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Regulation of Mouse Brain-Selective Sulfotransferase Sult4a1 by cAMP Response Element-Binding Protein and Activating Transcription Factor-2

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ABSTRACT

Sulfotransferase 4A1 (SULT4A1) is a novel cytosolic sulfotransferase that is primarily expressed in the brain. To date, no significant enzyme activity or biological function for the protein has been identified, although it is highly conserved between species. Mutations in the SULT4A1 gene have been linked to schizophrenia susceptibility, and recently, its stability was shown to be regulated by Pin1, a peptidyl-prolyl *cis-trans* isomerase implicated in several neurodegenerative diseases. In this study, we investigated the transcriptional regulation of mouse Sult4a1. Using a series of promoter deletion constructs, we identified three cAMP-responsive elements (CREs) that were required for maximal promoter activity. The CREs are located within 100 base pairs of the major transcription start site and are also present in the same region of the human SULT4A1 promoter. Electrophoretic mobility shift assays (EMSAs) identified two specific complexes that formed on each of the CREs. One complex contained cAMP response element-binding protein (CREB), and the other contained activating transcription factor-2 (ATF-2) and c-Jun. Overexpression of CREB or ATF-2 increased not only reporter promoter activity but also endogenous Sult4a1 mRNA levels in Neuro2a cells. Moreover, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) treatment increased both reporter promoter activity and Sult4a1 levels in μ-opioid receptor expressing Neuro2a/μ-opioid receptor cells, and EMSAs showed this to be due to increased binding of CREB and ATF-2 to the Sult4a1 promoter. We also show that DAMGO treatment increases Sult4a1 mRNA and protein levels in primary mouse neurons. These results suggest that Sult4a1 is a target gene for the μ-opioid receptor signaling pathway and other pathways involving activation of CREB and ATF-2.

Introduction

The cytosolic sulfotransferase SULT4A1 was first cloned from human brain in 2000 and was shown to have low sequence homology compared with other sulfotransferases (Falany et al., 2000). Nevertheless, it is highly conserved between species and is found primarily in specific regions of the brain (Liyou et al., 2003). In humans, the SULT4A1 gene is located on chromosome 22 in a region often deleted in schizophrenia (Minchin et al., 2008). Surprisingly, mutations in the coding sequence are extremely rare, and SULT4A1 has the lowest overall mutation rate of all of the known human sulfotransferases (Hildebrandt et al., 2007; Lewis and Minchin, 2009). These attributes suggest an important, albeit unknown, role for SULT4A1 in the human brain.

To date, no substrate for the enzyme has been identified. Crystallography studies suggest that the active site for SULT4A1 is smaller than that of other sulfotransferases and that it may not be able to accommodate the cofactor 3′-phosphoadenosine-5′-phosphosulfate (Allali-Hassani et al., 2007). We have shown that SULT4A1 is a binding partner for the peptidyl-prolyl *cis-trans* isomerase Pin1 and that Pin1 modulates the stability of SULT4A1 in a calpain-dependent manner (Mitchell and Minchin, 2009). Pin1 selectively binds to phosphoserine/threonine-proline motifs in the *trans* conformation (Verdecia et al., 2000). We identified two motifs in the N terminus of SULT4A1 that are required for Pin1 binding and for the Pin1-dependent destabilization of

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ABBREVIATIONS: SULT4A1, sulfotransferase 4A1; ATF-2, activating transcription factor 2; CRE, cAMP-responsive element; CREB, cAMP response element-binding protein; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; MOR, μ-opioid receptor; EMSA, electrophoretic mobility shift assay; bp, base pair; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline/Tween 20; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RLM-RACE, 5′-RNA ligase-mediated rapid amplification of cDNA ends; Neuro2a/MOR, Neuro2a cells stably transfected with the μ-opioid receptor; FP, forward primer; RP, reverse primer; DMSO, dimethyl sulfoxide.

the enzyme. These results provided evidence that post-translational modification of SULT4A1 occurs in cells. Whether these modifications are required for catalytic activity is yet to be established.

The localized expression of SULT4A1 in the brain suggests that the gene is regulated by brain-specific transcription factors and/or repressors. In mice, Sult4a1 mRNA levels are relatively constant in male but increase with age in female mice, suggesting the gene may be influenced by the level of sex hormones (Alnouti and Klaassen, 2006). Other than this interesting observation, very little is known about how SULT4A1 expression is regulated or why the protein is found predominantly in the brain. To address this, we isolated the murine Sult4a1 promoter and used reporter expression assays to identify potential regulatory sequences using murine Neuro2a cells, which constitutively express Sult4a1. We show that Sult4a1 expression is controlled by three cAMP-responsive elements (CREs) located within the first 100 bp upstream of the transcription start site.

Materials and Methods

5′-RNA Ligase-Mediated-RACE Analysis. Human brain RNA was obtained from the Queensland Brain Institute's Brain Bank and RNA from mouse brain was extracted using TRIzol reagent (Invitrogen, Mulgrave, VIC, Australia). RNA (10 μg) was processed using a FirstChoice 5′-RNA ligase-mediated (RLM)-RACE kit (Ambion, TX) and cDNA synthesized using random decamer primers. PCR was performed on the resulting cDNA using the RACE outer primer 5′-GCTGATGGCGATGAATGAACACTG-3′ with the *SULT4A1*-specific outer primer 5′-AAGTTGGCGATCTCCTCCATCTTC-3′. A nested PCR on the resulting product was then performed using the RACE inner primer 5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′ with the *SULT4A1*-specific inner primer 5′-TACTGAATTCTGGAACTCGAAGTACTTGCTCTCG-3′. Cycling conditions consisted of an initial 95°C step for 3 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final incubation at 72°C for 7 min. The resulting PCR products were cloned into the BamHI and EcoRI sites of pBlueScript KSII (Promega, Alexandria, NSW, Australia) and then sequenced to determine transcription start sites.

Cell Lines and Tissue Culture. The mouse neuroblastoma cell line Neuro2a was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air. Neuro2a cells stably transfected with the μ-opioid receptor (Neuro2a/MOR) (Chakrabarti et al., 1995) were a generous gift from Dr. MacDonald Christie (Brain and Mind Research Institute, University of Sydney, Sydney, Australia) and were cultured as above in medium supplemented with 0.2 mg/ml G418 (Invitrogen). Mouse neurons were isolated from C57BL6 at embryo day 16 and cultured as described elsewhere (Arumugam et al., 2007).

SULT4A1 Promoter Constructs. A 2981-bp fragment of the murine Sult4a1 promoter was amplified from Neuro2a genomic DNA using forward primer FP2981, 5′-TACTGGTACCAGGATCCCCATCCTCCAACG-3′, and reverse primer RP1, 5′-TACTGAAGATCTCTCCAGGGCGTGACGTCAC-3′. This fragment was digested with KpnI and BglII and cloned into the same sites of pGL3-enhancer (Promega), yielding pGL3-2981, where the number indicates bases upstream of the first detected transcription start site (−25 relative to the start codon) (Fig. 1A). A series of deletion constructs was made by PCR using the forward primers FP839 (5′-CCTAAGGGGTACCCCAGACCCTTG-3′), FP98 (5′-TACTGGTACCTCGGCAACCGACGTCAGG-3′), FP56 (5′-TACTGGTACCCATGACGTCATGC-3′), and FP47 (5′-TACTGGTACCCATGCCCGCGGAGCC-3′) together with RP1, yielding constructs pGL3-839, pGL3-98, pGL3-56, and pGL3-47, respectively. Site-directed mutagenesis was performed using a GeneTailor kit (Invitrogen) and pGL3-98 as template. Forward and reverse primers used were the following: CRE1-FP, 5′-GATAGGTACCTCGGCAACCGTGGTCAGGGGCG-3′; CRE1-RP, 5′-CGGTTGCCGAGGTACCTATCGATAGAGAAA-3′; CRE2-FP, 5′-CGTGCGTGGGTGCGGCCATGTGGTCATGCCCG-3′; CRE2-RP, 5′-CATGGCCGCACCCACGCACGCCGCGCCCCT-3′; CRE3-FP, 5′-AGCCCCGCGCCGGCGCCGTCTGGTCACGCCCT-3′; and CRE3-RP, 5′-GACGGCGCCGGCGCGGGGCTCCGCGGGCAT-3′. All clones were verified by DNA sequencing.

DNA Transfection and Dual Luciferase Assay. Cells were seeded at a density of 2.5×10^5 cells/ml in 24-well plates and were allowed to adhere overnight. Cells were transiently cotransfected with 0.5 μg of reporter plasmid DNA and 0.2 μg of pcDNA-rLuc (*Renilla reniformis* luciferase expression plasmid) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 24 h, the cells were washed with PBS, lysed in passive lysis buffer, and luciferase activity measured using the dual luciferase assay system (Promega) as outlined in the manufacturer's instructions. *Firefly* luciferase activities were normalized to *R. reniformis* luciferase activities and then expressed relative to the promoterless pGL3-enhancer plasmid.

The plasmids pFLAG-C2/cAMP response element-binding protein (CREB) and pFLAG-C2/activating transcription factor 2 (ATF-2) were kind gifts from Dr. Gerald Thiel (University of the Saarland

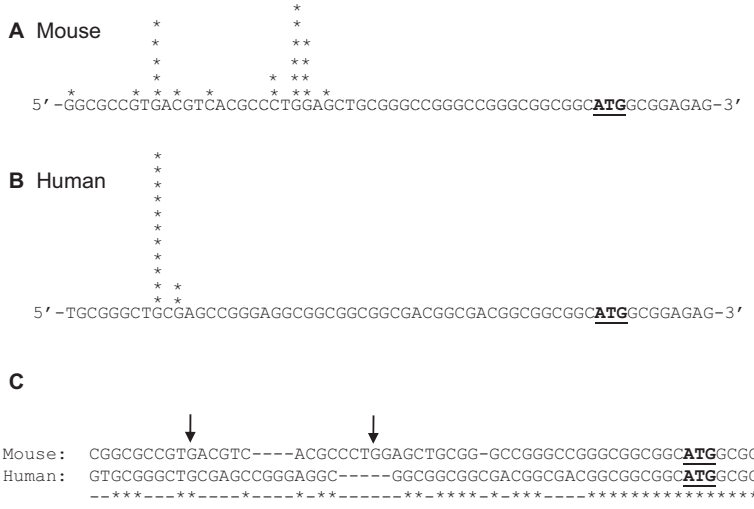

Fig. 1. Isolation of the mouse SULT4A1 promoter. A, the transcription start sites for the mouse Sult4a1 gene was determined by FirstChoice RLM-RACE. Two major sites were identified located at −28 and −41 bp upstream of the translation start site (*ATG*). B, in the human gene, only one major site was found located −41 bp upstream of the ATG. C, alignment of the human and mouse 5′-untranslated region shows a conserved sequence around the −41 bp transcription start site (left arrow) but not around the −28 bp site (right arrow).

Medical Center, Homburg, Germany) and express constitutively active forms of CREB and ATF-2, respectively (Thiel et al., 2005). Cells were transiently cotransfected as above with pGL3-98 and pcDNA-rLuc together with pFLAG-C2/CREB or pFLAG-C2/ATF-2. After transfection for 24 h, cells were lysed, and luciferase activities were measured as described above.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (Butcher et al., 2003) and incubated for 30 min at room temperature in binding buffer (2% Ficoll 400, 5% glycerol, 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, and 2.5 mM dithiothreitol) containing 0.5 μg of poly[D(I-C)] and ^{32}P-labeled oligonucleotide probe (10^5 dpm) in a final volume of 10 μl. Samples were then resolved on 5% polyacrylamide gels electrophoresed at 200 V in 0.25× Tris-borate-EDTA buffer for 45 min at 4°C. Gels were dried, placed on a screen overnight, and then analyzed with the use of filmless autoradiographic analysis (BAS-5000; Fujifilm, Tokyo, Japan; and Image Gauge version 3.0; Berthold Technologies, Bundoora, VIC, Australia).

Oligonucleotides were purified by reverse-phase chromatography and lyophilized (Geneworks, Thebarton, SA, Australia). Double-stranded oligonucleotides were prepared by heating both sense and antisense strands at 65°C for 5 min before slowly cooling to room temperature (sequences are shown in Fig. 3A). Oligonucleotides (5 pmol ends) were labeled using [γ-^{32}P]ATP (4500 Ci/mmol; MP Biomedicals, Seven Hills, NSW, Australia) and T4 polynucleotide kinase. Labeled probes were purified from unincorporated [γ-^{32}P]ATP using MicroSpin G-25 columns (GE Healthcare, Rydalmere, NSW, Australia).

For competition assays, unlabeled oligonucleotides were added to the binding reactions at a 100-fold molar excess 10 min before the addition of radiolabeled probe. Antibodies used for supershift analysis (CREB, ATF-2, and c-Jun) were from Cell Signaling Technology (Danvers, MA) and were added to binding reactions 10 min before the addition of probe.

Chromatin Immunoprecipitation. Cells were harvested, washed with PBS, and then treated with 1.5% formaldehyde in PBS for 15 min to cross-link chromatin. Cells were collected by brief centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 g/ml pepstatin A) and sonicated three times for 15-s bursts on ice. After centrifugation, the supernatants were transferred to new tubes and diluted 1:10 with dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). A 50-μl aliquot of each supernatant was removed and kept for positive PCR controls, and the remainder was divided into four lots and immunoprecipitated with either anti-CREB(1:50), anti-ATF-2 (1:25), anti-c-Jun (1:50), or rabbit IgG (negative control) and Protein A-Sepharose beads (Sigma, Castle Hill, NSW, Australia) overnight at 4°C. After extensive washing, immunoprecipitated DNA-protein complexes were eluted by a 15-min incubation in elution buffer (0.1 M $NaHCO_3$ and 1% SDS) and then heated at 65°C overnight to reverse cross-linking. DNA was purified using MinElute PCR purification spin columns (QIAGEN, Doncaster, VIC, Australia). PCR was performed using purified DNA as template with primers flanking the SULT4A1 promoter region (sense, 5′-ACAGGCAGTGATACGGACCCGACG-3′; antisense, 5′-TCTCCGCTTCGCTCTCCGCCATGC-3′) or GAPDH as a negative control (sense, 5′-AGTGCCAGCCTCGTCCCGTAGACAAAATG-3′; antisense, 5′-AAGTGGGCCCCGGCCTTCTCCAT-3′).

Quantitative Real-Time PCR. Total RNA was extracted from Neuro2a cells using TRIzol reagent (Invitrogen) and reverse-transcribed using oligo(dT)$_{15}$ primer (Promega) and Superscript II reverse transcriptase (Invitrogen).

Expression levels of SULT4A1 mRNA were determined using the iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Gladesville, NSW, Australia). First-strand cDNA was amplified using specific primers for mouse *SULT4A1* that spanned exons 5 to 7 (sense, 5′-CTACGGCTCCTGGTTTGAG-3′; antisense, 5′-ATGGAGACGGTGAAGATGTC-3′) and mouse β-actin that spanned exons 3 to 5 (sense, 5′-CCTAAGGCCAACCGTGAAAAG-3′; antisense, 5′-TCTTCATGGTGCTAGGAGCCA-3′). Reactions contained SensiMix Plus SYBR and fluorescein (Bioline, Alexandria, NSW, Australia) and 6 pmol of each primer in a total volume of 20 μl. Samples were amplified using the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 58°C for 15 s, and 72°C for 45 s. Melt curve analysis confirmed the specificity of the PCR, whereas agarose gel electrophoresis confirmed correct size of the PCR product. Relative expression levels of *Sult4a1* mRNA were determined by the comparative C_T method. C_T values for *Sult4a1* expression were normalized to C_T values for β-actin and then expressed relative to control.

Western Blot Analysis. Whole-cell extracts were boiled in Laemmeli buffer, electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (350 mA, 1 h), and immunoblotted with antibodies directed against SULT4A1 (Protein Tech Group, Chicago, IL) and α-tubulin (Merck, Kilsyth, VIC, Australia). In brief, membranes were blocked for 1 h with 5% skim milk powder in PBS, washed with 0.05% Tween 20 in PBS (PBST), and then incubated overnight at 4°C with primary antibody diluted 1:1000 in PBST. Membranes were washed with PBST and then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare) diluted 1:5000 in PBST. After a final wash with PBST, membranes were developed using Immun-Star enhanced chemiluminescence reagent (Bio-Rad Laboratories) and a ChemiDoc XRS system using Quantity One software version 4.5.0 (Bio-Rad Laboratories).

Data Analysis and Statistics. Data are expressed as mean ± S.E.M. Statistical comparisons between different treatments were assessed by Student's *t* tests assuming significance at a *P* value of 0.05 or less.

Results

Isolation of the Minimum Promoter Sequence for Murine Sult4a1. To isolate the minimum promoter of the Sult4a1 gene, we first identified the most common transcription start sites using murine brain RNA and 5′-RLM-RACE. For comparison, we also performed 5′-RLM-RACE on RNA isolated from human brain. Twenty-two independent clones were sequenced showing that transcription was initiated within the region −50 to −25 nucleotides relative to the start codon (Fig. 1A). Transcription initiation was most frequently observed at guanines located at nucleotides −41 (29%) and −28 (24%). For human *SULT4A1*, transcription of 11 (85%) from 13 clones was initiated from a single guanine located at nucleotide −41 relative to the start codon (Fig. 1B). Sequence alignment shows that the bases at −41 are similar between human and mouse, whereas the more proximal transcription start site in the mouse gene is not present in human the gene (Fig. 1C).

A 2981-bp sequence upstream of the first transcription start site was cloned into the luciferase reporter plasmid pGL3-enhancer. Deletion mutants were then generated, and promoter activity was determined in Neuro2a cells (Fig. 2A). Full activity was observed until the last 98 bp, suggesting that critical transcription factor elements were present between −25 and −98 bp upstream of the coding sequence. Analysis of this region using MatInspector (Quandt et al., 1995) identified three CREs located at −80, −45, and −10 bp relative to the first transcription start site (Fig. 2B), although the most distant motif was not consensus. Further deletions were then constructed to progressively remove each CRE

site, and promoter activity was determined. The results showed that each motif was required for optimal activity (Fig. 2C). In addition, site-directed mutagenesis studies showed that the CRE sites were essential for Sult4A1 expression, because mutation of all three CREs resulted in the abolition of promoter activity (Fig. 2D).

CREB and ATF-2 Bind to CREs in the Sult4a1 Promoter. CRE sites bind several families of transcription factors, including CREB, ATFs, and CRE modulators. Each has a bZip DNA binding domain in its C terminus and can form homodimers and heterodimers with other transcription factors (Foulkes and Sassone-Corsi, 1996). The two principal CRE-associated transcription factors are CREB and ATF-2. To identify possible transcription factors that bound to each of the CRE sites in the Sult4a1 promoter, oligonucleotides for each were labeled, incubated with Neuro2a nuclear extracts, and used in electrophoretic mobility shift assays (EMSAs). The sequence of each probe is shown in Fig. 3A. All three CRE sites formed two complexes (C1 and C2) that were abrogated by the addition of 100-fold excess of unlabeled probe but not by 100-fold excess of unlabeled mutant probe where two of the core nucleotides were altered (Fig. 3B). These results show that C1 and C2 are specific complexes involving protein binding to the CRE site.

To identify potential transcription factors in each complex, nuclear extracts from cells transiently transfected with CREB or ATF-2 constructs were used in an EMSA with the CRE2 and CRE3 probes (Fig. 3C). CREB increased the intensity of C2 for both probes (Fig. 3C, lane 3), whereas ATF-2 increased the intensity of the C1 complex (Fig. 3C, lane 4). Furthermore, supershifts with anti-CREB and anti-ATF-2 antibodies attenuated the intensity of C2 and C1, respectively. Taken together, these results indicate that CREB was a component of the C2 complex, whereas ATF-2 was a component of the C1 complex. Because ATF-2 has been shown to form stable heterodimers with c-Jun (van Dam and Castellazzi, 2001), we also performed a supershift with anti-c-Jun antibody, which caused a loss of the C1 complex (Fig. 3C, lane 7), suggesting that it contained both ATF-2 and c-Jun, possibly as heterodimers. To confirm that CREB, ATF-2, and

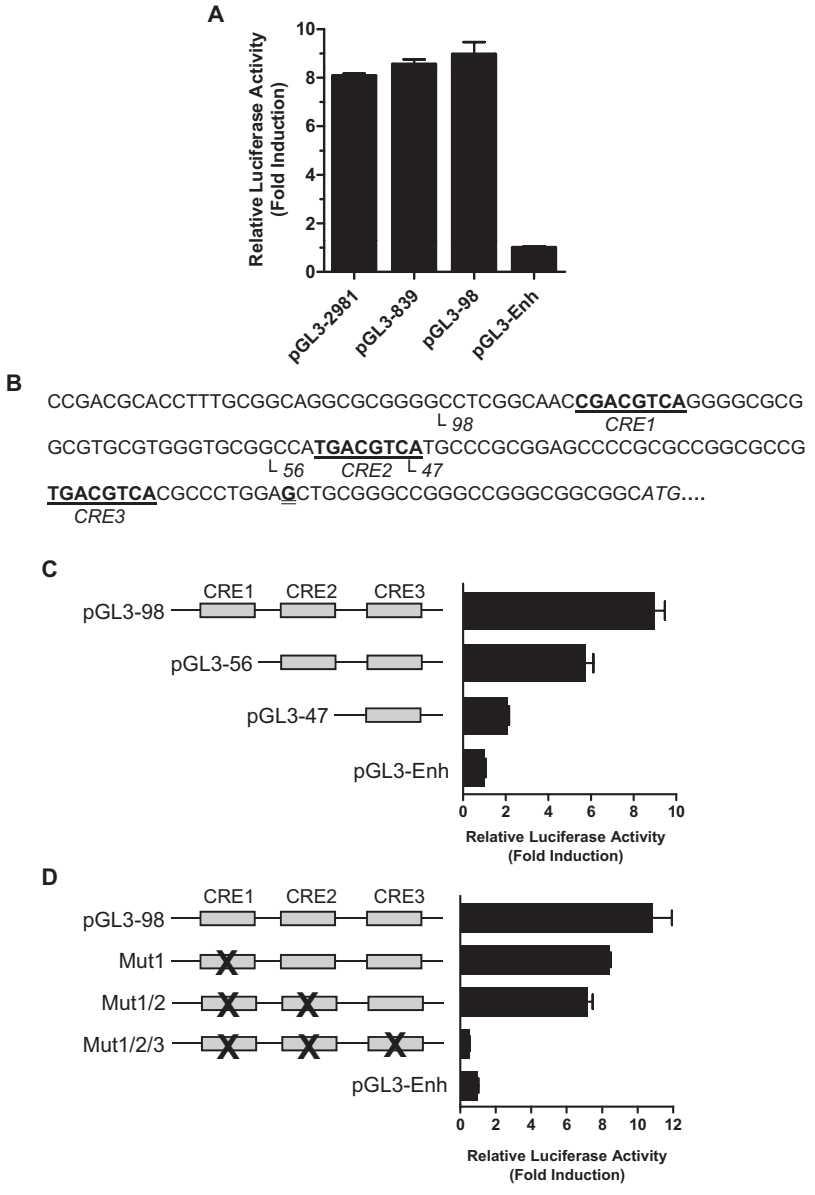

Fig. 2. Identification of the minimum promoter sequence for the mouse Sult4a1 gene. A, a 2981-bp sequence was initially cloned into the reporter vector pGL3-enhancer and gene expression was determined in Neuro2a cells using a dual luciferase assay. Deletion mutagenesis showed that full promoter activity required the first 98 bp of the promoter sequence. B, promoter sequence of the mouse Sult4a1 gene contains three putative CRE sites, labeled as CRE1, CRE2, and CRE3. C, the minimum promoter sequence isolated in A was further deleted to progressively exclude each CRE. The resulting luciferase activities showed that all three sites were required for optimal expression. D, progressive site-directed mutagenesis of each CRE showed that they are essential for Sult4a1 promoter activity.

c-Jun were recruited to the Sult4A1 promoter in vivo, chromatin immunoprecipitation (ChIP) analysis was performed using primary mouse neurons. These data showed that all three transcription factors bound to the region of the Sult4A1 promoter containing the CREs (Fig. 3D).

Overexpression of CREB and ATF-2 Increases Sult4a1 Promoter Activity and mRNA Levels. Neuro2a cells were transfected with CREB or ATF-2 along with the Sult4a1 promoter reporter plasmid pGL3-98. After 24 h, luciferase activity was determined. CREB induced a 4.6-fold increase in SULT4A1 promoter activity, whereas ATF-2 induced a 4.2-fold increase (Fig. 4A). To determine whether either or both transcription factors increased endogenous Sult4a1 expression in Neuro2a cells, mRNA was prepared for real-time PCR. CREB increased Sult4a1 mRNA 2.1-fold, whereas ATF-2 increased it 2.4-fold (Fig. 4B). These results show that both transcription factors are involved in Sult4a1 expression.

μ-Opioid Receptor Activation Increases Sult4a1 Expression. μ-Opioid receptors signal through the extracellular signal-regulated kinases to activate CREB in μ-opioid receptor-positive cells (Ligeza et al., 2008). To investigate whether physiological activation of CREB affected Sult4a1 expression, Neuro2a cells stably expressing the μ-opioid receptor were treated with the enkephalin analog DAMGO. Initially, the effect of DAMGO on CREB and ATF-2 DNA-binding ability was determined by EMSA (Fig. 5A). Both CREB (complex C2) and ATF-2 binding (complex C1) increased after DAMGO treatment for 30 min. However, by 24 h, the binding of transcription factors returned to basal levels or lower. When the Sult4a1 promoter reporter pGL3-98 was added to the cells, DAMGO increased luciferase activity by almost 2-fold (Fig. 5B). Finally, DAMGO also increased the expression of endogenous Sult4a1 after 24 h, as determined by real-time PCR (Fig. 5C). However, there was no evidence of increased Sult4a1 protein by Western blot (data not shown). The reason for this is discussed below. These experiments show that the μ-opioid receptor agonist DAMGO can increase Sult4a1 expression.

Finally, we used primary cultures from fetal mouse brains to investigate whether DAMGO could affect Sult4a1 expression in a normal nontransfected cell system. DAMGO increased Sult4a1 mRNA by almost 70% (Fig. 6A). In these cells, there was a significant increase in Sult4a1 protein as determined by Western blot analysis (Fig. 6B).

The lack of protein expression in the Neuro2a cells led us to investigate the Sult4a1 transcript produced in this cell type. SULT4A1 mRNA can exist as two different transcripts depending on the splicing of an aberrant exon between exons 6 and 7 (Minchin et al., 2008). In Neuro2a cells, the variant transcript was the major transcript detected, whereas in the mouse primary neurons, only the wild-type transcript was detected (Fig. 6C). This may explain why no Sult4a1 protein was detectable in the Neuro2a cells. The splice variant introduces a stop codon 85 bp upstream of the exon 7 junction,

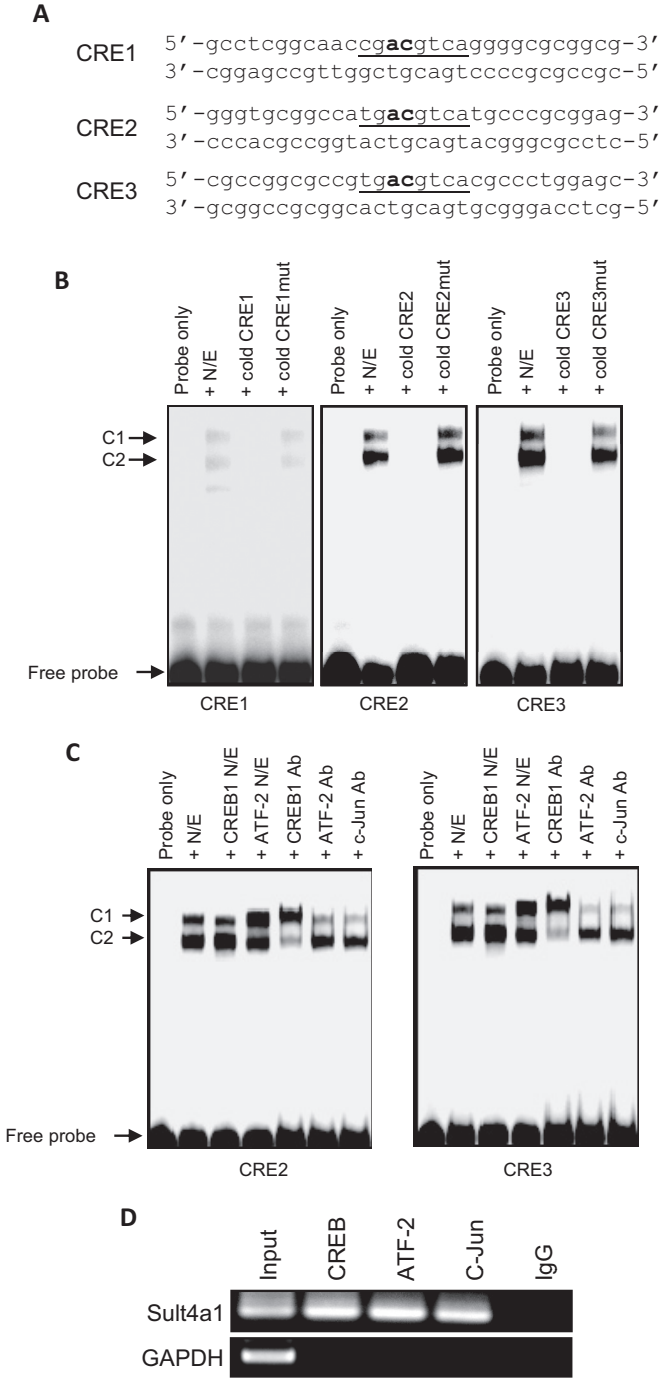

Fig. 3. Electrophoretic mobility shift assays of the CRE sites. A, the sequences used for the assays are shown with the respective CRE underlined. Mutant probes were generated by replacing the central AC (bold) with TG, which prevents binding of CREB/ATF complexes. B, each probe (left , CRE1; middle, CRE2; right, CRE3) was radiolabeled and incubated with nuclear extract (N/E). Complexes bound to the probes were separated by gel electrophoresis. Two major complexes (C1 and C2) were associated with each probe, although binding was much less for CRE1. Specificity was determined by the addition of 100-fold excess unlabeled probe (cold), which inhibited the formation of both complexes. By contrast, cold mutant probe did not affect binding. C, supershift assays were used to identify potential transcriptional factors associated with CRE2 (left) and CRE3 (right). Nuclear extracts from Neuro2a cells transiently transfected with CREB or ATF-2 expression vectors showed an increase in complex C2 and C1, respectively. The addition of anti-CREB antibody shifted the bottom band (C2), whereas anti-ATF-2 antibody shifted the top band (C1). Anti-c-Jun antibodies also shifted C1, suggesting that both ATF-2 and c-Jun were present in the C1 complex. D, ChIP assay shows that CREB, ATF-2, and c-Jun bind to the Sult4a1 promoter constitutively. ChIP was performed on Neuro2a cells using specific antibodies against CREB, ATF-2, and c-Jun. Rabbit IgG was used as a control for nonspecific binding. PCRs were performed on extracts before (input) or after immunoprecipitation using primers flanking the CREs in the Sult4a1 promoter or a region of the GAPDH promoter as a negative control.

which would produce a truncated protein (Minchin et al., 2008). The truncated protein may lack the antibody binding site(s), or the resultant protein may be rapidly degraded.

Discussion

SULT4A1 is primarily expressed in specific regions of the brain and, although its exact biological function is unknown, it is highly conserved among those mammalian species in which it has been studied to date. It shares low homology with other human cytosolic sulfotransferases but has retained the major secondary structural features of the sulfotransferase family of enzymes such as substrate and cofactor binding domains and dimerization domain (Minchin et al., 2008). Crystallography studies confirm that SULT4A1 has

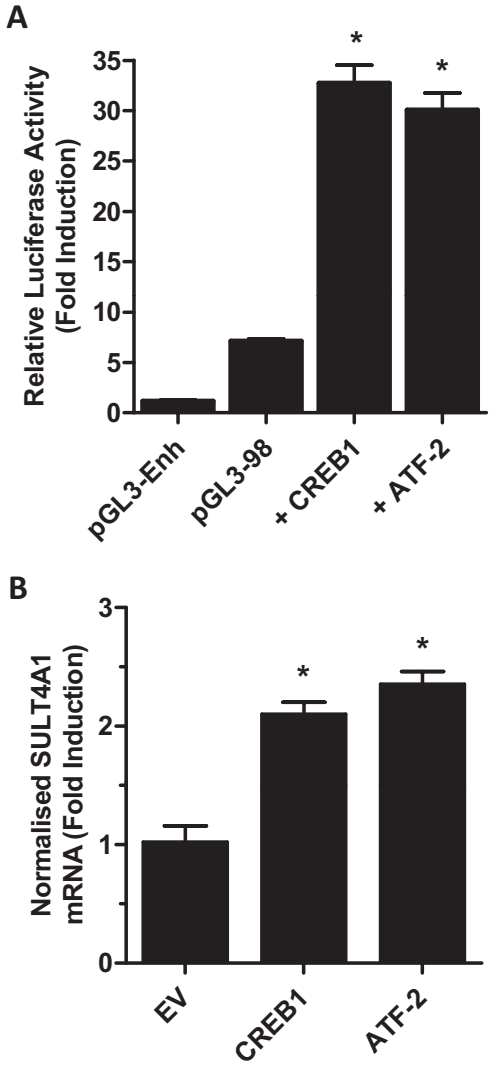

Fig. 4. The effect of overexpression of CREB and ATF-2 on Sult4a1 promoter activity. A, Neuro2a cells were transfected with pGL3-enhancer vector (pGL3-Enh), minimum promoter vector (pGL3-98), or the minimum promoter vector and CREB (+CREB) or ATF-2 (+ATF-2), and luciferase activity was determined after 24 h. Results are the mean ± S.E.M., $n = 3$. Asterisk indicates significant difference compared with pGL3-98 alone. B, expression of endogenous Sult4a1 was determined in Neuro2a cells after transfection with empty vector (EV), CREB vector, or ATF-2 vector. Sult4a1 mRNA was quantified by real-time PCR and normalized to β-actin. Results are the mean ± S.E.M., $n = 6$. Asterisk indicates significant difference compared with empty vector.

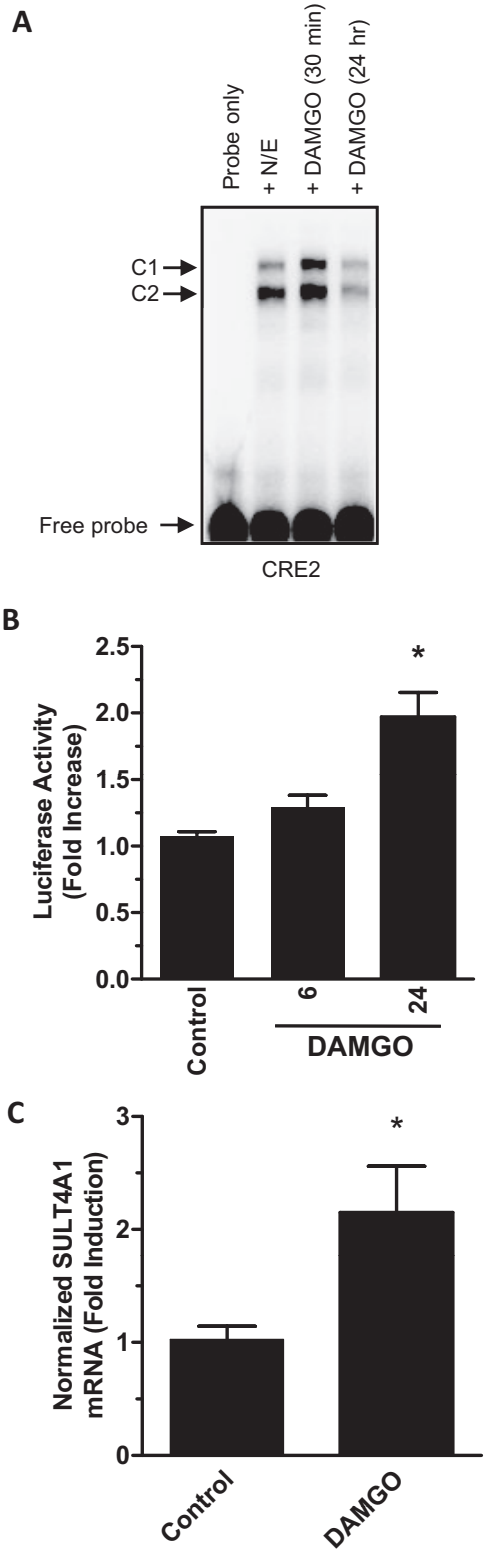

Fig. 5. Effect of DAMGO on Sult4a1 expression. A, Neuro2a cells stably transfected with the μ-opioid receptor (Neuro2a/MOR) were treated with 10 μM DAMGO for 30 min and 24 h. At the earlier time point, both C1 (ATF-2/c-Jun) and C2 (CREB) complexes were increased. However, by 24 h, only weak binding was seen for both complexes. B, DAMGO increased promoter activity in Neuro2a/MOR cells transfected with the pGL3-98 vector. Luciferase activity was determined 24 h after transfection. C, DAMGO increased Sult4a1 mRNA after 24 h treatment of Neuro2a/MOR cells. Results are the mean ± S.E.M., $n = 6$. Asterisk indicates significant difference compared with vehicle (0.1% DMSO)-treated cells (Control).

the same tertiary structure as the cytosolic sulfotransferases, but its active site is smaller (Allali-Hassani et al., 2007). The protein can bind substrates such as noradrenaline, isoprenaline, apomorphine, and resveratrol, although there is no evidence that it has catalytic activity toward any of these compounds.

This is the first study to investigate the transcriptional regulation of SULT4A1. The mouse and human SULT4A1 protein coding sequences share a greater than 98% nucleotide homology, but their 5′-untranslated regions show very little similarity. However, like the mouse, the human SULT4A1 promoter contains three consensus CRE sites positioned in a similar location relative to the major transcription start site and having the same spatial arrangement. This suggests that the CRE sites are also important in the regulation of human SULT4A1 expression.

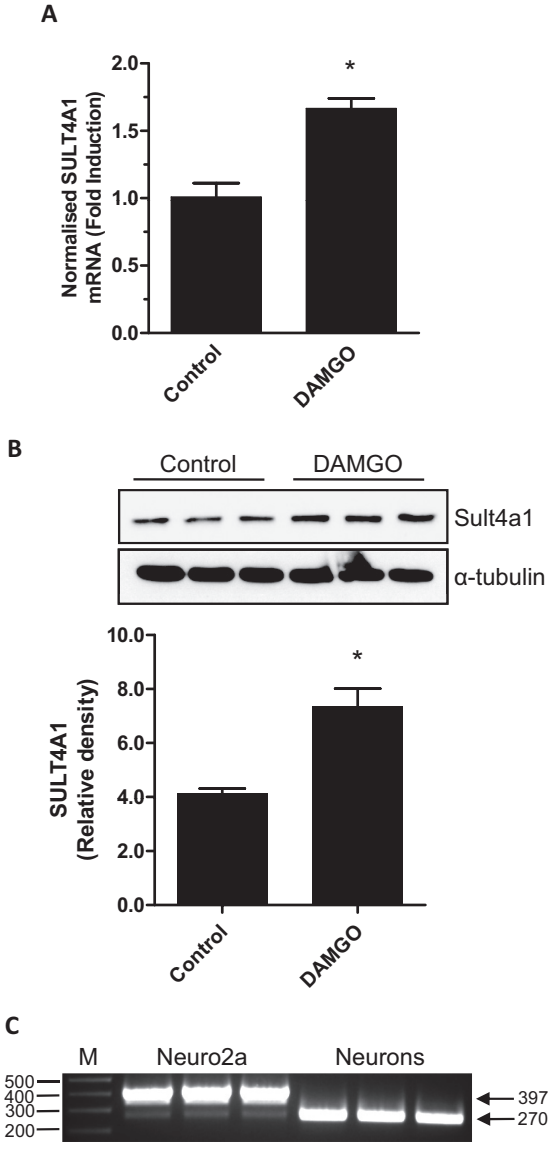

Fig. 6. Effect of DAMGO on SULT4A1 expression in mouse neuron primary cultures. Brain cells from embryonic day 15 to 16 embryonic mice were isolated as described elsewhere (Arumugam et al., 2007) and cultured under conditions to enrich neurons. A, after 7 to 9 days, the neurons were treated with 10 μM DAMGO for 24 h, and Sult4a1 mRNA was quantified by real-time PCR. Results are the mean ± S.E.M., $n = 6$. Asterisk indicates significant difference compared with vehicle (0.1% DMSO)-treated cells (Control). B, Sult4a1 protein was determined by Western blot in neurons treated with DAMGO for 24 h. Sult4a1 protein was quantified by densitometry and normalized to α-tubulin. Results are the mean ± S.E.M., $n = 3$. Asterisk indicates significant difference compared with vehicle (0.1% DMSO)-treated cells (Control). C, PCR of cDNA from Neuro2a cells and primary mouse neurons using primers located in exons 6 and 7 of the Sult4a1 gene. Primary mouse neurons contain only the wild-type transcript (270 bp), whereas the major transcript in Neuro2a cells is the splice variant (397 bp).

We identified two complexes by EMSA that bound to each of the CRE sites in the Sult4a1 promoter. The first complex was completely shifted by CREB antibodies, suggesting that it contained CREB homodimers or possibly CREB/ATF-1 heterodimers, because CREB can form heterodimers with ATF-1 but not any of the other ATF family members (van Dam and Castellazzi, 2001). The second complex was completely shifted by antibodies to either ATF-2 or c-Jun, suggesting that this complex contained ATF-2/c-Jun heterodimers. The preferred ATF-2 dimerization partner is c-Jun (Benbrook and Jones, 1990; Hai and Curran, 1991). Although CREB and ATF-2 are ubiquitously expressed, both are preferentially expressed in the brain (Kara et al., 1990; Carlezon et al., 2005). CREB is expressed in all cells of the brain, and CREB-mediated transcription plays a major role in adaptive neuronal responses, such as learning and memory, as well as outgrowth of neuronal processes, neurogenesis, mechanisms of psychotropic drugs, and the pathophysiology of neuropsychiatric and neurodegenerative disorders (Mayr and Montminy, 2001; Lonze and Ginty, 2002; West et al., 2002; Carlezon et al., 2005; McClung and Nestler, 2008; Pittenger and Duman, 2008). The role of ATF-2 in the brain is much less studied, but it has been demonstrated to be important in central nervous system development (Reimold et al., 1996), and more recently in the nucleus accumbens to regulate responses to emotional stimuli (Green et al., 2008).

CREB and ATF-2 both require phosphorylation before they become transcriptional activators. In the brain, neurotransmitters and neurotrophins act at membrane receptors, such as neurotrophin receptors, *N*-methyl-D-aspartate receptors, and G-protein-coupled receptors, to trigger intracellular signaling cascades that culminate in phosphorylation of CREB. Depending on the signaling pathway involved, phosphorylation of CREB at Ser^{133} can be by cAMP-dependent protein kinase A, Ca^{2+}/calmodulin-dependent kinase IV, or mitogen-activated protein kinase-activated ribosomal S6 kinases (Carlezon et al., 2005). ATF-2 is phosphorylated at Thr^{69} and Thr^{71} by stress-activated kinases such as c-Jun N-terminal protein kinase and p38 (Gupta et al., 1995; van Dam et al., 1995; Ouwens et al., 2002).

Activation of CREB and CRE-dependent gene transcription in neuronal cells via the μ-opioid receptor pathway has been extensively studied, and rather than cAMP-dependent protein kinase A, which is the key kinase that phosphorylates CREB at Ser^{133}, opioid-stimulated CREB phosphorylation depends on protein kinase C (Bilecki et al., 2004). Using the well characterized Neuro2a/MOR cell line, we show that SULT4A1 mRNA expression can be induced by treatment with DAMGO, a μ-opioid receptor agonist. Furthermore, primary mouse neurons treated with DAMGO showed not only an increase in Sult4a1 mRNA but also an increase in Sult4a1 protein levels.

SULT4A1 mRNA can exist as two different transcripts depending on the splicing of an aberrant exon between exons 6 and 7 (Minchin et al., 2008). It is noteworthy that mouse

primary neurons express only the wild-type Sult4a1 mRNA, whereas Neuro2a cells express primarily the variant transcript. The splice variant introduces a stop codon 85 bp upstream of the exon 7 junction, which would produce a truncated protein that lacks the dimerization motif (Minchin et al., 2008). The function of the splice variant and whether or not expression of the two different transcripts is altered under different conditions remains to be determined.

In conclusion, we have shown for the first time that Sult4a1 expression is regulated by CREB and ATF-2. We have identified three CRE sites within the first 100 bp of the promoter that are essential for optimal Sult4a1 expression. Furthermore, activation of CREB and ATF-2 via the μ-opioid receptor signaling pathway increases Sult4a1 expression. Further studies of other cAMP-related signaling pathways that activate CREB and/or ATF-2 may provide insight into the biological function of Sult4a1 in the brain.

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