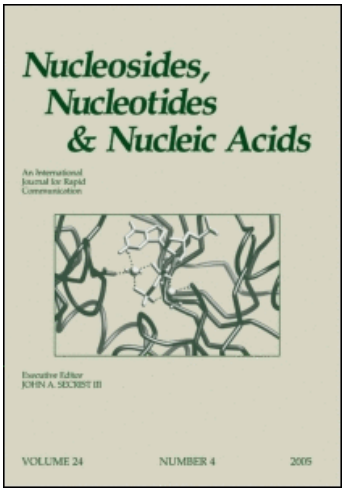

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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE PROMOTER FOR THE HUMAN EQUILIBRATIVE NUCLEOSIDE TRANSPORTER GENE, hENT1

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□ *Equilibrative nucleoside transporters (ENTs) are membrane proteins that transport nucleosides, nucleobases and analogs across membranes. ENT genes and the regulation of their expression are poorly understood. Therefore, we isolated and functionally characterized the promoter of the prototypic human ENT, hENT1. A single transcriptional initiation site 58 bp downstream of the TATA box and 272 bp upstream of the translation initiation site is present. Limited sequence similarity exists between the hENT1 and mouse ENT1 (mENT1) promoters suggesting conservation of ENT1 transcriptional regulators in mammals. Putative consensus sites for transcription factors exist within the hENT1 promoter. Reporter assays revealed similar but not identical transcriptional activity profiles in human cells.*

Keywords hENT1; gene; promoter

INTRODUCTION

Nucleoside transporters are integral membrane proteins that are responsible for the movement of nucleosides, nucleobases, and many nucleoside analog drugs across cell membranes.[1,2] Nucleosides are important in a number of cellular processes and can be synthesized de novo within the cell or salvaged from the extracellular medium via nucleoside transporters.[1] The purine nucleoside adenosine, acts as a paracrine hormone, particularly

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Abbreviations: ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; PCR, polymerase chain reaction; Acc. #, accession number; BSA, bovine serum albumin; ERE, estrogen response element; cDNA, DNA complementary to RNA; DMEM, Dulbecco's Modified Eagle's Medium; MEM/AM, Minimum Essential/Alpha Medium; βGal, β-galactosidase.

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within the cardiovascular and central nervous systems, to signal energy imbalances and stimulate compensatory cellular responses via G-protein coupled adenosine receptors. In addition, nucleosides are required for nucleotide synthesis and are therefore essential to dividing cells where nucleic acids are being synthesized and in cells where metabolism results in a rapid turnover of purine metabolites. The physiological relevance of nucleosides as substrates for nucleic acid synthesis has led to the development of nucleoside analog drugs, particularly for the treatment of cancer and viral infection. Nucleoside analogs have been widely used in treatment of hematopoetic cancers[3,4] and some solid tumors.[5] Nucleoside analogs are anti-metabolites, which, once phosphorylated, interfere with the de novo pathway of nucleoside and nucleotide biosynthesis (DNA repair and replication) acting largely to induce apoptosis. These drugs are typically hydrophilic and therefore require transport proteins to enable them to enter cells. Thus, nucleoside analogs are an important component of the anticancer armamentarium and the structure, function and regulation of nucleoside transport proteins all need to be better understood to optimize chemotherapies based on these drugs.

Two nonhomologous protein families are responsible for nucleoside transport, the equilibrative nucleoside transporters (ENTs, SLC 29) and the concentrative nucleoside transporters (CNTs, SLC 28).[1,2] ENTs and CNTs are differentially distributed within humans and also show striking variability in mRNA and protein levels between individuals and within tumor and normal cells.[6–9] The underlying reasons for variations in mRNA levels are not clear and an improved understanding of regulation of NT expression is needed. Various factors that influence ENT expression have been identified, including hypoxia,[10,11] passage number[12] and differentiation status.[13] Since relative expression of ENTs (particularly hENT1) may correlate with drug efficacy,[9] a better understanding of the ENT genes and their transcriptional regulation is needed. Moreover, since significant species and gender differences in ENT expression in rodents compared to humans have been described,[14] it is imperative that the human genes are fully understood. We previously have identified the chromosomal location of hENT1[15] and conducted a comparative genomic analysis of the ENT family.[16] We now extend these studies and report here the identification and functional characterization of the hENT1 promoter, the prototypic human nucleoside transporter. We demonstrate that the minimal promoter lies within a few hundred bases of the proximal promoter, which also contains negative regulatory elements that are cell-type specific. These data describe and functionally characterize the prototypic and widely expressed protein hENT1 and add to our understanding of the factors involved in the regulation of expression of this nucleoside analog drug transporter.

MATERIALS AND METHODS

Cloning of the Human ENT1 Genomic DNA

A lambda DASH II genomic library (Stratagene, La Jolla, CA, USA) was screened using standard methods with a PCR generated probe corresponding to the hENT1 open reading frame. Positive signals were screened to single plaque purity and DNA was amplified using Qiagen lambda Kits (Qiagen, Mississauga, ON, Canada) and subjected to Southern blot analysis. Positive clones were subcloned into pBluescript II SK^+, (Stratagene, La Jolla, CA).

DNA Sequencing and Analysis

DNA from positive clones was sequenced on both strands at the Molecular Core Facility, Department of Biology, York University, Toronto, Canada. To identify transcriptional motifs and putative transcription factor binding sites a number of web-based programs were used (e.g., MOTIF—http://motif.genome.jp, AliBaba 2.1—http://www.alibaba2.com, TRANSFAC 6.0, http://www.transfac.de/). Comparison of promoter region of hENT1 with other ENTs and mENT1 used the hENT1 sequence corresponding to Acc. # **AF495730** and the mENT1 sequence corresponding to Acc. # **AF218255** using WATER (http://www.ebi.ac.uk/emboss/align/). Default settings were used for all prediction programs.

Primer Extension Analysis

A primer extension system using AMV reverse transcriptase (Promega, Madison, WI, USA) was used to determine the location of 5′- end of the hENT1 mRNA. Total RNA from MCF- 7 cells was isolated using Trizol reagent (Invitrogen-Life Technologies, Burlington, ON, Canada) and human heart polyA RNA (Clontech, Palo Alto, CA, USA). An oligonucleotide with the sequence of 5′- AGCAGGCCAGTCCTGCGCGG-3′ (Sigma Genosys, Oakville, ON, Canada) was used for primer extension. The primer was end labeled with γ ^{32}P ATP using T4 polynucleotide kinase (30 minutes, 37°C). Labeled primer was hybridized to 30 μg of total RNA from MCF-7 and 1 μg of human heart $polyA^+$ RNA (45 minutes, 58°C) in the presence of deoxynucleotides and AMV RT. The resultant cDNA was analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflects the number of the bases between the labeled nucleotide of the 5′-end of the RNA and the quantity of cDNA product is proportional to the amount of targeted RNA.

Reporter Gene Constructions

The promoter region of hENT1 is GC-rich so reporter gene constructs were prepared by PCR amplification, using an Advantage-GC genomic PCR kit (Clontech, Palo Alto, CA, USA). Promoter regions were amplified and sub-cloned into the Kpn1/Xho1 sites of the promoterless luciferase reporter vector, pGL3-Basic (Promega, Madison, WI, USA).

The forward (sense) PCR primers used to isolate the promoter fragments were: 5′- GGGGTACCCCATGCCACCCAATGGGAAAGCTG-3′ (pLuc-756), 5′- GGGGTACCGTGGAGAGACCAGACTTG-3′ (pLuc-344), 5′- GGGGTACCCATCGATGTTGTCGCAGCTGCT-3′ (pLuc-282), and 5′-GGGGTACCTCTGCCTGTTCCGTGTCCTTC-3′ (pLuc-225A). The anti-sense PCR primers were, primer #1, containing a Xho1 site, 5′-CCGCTCGACGGGCTGTCCTGAGGCTGGTGACTGGTTG-3′ (used for the constructs described above), and primer #2, 5′-AGCAGGCCAGTCCT GCGCGG-3′, which was used to construct pLuc-225-short, containing a 270 bp fragment of the promoter. This PCR product was first subcloned into pGEM and a Kpn1/Xho1 fragment then cloned into the Kpn1/Xho1 site in pGL3-Basic. Another deletion construct (pLuc-558) was made by digesting the pLuc-756 construct with Kpn1/BstEII, releasing a 178 bp fragment to produce the shorter construct. Klenow enzyme was used to blunt-end the fragment which was then religated using T4 DNA ligase.

Transient Transfection and Luciferase Assay

MCF-7 cells (derived from an epithelial breast cancer) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO, USA). K562 cells (derived from a myelogenous leukemia) were cultured in Minimum Essential Medium/Alpha Medium (MEM/AM) supplemented with 10% (v/v) fetal bovine serum. Both cell lines were maintained at 37°C in 5% (v/v) CO_2. Cells were plated for transient transfection in six well plates at a density of ~6–8 × 10^5 cells/well. Cells were transfected at 70–80% confluency with LipofectAMINE (Invitrogen, Burlington, ON, Canada) and 2.5 μg of plasmid DNA (see below) according to the manufacturer's protocol. Cells were co-transfected with 2 μg of promoter construct (or the pGL3Basic vector as control) and 0.25 μg of pCMV-β-gal. Cells were lysed and luciferase activity measured using a Luciferase Assay System (Promega, Madison, WI, USA). Results from all transfections were normalized to the β-galactosidase activity expressed by the co-transfected control plasmid pCMV-β gal. The ratio between luciferase light units and β galactosidase activity was normalized for each cell line separately relative to the cells transfected with pGL3-Basic. All results are expressed relative to the luciferase activity of the pGL3-Basic, arbitrarily set to 100%. Each value represents the average ±

S.E. of at least 3 independent transfection experiments, each conducted in triplicate. Luminescence was measured using a Sirius Luminometer (Brethold Detection System).

RESULTS

Molecular Cloning of the Human ENT1 Gene and Promoter

We cloned the hENT1 gene by screening a lambda DASH II genomic library using a PCR generated probe corresponding to the hENT1 open reading frame. The identification and characterization of the hENT1 gene (without the promoter) has been described in more detail elsewhere (16). The entire hENT1 gene (promoter plus coding regions) as been deposited in GenBank (accession #. **AF495730**).

Sequence Analysis of the 5′-Flanking Region of the Human ENT1 Gene

About 2 kb of the 5′-flanking sequence were analyzed using web-based programs to identify putative transcription factors and other motifs. Analyses indicated that the promoter is GC-rich and several putative transcription factor-binding sites including AP-2, PTF-β, myogenin, and an ERE were identified. In addition, several Sp1 consensus sequences (GC- or GT-boxes) were identified in the promoter region (Figure 1). We performed primer extension analysis to determine the transcriptional initiation site using a complementary nucleotide probe corresponding from +26 to + 46 (solid underline, Figure 1). A single site was identified at the adenine nucleotide (A), 276 bp upstream of the translation initiation site (Figure 2). The 5′-flanking sequence of the ENT1 gene has a TATA box 58 bp upstream from the transcription initiation site (Figures 1 and 2).

Comparisons with the promoter sequences of other human ENT genes (hENT2, hENT3, and hENT4/PMAT) using WATER (http://www.ebi.ac.uk/emboss/align/) did not reveal any regions of conservation suggesting that each human gene possesses unique promoter characteristics. In contrast, comparison of the hENT1 promoter with the mouse ENT1 promoter found 3 regions of significant conservation (Figure 1, dotted underlines). One region flanked the predicted TATA box (−87 to +4), while the other two regions of conservation were upstream of the TATA box (−173 to −113) and within the predicted 5′ UTR (+215 to +279).

Promoter Activity of the Human ENT1 Gene

To functionally characterize the hENT1 promoter, we generated five promoter/luciferase gene reporter constructs from the 5′-flanking region of hENT1 (Figure 3). These constructs were transfected into MCF-7 and

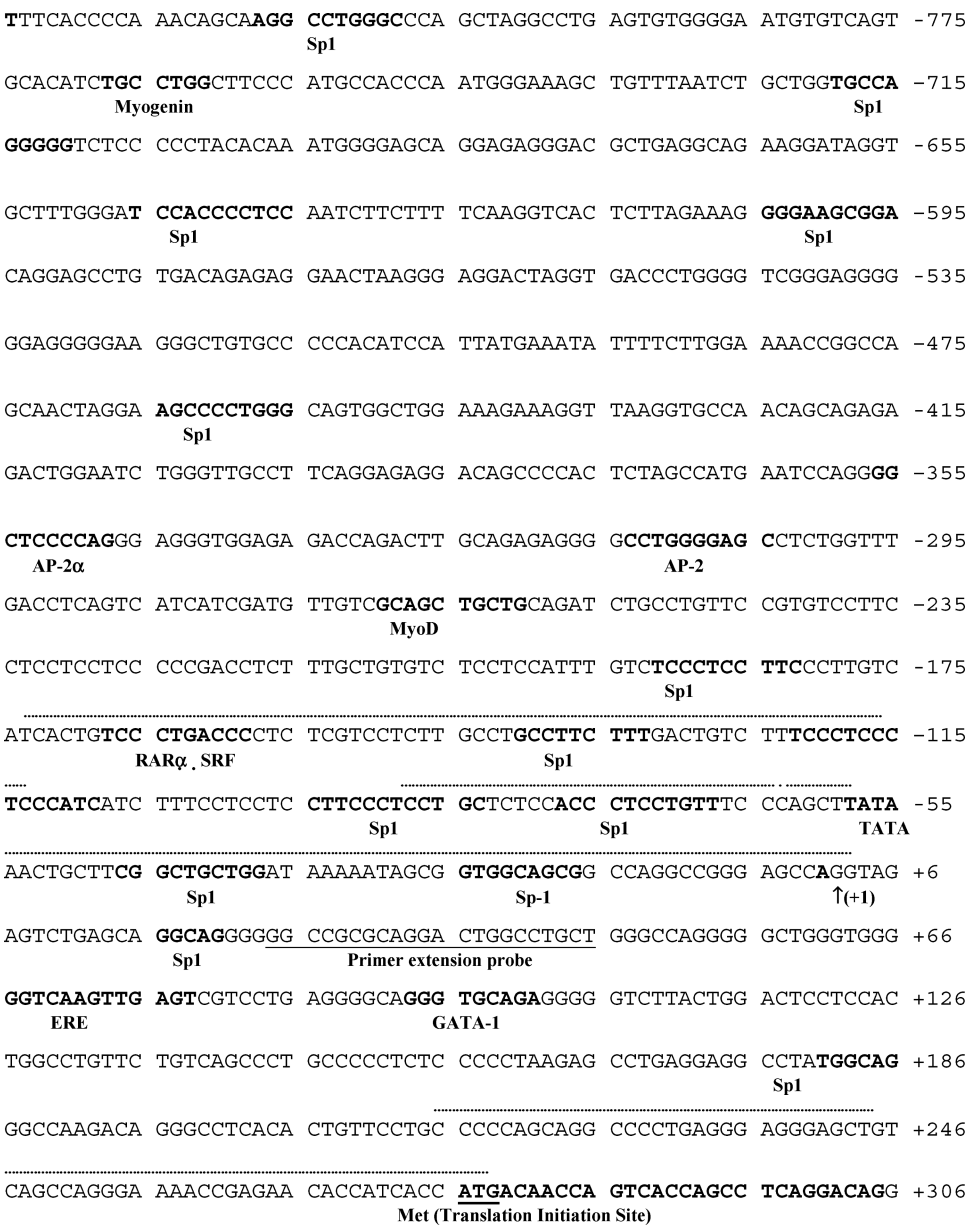

FIGURE 1 Human ENT1 promoter. The nucleotide sequence (GenBank Acc. # **AF495730**) is numbered from the initiation of transcription as +1. The translation initiation site is indicated in boldface and underlined (Met). The sequence used to generate the primer for the primer extension reaction is underlined (primer extension probe). Putative transcriptional regulatory elements are shown in boldface and named below the sequence. Two additional partially overlapping Sp1 sites were identified between −26 and −122 but are not shown for clarity. Sequences underlying dotted lines represent regions of the promoter that are conserved between hENT1 and mENT1.

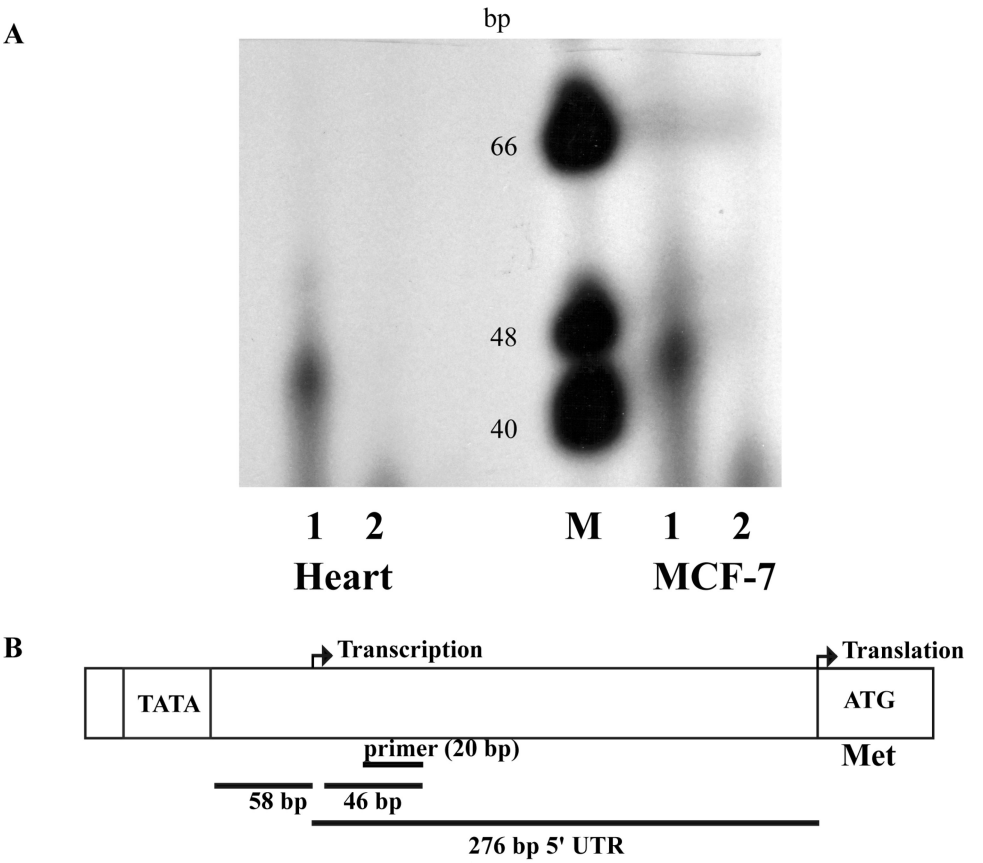

FIGURE 2 Identification of the transcriptional initiation site of hENT1. A) Primer extension analysis of human heart polyA$^+$ RNA (1 μg) and MCF-7 total RNA (30 μg). Lane 1 in each panel shows the test RNA, lane 2 is a negative control and M represents lane with markers. B) Transcription starts 58 bp downstream of the predicted TATA box and produces a 5′ untranslated region of 276 bp.

K562 cells to characterize putative regulatory elements of the human ENT1 promoter. We were interested in investigating possible regulators within the proximal region of the promoter including downstream of the transcriptional initiation site and also the potential role of the previously identified ERE (+ 67) since estrogen has been shown to regulate nucleoside transport in MCF-7 cells.[17] Therefore, one of our constructs (pLuc-225short) lacked the region of the promoter containing the ERE binding site.

Our results indicated that in MCF-7, the pLuc-225short construct represents a minimal promoter with significantly higher level of luciferase activity (15.5-fold increase over basic) than other constructs (Figure 3). In K562, the pLuc-255short construct also represents a minimal promoter although the overall activity is much lower than that seen in MCF-7 (5-fold above background). These data also suggest that negative regulatory *cis*-elements

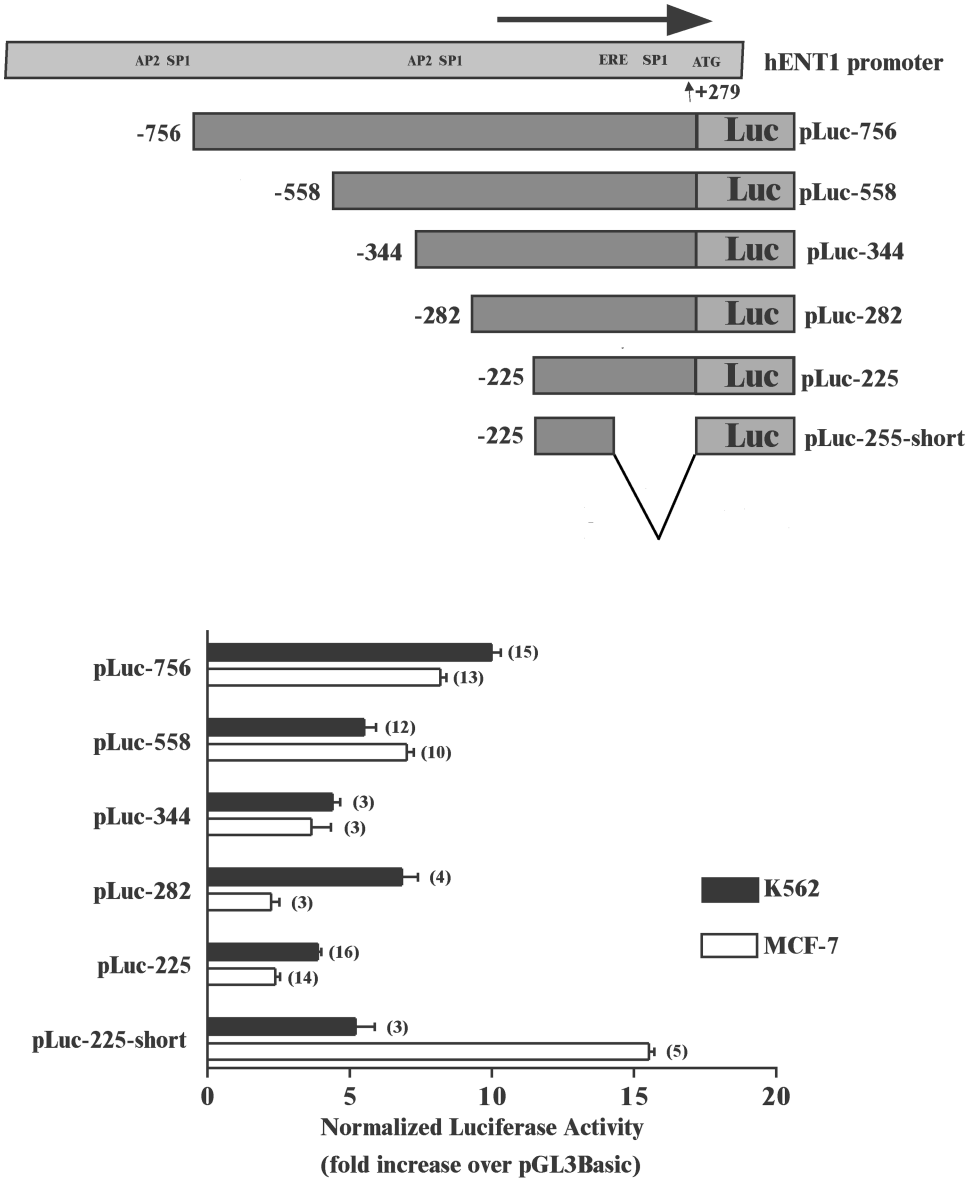

FIGURE 3 Effects of deletion on the constitutive transcriptional activity of the hENT1 promoter. MCF-7 and K562 cells were transfected with luciferase reporter gene fusion constructs of serially deleted hENT1 promoter sequences. Luciferase gene expression is shown relative to activity (mean ± SEM) of the promoterless vector pGL3Basic, (n) = number of experiments (each conducted in triplicate). Transfection efficiency was determined by co-transfection with pCMV-βGal.

exist within the region that was removed from the pLuc-255short construct since luciferase activity was higher in both cell lines in the absence of this region. Modest increases in activity are seen with longer constructs and the highest activity (except for pLuc-225 in MCF-7) was observed with pLuc-756

(10-fold increase over basic). These data suggest that cell type specific regulatory elements exist within hENT1 promoter.

DISCUSSION

In this study, we present a full description and functional analysis of the promoter sequence of prototypic equilibrative nucleoside transporter, hENT1. Despite the importance of hENTs in chemotherapeutic nucleoside analog drug uptake, the human ENT genes have not been fully characterized and the nature of transcriptional regulation of any ENT is almost completely unknown. Previously, we reported the chromosomal location of hENT1[15] and undertook a comparative genomic analysis of ENT genes in humans, *Drosophila* and *C. elegans*.[16] In addition, we have described highly variable hENT1 mRNA levels in different tissues.[6] These data suggested that active and complex regulation of hENT1 transcription and/or RNA stability exists in humans. Other workers have noted that levels of hENT1 protein also vary considerably between individuals[7,8] and that the absence of hENT1 mRNA correlates with a lack of response to cytotoxic nucleosides.[18,19] Taken together these data suggest that there is a pressing need to better understand the factors that regulate transcription of the drug transporter, hENT1, in order to improve chemotherapies based on cytotoxic nucleoside analogs.

Following on from our previous work on describing the hENT1 gene, we turned our attention upstream and isolated the promoter. The *hENT1* promoter is GC-rich which, in combination with the ubiquitous expression of hENT1 mRNA, would be suggestive of a housekeeping gene. However, the presence of single transcriptional start site, downstream from a classical TATA box and the previously described spatial and temporal variations in expression[6] suggest that *hENT1* is not a housekeeping gene but rather, is likely to be subject to transcriptional and/or post-transcriptional regulation. Standard analyses were used to identify regulatory elements within the promoter and a number of putative transcription factor consensus sequences were identified including ERE, MAZ, Sp1, AP-2, myogenin, IRF-2, CREB, and PTF-β. We also compared the promoter region with other ENT promoters (derived from Genbank) but only found conservation of sequence between the promoters of hENT1 and mENT1.[20] These data suggest that similar transcriptional regulators may have been conserved in the ENT1 lineage. Like mENT1,[20] there is one transcriptional initiation site in hENT1 (although we cannot rule out the possibility of additional transcriptional initiation sites in tissues not studied here) and there are three areas of similarity between mENT1 and hENT1 promoters, which contain predicted Sp1 consensus sites in both genes. In addition, the TATA box and transcriptional initiation site are in comparable locations relative to the translational initiation site in both hENT1 and mENT1. Taken together,

these data suggest that the proximal promoter region of ENT1 has been conserved in sequence in mammals and transcriptional regulation may be similar in mice and humans. Indeed, recent data have demonstrated this to be the case in the response of both mENT1[20] and hENT1 to hypoxia,[11] with both being down-regulated, apparently at the transcriptional level. This is additionally significant in that hypoxia is a feature of many tumor types,[21] including those that are often resistant to nucleoside analog treatment. Thus, transcriptional down-regulation of hENT1 (by hypoxia or other factors), either alone or in combination with the up-regulation of MDR1,[22] may represent a contributing factor to resistance of some tumors to chemotherapeutics. Thus, ENT1 homologs in mammals may possess similar transcriptional regulation, and comparative analyses of mammalian ENT genes may provide insight into transcriptional regulation in humans.

To further characterize *hENT1*, we made a series of luciferase-promoter constructs and transfected them into two cells lines, MCF-7 and K562, that have been well studied in terms of their nucleoside transport characteristics.[17,23] Our promoter analysis using luciferase constructs showed that the region from −255 bp and +1 bp contains positive *cis*-regulatory elements, although the overall levels of activity are quite different in the two cell lines. These data suggest that *hENT1* could be regulated by a wide spread transcription factor that controls basal expression in combination with cell-type specific regulatory factors to modulate local levels of expression. These findings are similar to those reported for the mENT1 promoter where fold differences in activity of identical constructs were noted in different cell lines, NG108-15 (mouse-rat neuroblastoma/glioma) and SH-SY5Y cells (human neuroblastoma).[20] Interestingly, the removal of a portion of the proximal promoter of hENT1 led to an increased activity in MCF-7 suggesting the presence of repressor elements in this cell line. We had anticipated that removal of the putative ERE in this region might lead to a decrease in activity in MCF-7 cells alone (and not K562) given that estrogen may be a positive regulator of ENT1 presence and/or activity in these cells.[17] However, this was not the case and, along with the observation for mENT1 of differential transcriptional regulation between NG108-15 and SH-SY5Y cells[20] suggests that further work is required to elucidate the regulatory elements that act in cell-specific ways.

In summary, we have functionally characterized the promoter for the prototypic equilibrative nucleoside transporter gene, *hENT1*. These studies lay the groundwork for future analyses of transcriptional regulators of the important drug transporter, *hENT1*.

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