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Genomic Organization and Expression of the Mouse Equilibrative, Nitrobenzylthioinosine-Sensitive Nucleoside Transporter 1 (ENT1) Gene

Doo-Sup Choi,*,†,[1] Masahisa Handa,* Hannah Young,* Adrienne S. Gordon,†,‡,§ Ivan Diamond,†,‡,§ and Robert O. Messing†,§

**Ernest Gallo Clinic and Research Center and †Department of Neurology, ‡Department of Cellular and Molecular Pharmacology, §Graduate Program in Neuroscience and Wheeler Center for the Neurobiology of Addiction, University of California, San Francisco, 5858 Horton Street, Suite 200, Emeryville, California 94608*

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We have cloned and characterized the genomic structure of the mouse gene for the NBMPR-sensitive equilibrative nucleoside transporter (mENT1), which is located on chromosome 17C. About 12-kb of genomic DNA was sequenced including the promoter region, 12 exons, 11 introns, and the 3′-untranslated region. All exon-intron junction sequences conform to the GT/AG rule. Primer extension analysis demonstrated a transcription initiation site located 252 bp upstream of the translation start site. Analysis of the 2.5-kb 5′-flanking sequence shows putative binding sites for several transcription factors, including GATA-1, IRF-2, Pit-1, myogenin, CREB, Sp-1, Ap-2, MAZ, and GR. We demonstrated that mouse ENT1 mRNA was highly expressed in heart, spleen, lung, liver, and testis. Lower levels of expression were detected in brain and kidney. Functional analysis of the 5′-flanking region showed that the nucleotide sequence from −652 to −111 contains *cis*-regulatory elements that promote gene expression. We found two Sp-1 binding sites (−296/−303, −307/−313) and two MAZ binding sites (−353/−359, −522/−528) in this region. Luciferase assay results suggest that MAZ and Sp-1 transcription factors are important positive regulators of transcription for the ENT1 gene in NG108-15 cells.

Key Words: **nucleoside transporter; gene structure; mRNA expression; chromosomal localization; Northern blot; luciferase assay; MAZ.**

Nucleoside transport plays an important role in salvaging extracellular nucleosides for intracellular nucleotide synthesis and for regulating endogenous nucleoside levels. Two main plasma membrane transporter families have been characterized. The sodium-independent equilibrative transporters (ENT) mediate nucleoside transport bidirectionally depending on concentration gradient across the plasma membrane, whereas sodium-dependent concentrative transporters (CNT) mediate inwardly directed transport driven by the sodium electrochemical gradient (1). Recently, two ENT subtypes have been cloned and characterized. They are designated ENT1 and ENT2 and both are expressed in several tissues. ENT1 is sensitive to nanomolar concentrations of NBMPR,[2] whereas ENT2 is resistant to NBMPR up to 1 μM (2–5). ENT1 and ENT2 are about 50% homologous in amino acid sequence and contain 11 putative transmembrane (TM) domains.

A major function of nucleoside transporters is to control intracellular and extracellular concentrations of adenosine (6). This may be important in coronary vasodilation, renal vasoconstriction, platelet aggregation, lipolysis, and neurotransmission. Adenosine interacts with four subtypes of adenosine receptors termed A1, A2a, A2b, and A3 (7). Adenosine acting at these receptors can alter cAMP formation, phosphatidylinositol turnover, and calcium mobilization. In the central nervous system, adenosine modulates release of norepinephrine, dopamine, serotonin, acetylcholine, GABA, and glutamate (8). Thus, it is postulated that adenosine plays a role in several neuropsychiatric dis-

The nucleotide sequences for the mouse ENT1 genomic DNA have been deposited in the GenBank database under the GenBank Accession No. AF218255.

[1] To whom correspondence should be addressed at Ernest Gallo Clinic and Research Center, 5858 Horton Street, Suite 200, Emeryville, CA 94608. Fax: 510-985-3101. E-mail: choids@itsa.ucsf.edu.

[2] Abbreviations used: NBMPR, nitrobenzylmercaptopurine riboside (= nitrobenzylthioinosine); ENT, equilibrative nucleoside transporter; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); DAPI, diaminophenylindole; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; BAC, bacterial artificial chromosome; dpc, days postcoitum.

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orders influenced by these neurotransmitters including sleep disturbances, anxiety, epilepsy, pain, depression, and addiction (8). Previously, our group (9) demonstrated that acute ethanol treatment inhibits ENT1-mediated adenosine influx, resulting in accumulation of extracellular adenosine in NG108-15 cells. This causes an initial increase in intracellular cAMP. After chronic exposure, there is a subsequent heterologous desensitization of cAMP signal transduction, in part through a decrease in amount of Gαs (10). These ethanol-dependent events do not occur if cells are pretreated with an adenosine receptor antagonist or if ENT1 is not present (11). Thus ENT1 is important for regulation of cAMP signaling by acute and chronic alcohol exposure in these cells.

Here, we present the first genomic cloning of a mouse nucleoside transporter. We sequenced about 12 kb of genomic DNA that encompass the coding region and 5′- and 3′-untranslated regions of ENT1. We identified the chromosomal localization of mouse ENT1, and analyzed mRNA expression patterns using quantitative RT-PCR and Northern blot analysis. In addition, we found that the transcription factors MAZ and Sp-1 appear to regulate expression of ENT1 in mouse–rat neuroblastoma × glioma (NG108-15) cells.

EXPERIMENTAL PROCEDURES

Cloning of the mouse ENT1 genomic DNA. The mouse ENT1 gene was cloned by screening a BAC (bacterial artificial chromosome) library (Genome Systems Inc., St. Louis, MO). A radiolabeled probe containing sequences within mouse ENT1 cDNA was generated by polymerase chain reaction (PCR) and used to probe a mouse 129-SvJ1 embryonic stem cell-derived BAC library. The probe was generated by PCR using the following oligonucleotides, 5′-ATGACAACCAGTCACCAGCCTC-3′ and 5′-TGCTTTCGCATGATTGATCAG-3′. Two positive clones containing 40–70 kb of genomic DNA were obtained. These were subjected to restriction enzyme mapping, Southern blot analysis, and subcloning into pBluescript II SK(+) (Stratagene, La Jolla, CA).

DNA sequencing. The subcloned DNAs were sequenced by the dideoxy chain termination method using a CEQ 2000 DNA analysis system (Beckman Coulter, Fullerton, CA). The DNA sequences were analyzed using MacVector 6.5.3 (Oxford Molecular, Madison, WI).

Chromosome localization of mouse ENT1. Fluorescence *in situ* hybridization was performed by Genome Systems (Genome Systems Inc., St. Louis, MO). DNA from a positive BAC clone containing the ENT1 sequence was labeled with digoxigenin dUTP by nick translation. The labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts in a solution containing 50% formamide, 10% dextran sulfate and 2× SSC. Specific hybridization signals were detected by incubating the hybridized samples on slides with fluoresceinated antidigoxigenin antibodies, followed by counterstaining with DAPI.

Primer extension analysis. Total RNA from NG108-15 cells was isolated using RNeasy (Qiagen, Valencia, CA). A 35-mer primer was made corresponding to a sequence that is about 80 bp downstream from the predicted transcription initiation site (http://www.fruitfly.org/seq_tools/promoter.html). The primer sequence was 5′-GAGCTGGACACAGAGTACGCAATGGAAGCCTCTGC-3′. The primer was end-labeled with]γ-^{32}P] ATP using T4 polynucleotide kinase for 30 min at 37°C. Labeled primers were ethanol-precipitated three times and resuspended in 0.3M sodium acetate. Primer (5×10^4 cpm) was annealed overnight to 45 μg of total RNA from NG108-15 cells and 45 μg of yeast tRNA as a control. cDNA was generated by incubating RNA with 50 units of monkey murine leukemia virus reverse transcriptase (NEB, Beverly, MA) at 42°C for 90 min. The RNA template was digested with RNase A, and the resulting products were precipitated. The protected fragments were analyzed by electrophoresis on a 6% acrylamide gel containing 7 M urea (Novex, San Diego, CA) at 1000 V. The DNA sequencing ladder was prepared using the same primer to size the protected fragment.

Quantitative PCR analysis and Northern blot analysis. To examine the mRNA expression pattern in mouse tissues, we used PCR on normalized first-strand cDNA from Clontech (12). We prepared PCR primers and performed two-step PCR according to the manufacturer's recommendations. The PCR primers were DS-200: 5′-TGGCAATCCTGCTGGTATTCCTTGTCAC-3′, and DS-201: 5′-GTTTCTGTTGGTGGGTGGAGAGTTGGG-3′. The size of the expected PCR product was 488 bp. Primers for PCR of G3PDH cDNA were used as a control.

Tissue expression of mouse ENT1 mRNA was examined using preblotted membrane (Clontech, Palo Alto, CA). Each lane contained approximately 2 μg of purified poly(A)$^+$ RNA that was adjusted to a consistent β-actin signal. The RNA was run on denaturing formaldehyde/1% agarose gel and blotted onto a positively charged nylon membrane. A ^{32}P-labeled *Xho*1–*Pst*1 fragment corresponding to nucleotides 1–711 of mouse ENT1 cDNA (GenBank Accession No. AF257188) was used as a probe. The probe did not contain any significant sequence homology with known sequences by BLAST analysis. The hybridization and washing conditions as well as the deprobing conditions were as described in the manufacturer's manuals.

Cell culture and transient transfection assays. NG108-15 cells were cultured in defined medium comprised of Dulbecco's modified Eagle medium/Ham's F-12 medium (3:1); 2 mM glutamine; 0.1 mM hypoxanthine; 1.0 μM aminopterin; 12 mM thymidine; 25 mM Hepes, pH 7.4; trace elements (0.5 nM $MnCl_2$, 0.5 nM $[NH_4]_cMo_7O_{24}$, 0.25 nM $SnCl_4$, 25 nM Na_3VO_4, 5 nM $CdSO_4$, 0.25 nM $NiSO_4$, 15 nM H_2SeO_3, 25 nM Na_2SiO_3); bovine insulin (5 μg/ml); human transferrin (50 μg/ml); and oleic acid (10 μg/ml) complexed with fatty acid-free bovine serum albumin (2 mg/ml). Posttransfection cells were cultured in growth medium containing Dulbecco's modified Eagle medium with 10% Serum Plus Medium Supplement (JRH Biosciences), 0.1 mM hypoxanthine, 1.0 μM aminopterin, 12 mM thymidine, and 2 mM glutamine. SH-SY5Y human neuroblastoma cells were cultured before and after transfection in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Both cells lines were maintained at 37°C in 10% CO_2.

Cells were plated for transient transfection on 60-mm dishes at a density of $5–8 \times 10^5$ cells/dish. Cells were transfected once they became 70–90% confluent (24–48 h after plating). Superfect (Qiagen) was used to transfect each dish with 5 μg of plasmid DNA (see below) according to the manufacturer's protocol. Cells were returned to their respective medium for 24 h after which time they were prepared for luciferase assay.

Reporter gene construction and luciferase assays. Reporter plasmids were constructed using the promoterless luciferase reporter vector, pGL3 basic (Promega, Madison, WI). Five different serial deletion fragments were generated using the *Pfu* DNA polymerase chain reaction (Stratagene, La Jolla, CA). Each fragment was created with *Spe*1 (5′-end) and *Xho*1 (3′-end) restriction enzyme sites and was subcloned into the *Nhe*1 and *Xho*1 site in the pGL3 basic vector. The PCR primers used to isolate the fragments were, 5′-GACTAGTCACCCCCA-TTCACCAGCTCTGC-3′ (pLuc-2121, 2402 bp fragments from −2121 to +281; the transcription initiation site was numbered as +1), 5′-GACTAGTCCTAGATAAATAGCAGTGCTAC-3′ (pLuc-1479, 1761 bp), 5′-GACTAGTCCACTCTGGACCCTCACCTAGGACC-3′ (pLuc-994,

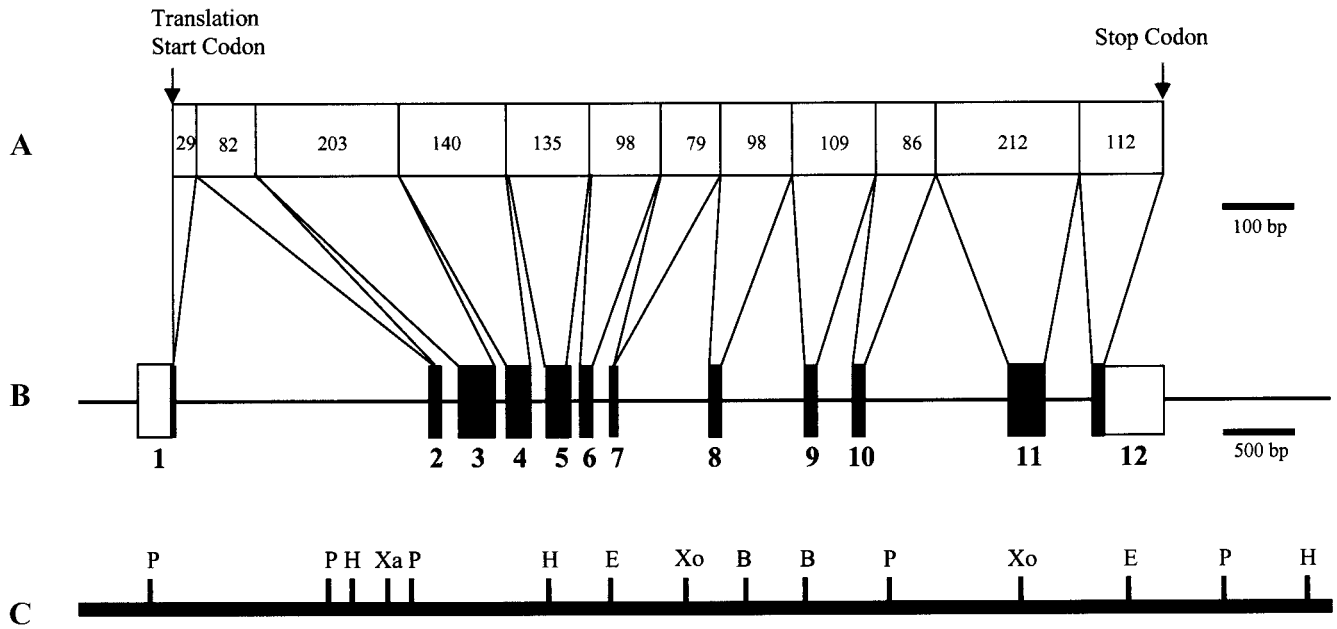

FIG. 1. Genomic structure of mouse ENT1 gene. (A) Numbers of nucleotide base pairs in each exon in the open reading frame of mouse cDNA (GenBank Accession No. AF257188). (B) Schematic representation of the genomic structure of the mouse ENT1 gene showing relative positions of the 12 exons. The white boxes represent noncoding exons, and the black boxes represent translated regions. (C) Recognition sites for restriction endonucleases. P, *Pst*1; H, *Hin*dIII; Xa, *Xba*1; E, *Eco*R1; Xo, *Xho*1; B, *Bam*H1.

1276 bp), 5′-GACTATCTAGACAACGGGAAGAGGATAG-3′ (pLuc-652, 934 bp), 5′-GACTAGTCCTCCTCCTCCTCGATTGCTC-3′ (pLuc-112, 394 bp), and the antisense PCR primer containing the *Xho*1 site was 5′-CCGCTCGAGCGGCTGTCCTGAGGCTGGTGACTGGTTG-3′. Cells were transfected with one of the constructed vectors or the pGL3 basic vector. Cell lysis and measurement of luciferase activity was performed using the Luciferase Assay System (Promega, Madison, WI). Protein concentrations were measured by the Bio-Rad protein assay based on the method of Bradford (13).

RESULTS

Molecular cloning and genomic structure of mouse ENT1 gene. A BAC library of genomic DNA from SvJ1 embryonic stem cells was screened with a 178-bp probe corresponding to the N-terminal portion of mouse ENT1 cDNA. BLAST analysis showed that this probe has no significant homology with known sequences. We isolated BAC plasmids from two positive BAC clones. The restriction enzyme digestion pattern with *Bam*H1, *Eco*R1, *Hin*dIII, *Not*1, *Pst*1, *Xba*1, and *Xho*1 showed that the two positive clones were identical by Southern blot analysis. We detected positive bands with a 1.1-kb probe corresponding to most of the ENT1 cDNA. We subcloned 6 different BAC fragments into pBluescript II SK(+) and sequenced about 12 kb of genomic DNA including the entire coding region and 5′-flanking region (Fig. 1, GenBank Accession No.

TABLE 1

Exon–Intron Boundary of Mouse ENT1 Gene

Exon	Exon size (bp)	Exon–Intron boundary Splice donor-----Splice acceptor	Intron size (bp)
1	280	CTCAGGACAG **gt**actgggtg-----tcctacgc**ag** GTATAAGGCA	1836
2	82	CGCAACCAAG **gt**gaggttgg-----aaccccac**ag** TATTTCACAA	131
3	203	TGCATCAGCG **gt**gagcctcc-----cttctcct**ag** GATCTCTCAA	103
4	140	CTCATCAATT **gt**aatcggga-----actccccc**ag** CATTTGGTGC	132
5	135	GCCATTGCCA **gt**gagtccaa-----tctgcgcc**ag** GTGGTTCTGA	108
6	98	GCCTCGGACG **gt**gagcaaat-----tgctgaac**ag** GAATTCTATC	134
7	79	ATAAATAAAG **gt**cttaatta-----cacccgac**ag** GAGAGGAGCC	609
8	98	ACTTAAGAGT **gt**acgtgggc-----ctctctcc**ag** ATCTGTGTCC	614
9	109	AGTCCCTGGA **gt**atgtgtgt-----tctttcct**ag** AAAGCTACTT	247
10	86	CTGCATGTGG **gt**gagtacag-----gtgtccgc**ag** CCTGGCCAGG	919
11	212	TCGGGCCCAA **gt**gagtcggg-----ttcctttt**ag** GAAAGTCAAA	310
12	566		

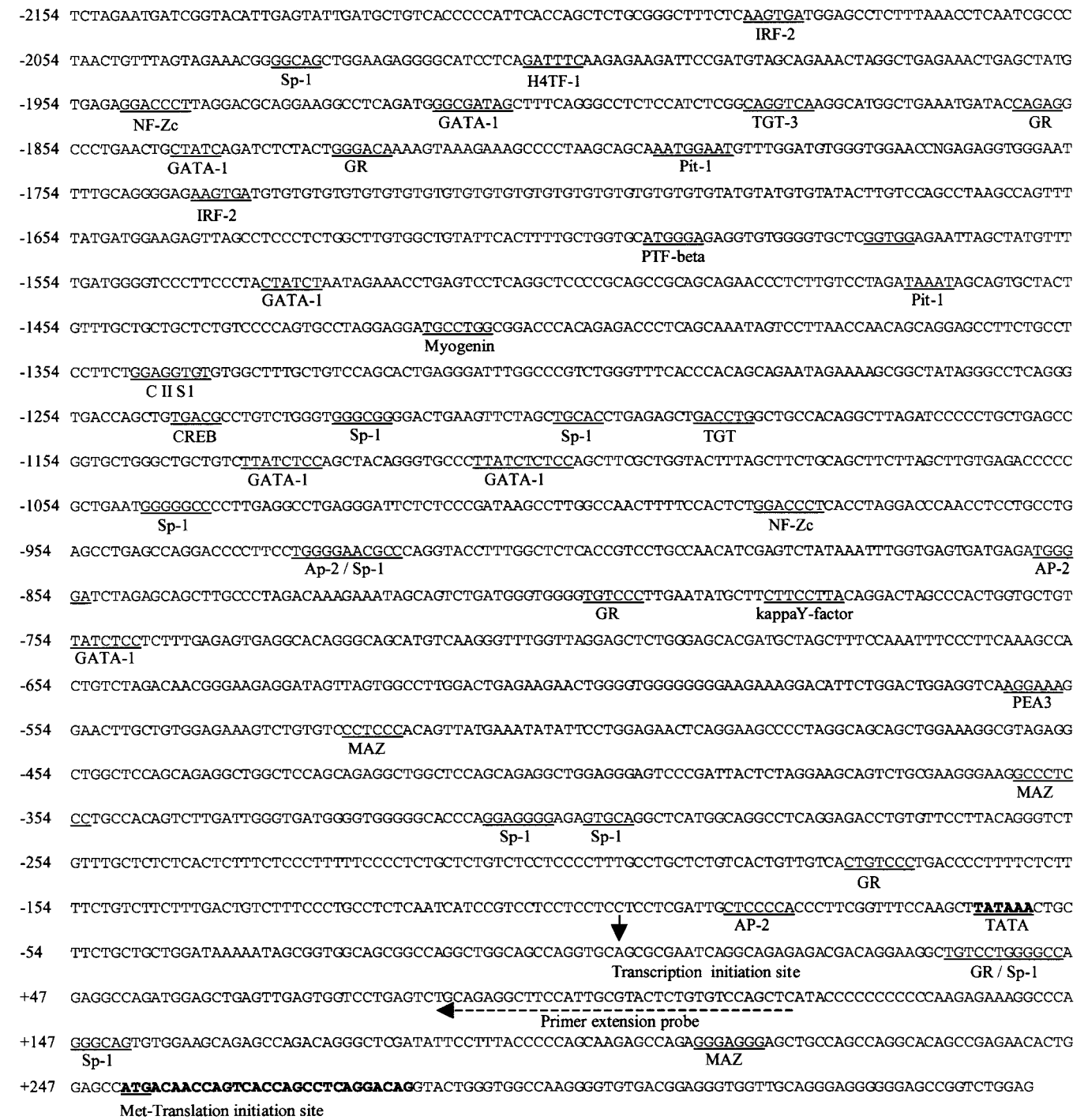

FIG. 2. Putative promoter elements in the 5′-untranslated region of the mouse ENT1 gene. The nucleotide sequence (GenBank Accession No. AF218255) is numbered from the initiation of transcription as +1. The translation initiation site and translated nucleotide sequence in the first exon is indicated in boldface. Dashed arrow indicates primer used in primer extension experiments to identify the transcription initiation site. Putative regulatory elements are underlined.

AF218255). Exon–intron boundaries were determined by identifying the differences between the genomic sequence and the cDNA sequence of mouse ENT1 (GenBank Accession No. AF257188). There were 12 exons and 11 introns within 9.7 kb of the mouse genomic DNA (Fig. 1 and Table 1).

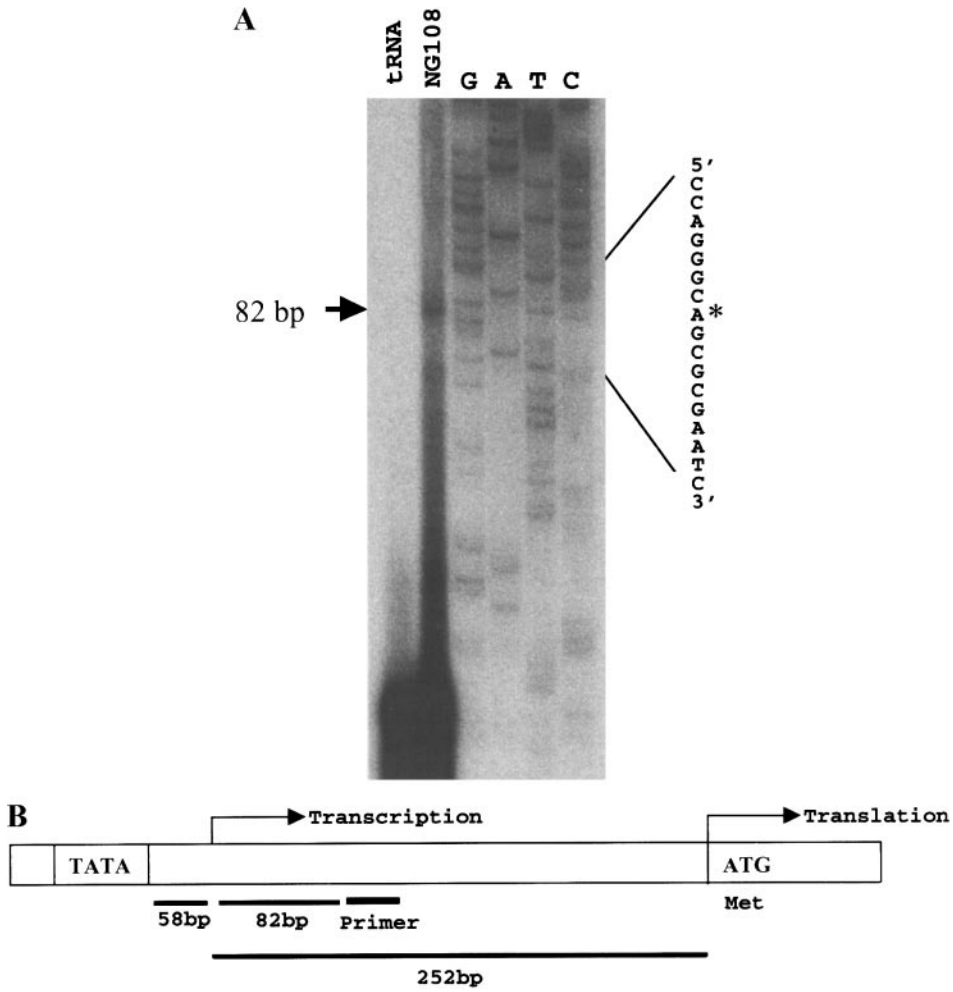

FIG. 3. Identification of transcription initiation site by primer extension experiment. (A) [γ-^{32}P]ATP-labeled 35-mer oligonucleotide probe was hybridized to 30 μg of total RNA from NG108-15 cells and control yeast tRNA. The sample was size-fractionated on a 6% polyacrylamide gel together with a sequencing ladder for size marker. Arrow and asterisk indicate the site of initiation of transcription of the mouse ENT1 gene. (B) The transcription initiation site is located 58 bp downstream of the TATA consensus sequence and 252 bp upstream of the translation initiation site.

Sequence analysis of the 5′-flanking region of the mouse ENT1 gene. About 2.5 kb of the 5′-flanking sequence was analyzed using the "signal scan" web program (http://bimas.dcrt.nih.gov/molbio/signal/). We identified several known transcription factor-binding sites including GATA-1, IRF-2, Pit-1, GR, CREM, myogenin, MAZ, Sp-1 and AP-2 (Fig. 2). Interestingly, there were three MAZ consensus sequences (GGGAGGG) and four Sp-1 consensus sequences in the promoter region proximal to the translation initiation site (Fig. 2). The 5′-flanking sequence of the ENT1 gene had a TATA box 58 bp upstream from the transcription initiation site (Figs. 2 and 3). Primer extension analysis using a complementary nucleotide probe corresponding to +83 to +118 (underlined with arrow, Fig. 2) was performed to identify the transcription initiation site of the mouse ENT1 gene. A single site was identified at the adenine nucleotide (A), 252 nucleotides upstream of the translation initiation site (Fig. 3). This transcription initiation site matched the one predicted by software-assisted promoter analysis (http://www.fruitfly.org/seq_tools/promoter.html).

Mouse ENT1 gene is localized to mouse chromosome 17C. We determined the chromosomal location of the mouse ENT1 gene by *in situ* hybridization with the ENT1 BAC clone. Hybridization signals were observed on chromosome 17C (Fig. 4). This experiment resulted in the specific labeling of the middle portion of chromosome 17. Measurements of 10 specifically hybridized chromosomes demonstrated that ENT1 is located at a position which is 44% of the distance from the heterochromatic–euchromatic boundary to the telomere of chromosome 17, an area that corresponds to band 17C.

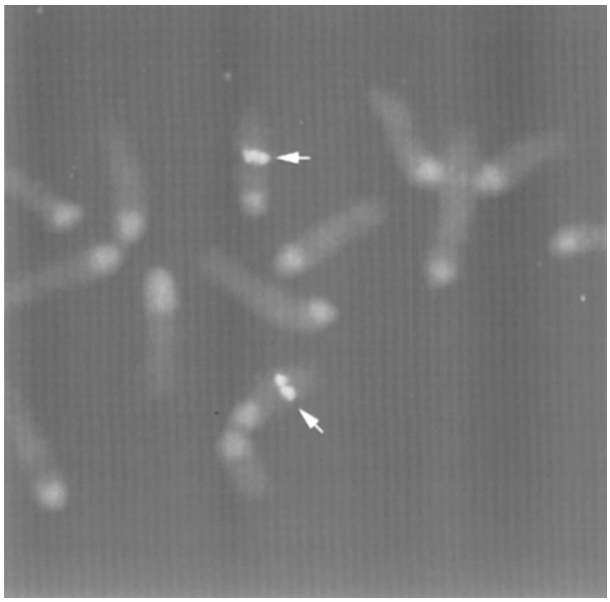

FIG. 4. Fluorescence *in situ* hybridization mapping with a BAC clone of the ENT1 gene at chromosome 17C. The digoxigenin dUTP-labeled BAC-ENT1 clone was used for fluorescence *in situ* hybridization of metaphase chromosomes derived from mouse embryo. Specific hybridization signals were detected by fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. Initial experiments resulted in specific labeling of the middle portion of a small sized chromosome that was believed to be chromosome 17 on the basis of DAPI staining. A second experiment was conducted using a probe that is specific for the telomeric region of chromosome 17, which was cohybridized with ENT1 BAC clone. A total of 80 metaphase cells were analyzed with 78 exhibiting specific labeling. Arrows indicate position of ENT1 gene in chromosome 17.

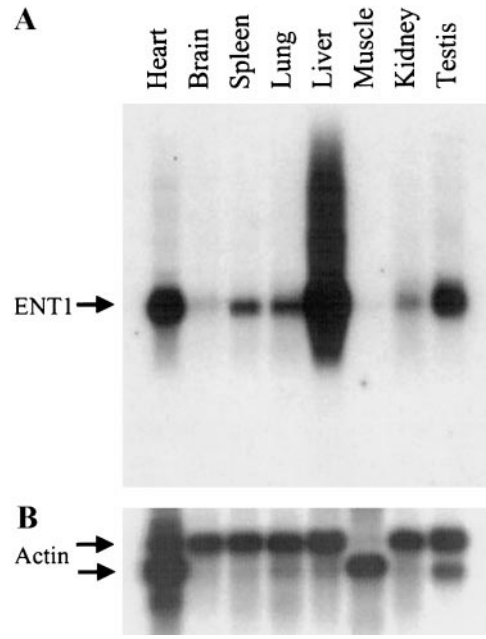

FIG. 6. Northern blot analysis of ENT1 expression in various mouse tissues. A premade mouse multitissue Northern blot was purchased from Clontech. Each lane contains approximately 2 μg of purified poly(A)$^+$ RNA. (A) The mRNA expression results obtained with the ENT1-specific probe. (B) Control: the same blot was reprobed with β-actin cDNA to check the integrity of the RNA.

Expression pattern of mouse ENT1 in various tissues. Quantitative RT-PCR analysis demonstrated that ENT1 mRNA was highly expressed in heart, spleen, lung, liver, and testis in adult mice. Lower levels of expression were detected in adult brain, muscle, and kidney. Interestingly, ENT1 mRNA showed biphasic expression during embryonic development being present at 7 dpc and again during late development at 15-17 dpc (Fig. 5).

Northern blot analysis from various mouse tissues showed 2.6 kb ENT1 mRNA in all tissues except skeletal muscle, with highest levels in liver, heart, testis, spleen, lung, kidney, and brain (Fig. 6). This is similar to the results from RT-PCR (Fig. 5).

Promoter activity of the mouse ENT1 gene. We generated a series of deletion ENT1 promoter/luciferase gene reporter constructs from the 5′-flanking region of ENT1 (Fig. 7). We used these constructs to transfect NG108-15 and SH-SY5Y cells to characterize putative regulatory elements of the mouse ENT1 promoter. In NG108-15 cells, construct pLuc-652 which contains MAZ, Sp-1, AP-2, PEA3 and GR elements produced the highest level of luciferase activity (about a 27-fold increase relative to the pGL3 basic vector), whereas deletion of sites between −652 and −112 in pLuc-112 reduced this activity. This suggests the two MAZ, two

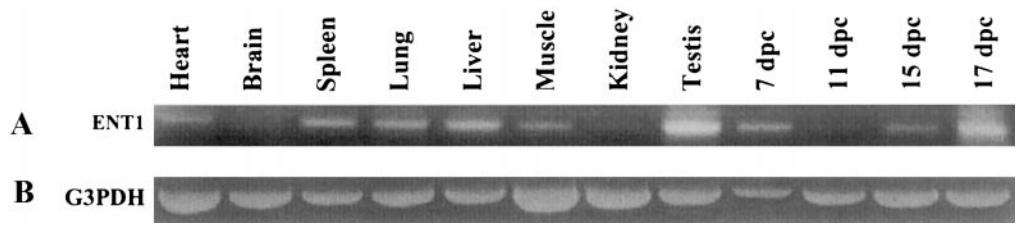

FIG. 5. Tissue expression patterns of mouse ENT1 mRNA in normal mouse tissues. cDNAs prepared from various normal mouse tissues purchased from Clontech were used as templates for PCR. (A) Pattern of mouse ENT1 mRNA abundance using 25 cycles of two-step PCR with primers DS 200 and DS 201 as described under Experimental Procedures. The actual size is 488 bp. (B) Housekeeping gene, G3PDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA used as control.

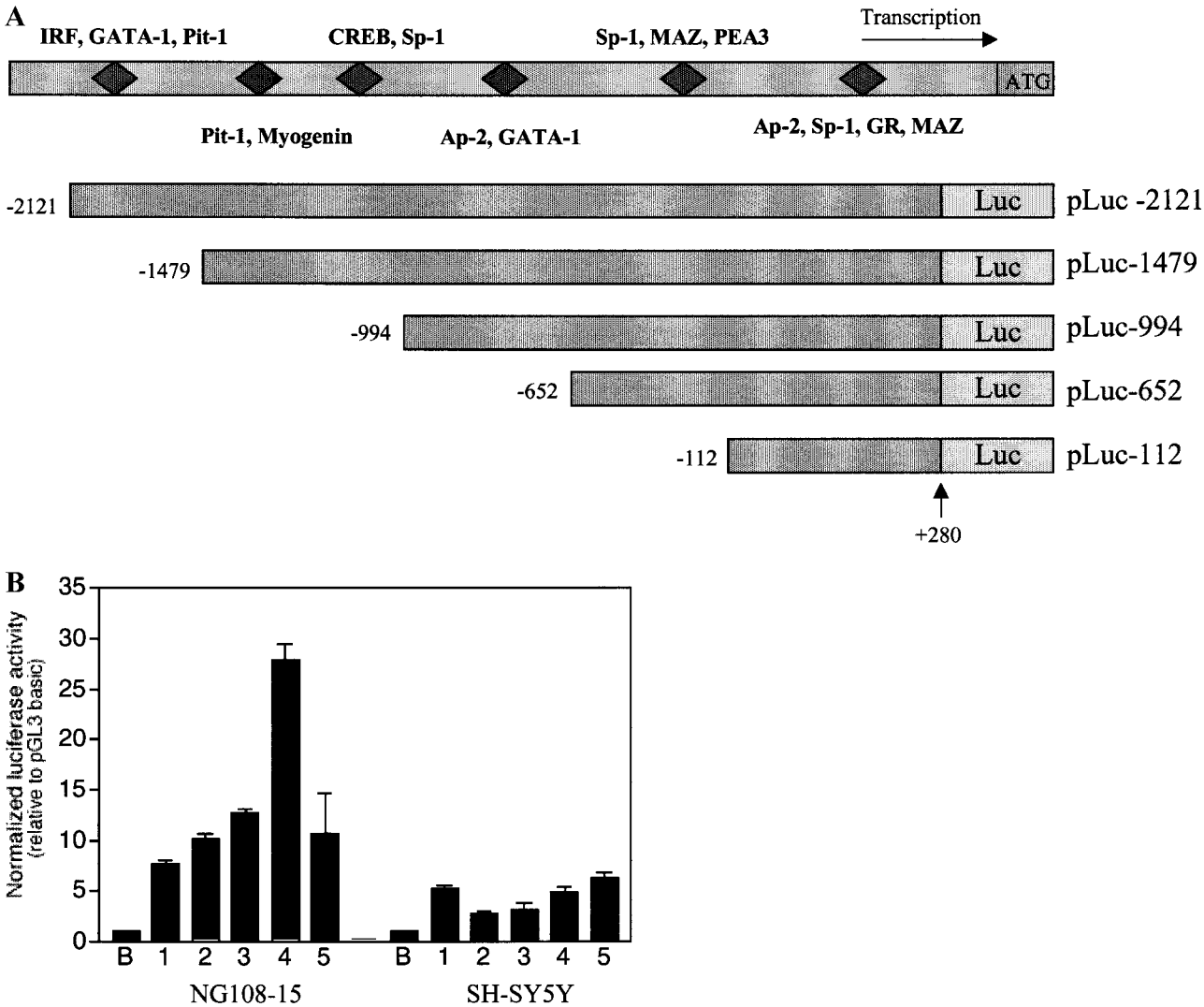

FIG. 7. Expression analysis of the 5′-flanking region of the mouse ENT1 gene. 5′-deletion analysis of the promoter region was performed in NG108-15 cells and SH-SY5Y cells. Structures of the ENT1 promoter–luciferase (Luc) reporter deletion series (A) are shown, and their relative luciferase activities in NG108-15 cells and SH-SY5Y cells (B) are shown, respectively. Each value was normalized for total protein and divided by pGL3 basic vector luciferase levels. Results are means ± SE values for at least three independent experiments. B, pGL3 basic; 1, pLuc-2121; 2, pLuc-1479; 3, pLuc-994; 4, pLuc-652; 5, pLuc-112.

Sp-1 and GR sites between −652 and −112 are important positive regulators of promoter activity in NG108-15 cells. Luciferase activity in SH-SY5Y cells was generally lower than in NG108-15 cells. However, the same construct, pLuc-652, produced relatively high levels of luciferase activity (about a 5-fold increase relative to the pGL3 basic vector) in SH-SY5Y cells, but further deletion did not diminish luciferase activity. This suggests that the most proximal MAZ and Sp-1 sites are more important in these cells.

DISCUSSION

In this study, we present the first description of a complete genomic sequence for a member of the mammalian nucleoside transporter family. We cloned ENT1 and sequenced 11873 bp of mouse ENT1 genomic DNA, finding that ENT1 contains 12 exons and 11 introns. All exon–intron boundaries begin with GT at the 5′-end and terminate with AG at the 3′-end, conforming to the GT-AG rule (14). BLAST analysis showed that there was no significant sequence homology with other known genes, including transporter genes, in the 5′- and 3′-untranslated regions and intronic regions. We also found that the mouse ENT1 gene is located on chromosome 17C. Human ENT1 is located at 6p21.1-21.3 (15). The receptor tyrosine kinase gene, Ptk-3, also maps to human chromosome 6p21.3 and mouse chromosome 17C (16), demonstrating, together with our findings, that these regions are syntenic. In humans, a gene responsible for juvenile myoclonic epilepsy (JME) maps to 6p21.2-p11 (17). JME is a heritable epileptic syndrome

occurring in children and young adults. Except for ENT1, no genes that might affect neuronal excitability, such as genes encoding ion channels, neurotransmitters, or neurotransmitter receptors, have been identified in this portion of human chromosome 6 (18). Adenosine receptor antagonists, such as theophylline, can cause seizures in mice and humans (19). In addition, adenosine can suppress seizures in rodents (20). Therefore, defective regulation of adenosine transport might be epileptogenic if it leads to decreased levels of extracellular adenosine.

The rank order of mouse ENT1 mRNA abundance in mouse tissues is liver $\gg$ heart $>$ testis $\gg$ lung $\geq$ spleen $\geq$ kidney $\gg$ brain $>$ skeletal muscle (Figs. 5 and 6). The highest level of ENT1 expression in liver suggests that the ENT1 transporter plays a role in the re-uptake process in metabolically active liver cells. Recently, it has been demonstrated that ENT1 mRNA is expressed widely in rat brain including pyramidal neurons of the hippocampus, granular neurons of the dentate gyrus, cerebellar granule cells, Purkinje cells, parietal cortex neurons, and dorsolateral striatum (21). In contrast, human ENT-2 is expressed very highly in skeletal muscle and much less in kidney, heart, spleen, liver, brain, and testis (4). The abundant expression of ENT2 in skeletal muscle suggests it may play a major role in transporting adenosine and its metabolites, such as inosine and hypoxanthine, across muscle cell plasma membrane during strenuous exercise and during exercise recovery (4). This idea is supported by evidence that ENT2 has especially high affinity for inosine and hypoxanthine (22). These differences in expression suggest that the subtypes of the ENT family, ENT1 and ENT2, have distinct functions.

A single transcription initiation site for ENT1 was identified 252 bp from the translation initiation site (Figs. 2 and 3). Several putative transcription factor consensus sequences were identified including Sp-1, Pit-1, IRF-2, myogenin, CREB, GATA-1, AP-2 and MAZ (Fig. 2). GATA-1, a zinc finger transcription factor essential for erythroid differentiation (23), was found six times within the 2-kb 5′-flanking region (Fig. 2). Interestingly, human ENT1 protein has been purified from erythrocytes, suggesting that these GATA-1 sites may be important for regulation of ENT1 expression in erythroid cells (24). Our promoter analysis using luciferase constructs showed that the -652 bp to -112 bp region contains positive *cis*-regulatory elements (Fig. 7). This region contains two MAZ and two Sp-1 binding sites (Fig. 2). MAZ, a Myc-associated zinc protein has been identified as a transcription factor that binds to a GA box (GGGAGGG) (25, 26). Recently, we cloned mouse ENT1 and ENT2 cDNAs, and found a splice variant that lacks a putative casein kinase II (CKII) phosphorylation site.[3] Tsutsui *et al.* (27) demonstrated that CKII phosphorylates MAZ, and this phosphorylation increases MAZ activity. This suggests that casein kinase II may not only regulate ENT1 function but may also stimulate ENT1 expression through MAZ activation. Furthermore, expression of MAZ is increased during the terminal phase of chronic myelogenous leukemia (28). Therefore, we postulate that salvage of extracellular nucleosides through ENT1 may be important in rapidly growing cells. Further work will be required to define the role of MAZ and Sp-1 in regulation of ENT1 expression.

[3] Handa, M., Choi, D.-S., Caldeiro, R. M., Messing, R. O., Diamond, I., and Gordon, A. S., submitted.

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