assessed by luciferase activity in JEG-3 cells. Data is shown as fold induction to luciferase activity of the pMetLuc2 reporter, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to constructs containing the −3004 fragment. (B) Transcriptional activity of the pMetLuc2-380 vector containing wildtype (*filled symbols*) and individually mutated motifs (*unfilled symbols*): ZIC2/HEN1, AP1, NFY, SP1-KLF7 and HNF1-MYT1. Data is shown as fold induction to luciferase activity of the pMetLuc2 reporter, $n = 3$.

3.3. Transcriptional activity of motifs in the 5′-flanking region of human SLC13A4

To evaluate the contribution of putative ZIC2, HEN1, NFY, SP1, KLF7, HNF1, MYT1 and AP1 binding motifs to *SLC13A4* mRNA expression, we mutated the core sequences of each motif in the pMetLuc2-380 vector and compared luciferase activities to the control sequence (Fig. 2B). Luciferase expression from the mutated AP1 and overlapping HNF1 and MYT1 sequences was similar to the wildtype sequence, suggesting that these sites are not responsible for the transcriptional activity of the pMetLuc2-380 vector. These findings are also consistent with HNF1 and MYT1 transcription factors not being detected in the placenta [31,32], as well as the AP1 motif in human *SLC13A4* not found in the *SLC13A4* 5′-flanking region of other species. Mutational analysis of the overlapping ZIC2 and HEN1 sites showed ≈50% increase in luciferase activity, when compared to wildtype sequence, indicating that the ZIC2 and/or HEN1 motifs may negatively regulate transcription of *SLC13A4* in placental cells. Mutations in the core sequences of NFY and overlapping SP1 and KLF7 sites, led to partial (≈70%) and complete loss of transcription, respectively, induced by the *SLC13A4* sequences in pMetLuc2-380. These findings suggest that NFY and the SP1 and/or KLF7 sites may play important roles in the transcriptional activity of the human *SLC13A4* promoter. This finding is supported by the expression of the NFY, SP1 and KLF7 transcription factors in placental trophoblasts [27,28], where *SLC13A4* is expressed [9,10].

This is the first study to identify sequence elements in the *SLC13A4* 5′-flanking region that mediate enhanced or decreased transcriptional activity, and therefore may contribute to regulating sulfate supply to the developing fetus.

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