Molecular cloning and characterization of the mouse Na⁺ sulfate cotransporter gene (*Slc13a4*): structure and expression

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Sulfate is an essential ion required for numerous functions in mammalian physiology. Due to its hydrophilic nature, cells require sulfate transporters on their plasma membranes to allow entry of sulfate into cells. In this study, we identified a new mouse Na⁺-sulfate cotransporter (mNaS2), characterized its tissue distribution and determined its cDNA and gene (*Slc13a4*) structures. mNaS2 mRNA was expressed in placenta, brain, lung, eye, heart, testis, thymus and liver. The mouse NaS2 cDNA spans 3384 nucleotides and its open frame encodes a protein of 624 amino acids. *Slc13a4* maps to mouse chromosome 6B1 and contains 16 exons, spanning over 40 kb in length. Its 5'-flanking region contains CAAT- and GC-box motifs and a number of putative transcription factor binding sites, including GATA-1, MTF-1, STAT6 and HNF4 consensus sequences. This is the first study to define the tissue distribution of mNaS2 and resolve its cDNA and gene structures, which will allow us to investigate mNaS2 gene expression *in vivo* and determine its role in mammalian physiology.

Key words: sodium sulfate cotransport, tissue distribution, placenta, brain, lung

INTRODUCTION

Inorganic sulfate (SO₄²⁻) is a physiologically essential ion involved in many metabolic and cellular processes (Markovich, 2001). It is required for the biotransformation of xenobiotics and the activation of endogenous compounds such as heparin and heparan sulfate (Falany, 1997). In addition, sulfation of structural compounds such as glycosaminoglycans and cerebroside sulfate is essential for the maintenance of normal structure and function of tissues (Mulder and Jakoby, 1990). Special interest has focused on two sulfate transporters, DTDST (Hästbacka et al., 1994) and DRA (Hoglund et al., 1996), which have been associated with human disease: 1) mutations in the DTDST gene (*SLC26A2*) lead to four different chondrodysplasias (multiple epiphyseal dysplasia, diastrophic dysplasia, atelosteogenesis type II and achondrodysplasia type IB); and 2) the DRA gene (*SLC26A3*) is defective in congenital chloride diarrhea (reviewed in (Dawson and Markovich, 2005)). These disorders highlight the important roles of sulfate in mammalian physiology and underscore the need to further our understanding

of sulfate transporters, including the solute linked carrier 13 (SLC13) transporters, NaS1¹ and NaS2², yet to be identified in any human disease (reviewed in (Markovich and Murer, 2004)).

NaS1 (for Na⁺-Sulfate cotransporter 1) was the first sulfate transporter isolated by expression cloning using *Xenopus* oocytes (Markovich et al., 1993). NaS1 encodes a Na⁺-dependent sulfate transport protein of 595 amino acids (66 kDa), with the hydropathy profile suggesting 13 transmembrane domains (TMD). We have isolated the orthologs of NaS1 from mouse (mNaS1; (Beck and Markovich, 2000)) and humans (hNaS1; (Lee et al., 2000)), and recently generated a NaS1 knock-out (*Nas1*^{-/-}) mouse that lacks a functional NaS1 protein (Dawson et al., 2003). *Nas1*^{-/-} mice exhibit hypersulfaturia and hyposulfatemia, highlighting the essential role of NaS1 in maintaining blood sulfate levels.

The second Na⁺-sulfate cotransporter, NaS2, was identified in human high endothelial venules (Girard et al., 1999), and we recently demonstrated that its mRNA was expressed in placenta, testis, brain, heart, thymus and liver (Markovich et al., 2005). Human NaS2 (hNaS2) encodes a 627 amino acid protein with 12 putative trans-

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¹ Initially designated NaS₁-1; ² Initially designated SUT-1

membrane domains (Girard et al., 1999) and exhibits >40% amino acid identity with the mammalian NaS1 orthologs (Markovich and Murer, 2004). Both hNaS1 (*SLC13A1*) and hNaS2 (*SLC13A4*) genes are localized to human chromosome 7q31-q33 (Lee et al., 2000), in a region of conserved synteny with *Slc13a1* and *Slc13a4* on rat chromosome 4q22 (Dawson et al., 2005a). In contrast to numerous studies showing the structure of human and mouse NaS1 genes (reviewed in (Lee et al., 2005)), there is no information available on the genomic organization of murine NaS2. In order to gain a better understanding of the physiological roles of NaS2, in this study, we determined its murine tissue distribution, mRNA transcript and gene structures, and identified putative response elements in its 5' flanking region.

MATERIALS AND METHODS

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) Studies were performed on samples derived from mice, at 10 weeks of age, with a mixed genetic background (C57BL/6J x 129/Sv)F₁. Total RNA was extracted from various mouse tissues (brain, colon, eye, kidney, thymus, spleen, heart, ileum, testis, lung, placenta, liver and prostate) by using standard procedures (TRIzol reagent; Invitrogen) and treated with 5 units of RNase-free DNase I enzyme (Promega) for 30 minutes at 37°C. After denaturation at 65°C for 5 minutes, total RNA (2 µg) was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Tissue distribution by PCR analysis Primer 5'-TG-GTCATCTGTGTGCCGCTG-3' in exon 1 and antisense primer 5'-CTCCACGATGGGCATCACCA-3' in exon 4, were used to amplify 413bp mNaS2 cDNA fragments using cycle parameters: 94°C for 1 minute; followed by 22 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. In a separate tube, primers 5'-TGAAGGT-CGGTGTGAACGGATTGCG-3' and 5'-CATGTAGC-CATGAGGTCCACCAC-3' were used to amplify 983bp GAPDH cDNA fragments using cycle parameters: 95°C for 1 minute; followed by 18 cycles of 95°C for 1 minute, 66°C for 2 minutes, and 72°C for 2 minutes.

Northern blot analysis Total RNA (10 µg) was separated on a 1.2% agarose formaldehyde gel in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and transferred to Hybond-XL nylon membranes (Amersham Pharmacia). The blots were probed with a ³²P-labeled 1.8 kb mouse NaS2 cDNA and a 600 bp mouse GAPDH probe.

Mouse NaS2 cDNA cloning The open reading frame of mNaS2 mRNA was RT-PCR amplified from mouse

brain RNA, using primer 5'-gaagatctATGGGCTTGCTG-CAGGGCCTTC-3' and antisense primer 5'-ggactagtT-TAGGTCTGATCAGTGATGTTGCTGAC-3', containing artificial restriction sites (lower case letters) and then subcloned into the pT7TS vector (pT7TS plasmid was kindly provided by Dr. Vize, University of Texas at Austin). The clones were confirmed by sequencing using the ABI Prism Big DyeTM Terminator kit (Applied Biosystems) following the manufacturer's protocol and gel separation was performed using a ABI 3730xl automatic capillary sequencer at the Australian Genome Research Facility (AGRF), University of Queensland.

RESULTS

Tissue distribution of mNaS2 mRNA Using RT-PCR, mNaS2 mRNA was amplified in mouse placenta, brain, lung, eye, heart, testis, thymus and liver (Fig. 1A). A search of the NCBI mouse EST database (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.23666> on 3 July 2006) revealed numerous mNaS2 ESTs from brain (*n* = 38), placenta (*n* = 9), lung (*n* = 1), heart (*n* = 2), eye (*n* = 1), pre-implanted embryos (*n* = 11) and whole embryos 5–16 dpc (*n* = 35), indicating that mNaS2 is expressed in the adult mouse brain, placenta, lung, heart and eye, and also during embryonic development. Northern hybridization revealed the presence of a single 3.4 kb mRNA transcript in placenta, brain, lung and eye (Fig. 1B).

Fig. 1. mNaS2 mRNA tissue distribution. (A) RT-PCR amplification of 413 bp mNaS2 and 983 bp GAPDH cDNA fragments. Data are representative of three separate experiments. (B) Northern analysis of mouse RNA, hybridized with a ³²P-labeled mNaS2 cDNA (Upper) showing the 3.4kb NaS2 transcript, and GAPDH cDNA (Lower).

mNaS2 protein Amino acid sequence alignments (Fig. 2) revealed mNaS2 to have 89% and 97% identity with human and rat NaS2 proteins, respectively. The mouse NaS2 protein contains 624 amino acids (Fig. 2) with a cal-

TM1		
mNaS2	MELLOGLLQARKLLLVICVPLLLPLPTIYPTS-----	33
rNaS2	MELLOGLLQARKLLLVICVPLLLPLPTIYPTSGGVELYFQWEMSRETFPNLTRMEFYL	60
hNaS2	MELLOGLLRVRKLLLVVCPVLLPLPLVLPSS-----	33
mNaS2	-----EACAYVLLVTAVYWSEAVPLGAAA	59
rNaS2	HCRMSLVHQATGMASKDKEHEEAISLNFILFQKEAACAYVLLVTAVYWSEAVPLGAAA	120
hNaS2	-----EASCAYVLLVTAVYWSEAVPLGAAA	59
TM2		TM3
mNaS2	LVP AFLYPFFGVLRSEVAREYFKNTLLLVGVICVAAAVEKYNLHKRIALRMVLMAGAK	119
rNaS2	LVP AFLYPFFGVLRSEVAREYFKNTLLLVGVICVAAAVEKYNLHKRIALRMVLMAGAK	180
hNaS2	LVP AFLYPFFGVLRSEVAREYFKNTLLLVGVICVAAAVEKYNLHKRIALRMVLMAGAK	119
TM4		
mNaS2	PGMLLLCFMCCTTMSMLSNSTSTTAMVMPIVEAVLQELINAEELQAAAG---TEEAELM	176
rNaS2	PGMLLLCFMCCTTMSMLSNSTSTTAMVMPIVEAVLQELINAEELQVAGNSSTEEAELM	240
hNaS2	PGMLLLCFMCCTTLLSMVLSNTSTTAMVMPIVEAVLQELVSAEDEQLVAGNSNTEEAPEI	179
mNaS2	GLDVNRRQTSMELEIFVNETSAADFTSLMQSKNLNGVPMVTKSINTANQQQKKQKQPSQE	236
rNaS2	GVDVSNRRQTSVELIFVNETSAADFTSLMQNKNLNGVPMVTKSIKTANQQQKKQKQHSQE	300
hNaS2	SLDVKNSSQPSLELEIFVNEE-SNADLTLLMHNENLNGVPSITNPDKTANQHGGKKQKQPSQE	238
* ●		TM5
mNaS2	KPGVPTPSSKTQELNKKKYRSHHDQMI CKQLSLSISYANTIGGLTTIIGTSTSLIFLEHF	296
rNaS2	KPGVPTPSSKNQELNKKKYRSHHDQMVCKQLSLSISYANTIGGLTTIIGTSTSLIFLEHF	360
hNaS2	KPQVLTSPRKQKLN-RKYRSHHDQMI CKQLSLSISYSATIGGLTTIIGTSTSLIFLEHF	297
TM6		
mNaS2	NNQYPAAEVVNF GTWFLFSEPISLIMLVVSMFWHWFELGCNFKETCSLSKKKKTRREEL	356
rNaS2	NNQYPAAEVVNF GTWFLFSEPISLIMLVVSMFWHWFELGCNFKETCSLSKKKKTRREEL	420
hNaS2	NNQYPAAEVVNF GTWFLFSEPISLIMLVVSMFWHWFELGCNFKETCSLSKKKKTKREQL	357
* ●		TM7
mNaS2	SEKRIREEYEKLGDISYPEMVTAEFFILMTVLVFTREPGEVPGWD SFFEKKGYRTDATVS	416
rNaS2	SERRIREEYEKLGDISYPEMVTAEFFILMTVLVFTREPGEVPGWD SFFEKKGYRTDATVS	480
hNaS2	SEKRIQEEYEKLGDISYPEMVTGFFFILMTVLVFTREPGEVPGWD SFFEKKGYRTDATVS	417
TM8		TM9
mNaS2	VFLGFLLEFLIPAKKPCFGKKS DGTGQEASKGIEPIITWKDFQKTPWEIVILVGGGYALA	476
rNaS2	VFLGFLLEFLIPAKKPCFGKKS DGTGQEASRGIEPIITWKDFQKTPWEIVILVGGGYALA	540
hNaS2	VFLGFLLEFLIPAKKPCFGKKN DGENQEHSLGTEPIITWKDFQKTPWEIVILVGGGYALA	477
TM10		TM11
mNaS2	SGSKSSGLSTWIGHQMLSLSSLPPWATILLACVLVSIVTEFVSNPATITITFLPILCTLSE	536
rNaS2	SGSKSSGLSTWIGHQMLSLSSLPPWATILLACVLVSIVTEFVSNPATITITFLPILCTLSE	600
hNaS2	SGSKSSGLSTWIGNQMLSLSSLPPWAVTLLACILVSIVTEFVSNPATITITFLPILCSLSE	537
TM12		TM13
mNaS2	TLHINPLYTLVPVTMSISFAVMLPVGNPPNAIVE SYGHQIKDMVKAGLVNVI GLVTVM	596
rNaS2	TLHINPLYTLVPVTMSISFAVMLPVGNPPNAIVE SYGHQIKDMVKAGLVNVI GLVTVM	660
hNaS2	TLHINPLYTLIPVTMCISFAVMLPVGNPPNAIVE SYGHQIKDMVKAGLVNVI GLVTVM	597
Y		
mNaS2	VAINTWGVSLHLDAPPAWAKVSNITDQ-	624
rNaS2	VAINTWGVSLHLDTPPAWAKVGNITDQT	689
hNaS2	VAINTWGVSLHLDTPPAWAVSNITDQA	626

Fig. 2. mNaS2 sequence alignment with rat (rNaS2) and human (hNaS2) proteins. Alignments were generated using the Clustal W program (Higgins et al., 1992). Identical amino acids are depicted in grey shading. Putative transmembrane domains (TM1 to TM13) are indicated by bold face lines. Potential phosphorylation (*, protein kinase C; ●, casein kinase II) and N-glycosylation (Y) sites are indicated.

Fig. 3. mNaS2 cDNA structure. (A) A schematic showing exons 1–16 (boxes) and protein coding sequences (white portions) spread over 3384 nucleotides. (B) The predicted 13 transmembrane spanning domains (shaded boxes) of mNaS2 protein aligned with the mNaS2 exon boundaries (dotted lines).

Fig. 4. Mouse *Slc13a4*, human *SLC13a4* and rat *Slc13a4* structures. *Slc13a4* exon-intron organization showing exons (vertical lines; *optional exon 2 in rat) and introns (horizontal lines) spread over approximately 40.1 (mouse), 47.0 (human) and 46.5 (rat) kb.

Table 1. Exon-intron organization of the mouse *Slc13a4* gene

Intron No.	Phase	5' splice donor ^a	Intron size (bp) ^b	3' splice acceptor ^a	Exon No.	Exon size (bp)
1	0	ACCAGC/gtaagtggca	5504	ccccttcag/GAAGCT	1	772
2	0	AGTGAG/gttagacccc	11794	ccccctacag/GTGGCG	2	89
3	2	TGGCAT/gtgagtca	1876	tgccctcag/GCTCCT	3	137
4	1	TCATGG/gtacagttca	352	accacttag/GTC TTG	4	164
5	2	TGAAGA/gtgagtatga	2677	ttgttttcag/CACGTC	5	55
6	0	AGCAAG/gtatgacctt	1310	gtcccagcag/AATCTG	6	40
7	0	TCACAG/gtaactgaac	2742	ccctggcag/GAAAAG	7	81
8	2	CAACAA/gtaagtaatt	778	taccttcag/CCAGTA	8	188
9	2	CTGCAA/gtgagtacca	1072	cccttcttag/CTTCAA	9	120
10	2	CATTAG/gtaagagcta	1686	cctttgacag/CTACCC	10	102
11	2	TGAAAA/gtaagtata	710	tctttgctag/GAAAGG	11	102
12	1	GTGATG/gtgagaccg	600	tgtgatgcag/GAACAG	12	98
13	0	AGCAAG/gtaactagtt	3145	cttcccgcag/AGCTCT	13	125
14	0	ACCCTG/gtgagtggat	1127	tctctctag/TCCGAA	14	162
15	0	GACATG/gtgagctacg	1375	tgtctacag/GTGAAA	15	138
					16	1011

^a Exon sequences are indicated by uppercase letters and intron sequences by lowercase letters. ^b Intronic sequences were determined from the alignment of genomic DNA (NT_039341.4) and mNaS2 cDNA (AK038937; NM_172892; AK036354 and BC089161) sequences.

culated molecular mass of 68.9 kDa, containing 13 putative transmembrane domains (TMD), 3 potential protein kinase C sites (Ser²⁴⁴, Ser³⁴⁶ and Ser³⁵⁷), 5 casein kinase II sites (Ser¹⁹⁷, Ser²⁵⁷, Thr³⁵¹, Ser³⁷² and Thr⁴⁵³), and 1 N-glycosylation site (Asn⁶²⁰).

mNaS2 cDNA By using a combination of RT-PCR and NCBI database mining, we cloned the entire mNaS2 (3384 bp) cDNA (Fig. 3A). The mNaS2 transcript includes a predicted 673 bp 5'-UTR, 1838 bp open reading frame, and a 873 bp 3'-UTR, containing a polyadenylation signal (AATAAA) located 23 nucleotides upstream of the poly(A) tail (NCBI accession no. AK038937). The putative *Slc13a4* transcription initiation site was determined by alignment of mouse (AK038937) rat (AY911718) and

human (BC030689) NaS2 cDNA sequences, and agrees with our Northern blot data showing a single 3.4kb mRNA transcript (Fig. 1B). A comparison of the predicted TMDs to the exon structure of the mouse NaS2 gene (Fig. 3B), showed that each TMD is encoded by a separate exon, with the exception of TMDs 10 and 11, which are encoded by exon 14.

***Slc13a4* organization** To determine the *Slc13a4* structure, we aligned *Slc13a4* genomic (NT_039341.4) and its cDNA (AK038937; NM_172892; AK036354 and BC089161) sequences. *Slc13a4* maps to mouse chromosome 6B1 and spans approximately 40.1 kb, with 16 exons and 15 introns (Fig. 4). Exon sizes vary from 40 bp to 1011 bp and intron sizes range from 352 bp to 11,794 bp (Table 1). The translation initiation site is present in exon 1, and the TAA stop codon is situated in exon 16 (Fig. 3A). Nucleotide sequences at the intron-exon boundaries of mouse *Slc13a4* conform to the GT/AG rule for intron donor and acceptor sites, and the codon phase is mainly 0 or 2 (Table 1), which is similar to human *SLC13A4* (Markovich et al., 2005) and rat *Slc13a4* (Dawson et al., 2005a). The 5'-flanking region of *Slc13a4* contains numerous putative transcription factor binding sites (Fig. 5), including MTF-1 (at -88 nt), GATA-1 (at -118 nt), BARBIE (at -194 nt), CEBPB (at -234 nt), RORA2 (at -263 nt and -367 nt), XBP1 (at -283 nt), PPARA (at -323 nt), STAT6 (at -406 nt and -466 nt), HNF4 (at -486 nt), FREAC2 (at -620 nt), NFkB (at -703 nt and -716 nt), GATA-3 (at -733 nt), SRF (at -828 nt). P53 (at -944 nt) and PRE (at -990 nt). It also contains potential CAAT- (at -69 nt and -171 nt) and GC-box (at -1020 nt) motifs, but lacks any canonical TATA-box.

DISCUSSION

In the present study, we cloned the mouse Na⁺-sulfate cotransporter mNaS2, characterized its tissue distribution and determined its cDNA and genomic structures. mNaS2 mRNA expression is similar to hNaS2 (Markovich et al., 2005), with the exception that mNaS2 mRNA is expressed additionally in lung. We also detected mNaS2 mRNA in the eye, which was not tested in our previous studies of hNaS2 and rNaS2 (Dawson et al., 2005a; Markovich et al., 2005). Interestingly, rNaS2 mRNA expression was only detected in placenta, brain and liver, demonstrating a different tissue distribution of NaS2 between rodent species. The NaS1 orthologs also exhibit different tissue distributions between species, with hNaS1 found only in kidney (Lee et al., 2000), whereas rNaS1 is expressed in both kidney and intestine (Markovich et al., 1993), and mNaS1 also detected in the duodenum, jejunum, cecum, colon, testis, adrenal gland and adipose tissue (Beck and Markovich, 2000). The tissue distribution of mNaS2 mRNA is different to other cloned

Fig. 5. Location of putative transcription factor binding sites in the mouse *Slc13a4* 5'-flanking region. Nucleotide sequence of the predicted *Slc13a4* 5'-flanking region (from -1026 to +24 nt) is shown. Position +1 (arrow) denotes the putative transcription initiation site. The DNA sequence was scanned for elements that share homology to known transcription factor binding sites using MatInspector (Quandt et al., 1995). The GC- (SP1 binding site) and CAAT-boxes, as well as putative transcription factor binding motifs are boxed, with core sequences underlined. MTF-1, metal transcription factor 1; BARBIE, barbiturate-inducible element; CEBPB, CCAAT/enhancer binding protein beta; RORA2, RAR-related orphan receptor alpha2; XBP1, X-box-binding protein 1; PPARA, PPAR/RXR heterodimers; STAT6, signal transducer and activator of transcription 6; HNF-4, hepatic nuclear factor 4; FREAC7, fork head related activator-2; NFKAPPAB, NF-κB; GATA-3, GATA binding factor 3; SRF, serum response factor; P53, Tumor suppressor p53; and PRE, progesterone receptor binding site.

mouse sulfate transporters: *msat-1* (*slc26a1*) is expressed in kidney, liver, calvaria, cecum, brain and skeletal muscle (Lee et al., 2003), whereas *ddtst* (*slc26a2*) is expressed ubiquitously (Sato et al., 1998). The expression of mNaS2 mRNA in the placenta, suggests that mNaS2 would be responsible for sulfate transfer from mother to fetus, facilitating placental steroid hormone metabolism (Pasqualini, 2005), xenobiotic metabolism (Reynolds and Knott, 1989) and the homeostasis of thyroid hormones (Visser, 1994). Recently, hNaS2 and mNaS2 expression was localized to placental trophoblast cells (Miyachi et al., 2006), which is consistent with our findings of placental expression of hNaS2 (Markovich et al., 2005) and mNaS2. The importance of maintaining sufficiently high levels of sulfate during pregnancy, was highlighted in our previous study which showed miscarriages in hyposulfatemic *Nas1*^{-/-} mice (Dawson et al., 2003). In addition, disruption of the mouse sulfotransferase gene, *sult1e1*, leads to placental thrombosis and spontaneous fetal loss (Tong et al., 2005). Sulfate is also required in the placental trophoblast cells for the synthesis of proteoglycans, including heparan sulfate, dermatan sulfate and chondroitin sulfate, which prevent blood coagulation in the intervillous space, protect the fetus from the maternal immune system, and enable the implantation of the fetus in the uterine epithelium (Delorme et al., 1998; Slater and Murphy, 1999; Achur et al., 2000; Genbacev et al., 2003). Taken together, these findings highlight the essential role of sulfate during fetal development, and suggest that NaS2 may play an important role in maintaining placental sulfate levels for fetal development.

Our data showed that mNaS2 mRNA is also expressed in the brain. These findings, together with a previous study showing its expression in the mouse choroid plexus (Matsumoto et al., 2003), suggests that mNaS2 may play a role in the transport of sulfate into cells of the central nervous system (CNS). Our previous studies which showed the expression of mouse, rat and human *sat-1* mRNA in brain (Lee et al., 1999; Lee et al., 2003; Regeer et al., 2003), together with mNaS2, rNaS2 (Dawson et al., 2005a) and hNaS2 (Markovich et al., 2005) mRNA detected in brain, may suggest an important physiological role for sulfate transporters in the CNS. Sulfate plays an important role in the brain through the biosynthesis of sulfate proteoglycans, which are involved in modulating cell interactions in developing nervous tissues (Dow and Riopelle, 1994). In addition, sulfate conjugation is an important step in the metabolism of certain neurotransmitters, including serotonin, dopamine and norepinephrine (reviewed in (Strott, 2002)). In addition, we have recently shown behavioral abnormalities in the hyposulfatemic *Nas1*^{-/-} mouse, which lacks a functional Na⁺-sulfate cotransporter mNaS1, highlighting the consequences of disturbed sulfate homeostasis on neurological function (Dawson et al., 2004; Dawson et al., 2005b). The

physiological importance of mNaS2 expression in liver, lung, heart, thymus, testis and eye, is yet to be determined. However, its expression in liver may be important for sulfotransferase activity or bile synthesis, as proposed for the expression of *sat-1* in the rat liver (Bissig et al., 1994).

The mNaS2 protein model of 13 transmembrane domains is in agreement with the prediction for hNaS2 (Markovich et al., 2005) and rNaS2 (Dawson et al., 2005a). Analysis of mNaS2 amino acid sequence revealed a potential extracellular *N*-glycosylation site, several putative intracellular protein kinase C (PKC) sites and casein kinase II (CKII) sites, which are all conserved between mNaS2, hNaS2 and rNaS2 (Fig. 2). The mouse, rat and human NaS1 orthologs have a similar arrangement of potential *N*-glycosylation, PKC and CKII sites, when compared with NaS2. In addition, mNaS2 shows high protein identity (49%) and chromosomal localization (6B1) with mNaS1, suggesting that the *Slc13a1* and *Slc13a4* genes may be derived from a gene duplication event through evolution.

The general arrangement of mouse *Slc13a4* is very similar to that of human *SLC13A4* (Markovich et al., 2005) and rat *Slc13a4* (Dawson et al., 2005a), with the exception of optional exon 2 found in rat *Slc13a4* (Fig. 4). Despite mNaS2 sharing a high protein identity (97%) and similar gene structure to rNaS2 (Fig. 2 & 4), an mNaS2 optional exon 2 cDNA sequence was not detected by RT-PCR (Fig. 1), and has yet to be identified in the NCBI EST database, suggesting that this additional exon found in rNaS2, is not conserved in all mammals. Similarly, we found an optional exon (exon 2) in human *SLC26A1* which is not present in rat *slc26a1* (reviewed in (Lee et al., 2005)). The positioning of mouse *Slc13a4* exons is particularly well conserved when compared with human *SLC13a4* and rat *Slc13a4*, whereas the variation in size of introns 8 and 13, accounts for the major size differences between mNaS2, hNaS2 and rNaS2 (Fig. 4). Within the first 1026 nucleotides of the 5'-flanking region of mouse *Slc13a4*, are a number of potential cis-acting elements recognized by well-known transcription factors, that may play a role in the basal or chronic regulation of *Slc13a4*. These include PRE, P53, SRF, SF1, BARBIE, STAT6, RORA2, PPARA, XBP1, CEBPB, GATA-1, GATA-3 and MTF-1. The significance of these consensus sites and their involvement in the transcriptional regulation of mNaS2 mRNA is yet to be determined. We also identified consensus sequences for the binding of transcription factors (HNF-4 and FREAC2), which have important roles in embryonic development (Lehmann et al., 2003; Watt et al., 2003). This finding, together with numerous embryonic mNaS2 ESTs reported in the NCBI database, suggests that mNaS2 could be transcriptionally regulated during development. The identification of phylogenetically conserved motifs for

CAAT- and GC-boxes, and transcription factors (GATA-1, STAT6, HNF4, and BARBIE) in mouse, rat *Slc13a4* (Dawson et al., 2005a) and human *SLC13A4* (Markovich et al., 2005), suggest similar regulatory mechanisms may exist for these three genes. However, the presence of several putative transcription factor motifs (XBP1, SRF, PRE and RORA2) in mouse *Slc13a4*, but not in rat *Slc13a4* or human *SLC13A4*, could contribute to tissue specific differences in the way these genes respond to hormonal and/or environmental stimuli. This is consistent with varied mRNA expression patterns for mNaS2 (placenta, brain, lung, eye, heart, testis, thymus and liver), rNaS2 (placenta, brain and liver) and hNaS2 (placenta, brain, eye, heart, testis, thymus and liver). Functional analysis of the putative transcription factor binding motifs in mouse *Slc13a4*, will provide important insights into the transcriptional regulation of mNaS2 and its specific pattern of tissue distribution.

In conclusion, this is the first study to isolate the mNaS2 cDNA, define its mRNA tissue distribution and determine its cDNA and gene structures. This information provides the necessary tools to generate an mNaS2 null mouse, which will further our understanding of the role of NaS2 in mammalian physiology.

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