Results

Expression profile of hENT1 mRNA isoforms in human hepatocytes and hepatoma cells

When examining the expression profile of hENT1 mRNA isoforms in the pooled (50-donor) human hepatocytes, HepG2 cells, Huh-7 cells, and FLC7 cells, qPCR was performed using the GAPDH mRNA as an internal control (Fig. 2A). Pooled hepatocytes were used based on the expectation that the individual variability of mRNA expression levels would be masked. The results showed that hENT1c and hENT1d mRNA isoforms were abundantly expressed in all the cells examined, while the hENT1a1 mRNA expression level was significantly lower, and the hENT1b mRNA isoforms expression was only detected at trace levels. To confirm mRNA expression level difference, the copy numbers of hENT1a1, c, and d mRNA isoforms in pooled human hepatocytes and hepatoma cells were determined using an absolute qPCR method. Consistent with the results described above, the hENT1c and hENT1d mRNA copies in human hepatic cells were 62~172-fold, and 64~93-fold larger than that of hENT1a1 mRNA, respectively (Table 1).

To enhance verification of the predominant expression of hENT1d mRNA in hepatic cells, an mRNA knockdown experiment was performed using the hENT1d mRNA-specific shRNA in HepG2 cells. This experiment was, unfortunately, not applicable to hENT1c mRNA, as described in the materials and methods section. As can be seen in Fig. 2B, the results showed that the hENT1d shRNA, but not the scramble shRNA, reduced the hENT1d mRNA level to 25 ± 3 (%) without significant effects on the hENT1c mRNA levels in HepG2 cells. Consequently, the total hENT1 mRNA level in dKD-HepG2 cells was 60 ± 19 (%) of that of the control-HepG2 cells, indicating that the hENT1d mRNA amount apparently accounted for approximately half of the total hENT1 mRNA amount.

To determine how hENT1c and hENT1d mRNA expression levels varied among hepatocyte lines and to determine if significant expressions of hENT1a1 or 1b mRNA isoforms could be observed in a single donor, qPCR was performed using cDNA obtained from seven human hepatocytes lines. As with the case of pooled human hepatocytes, hENT1c and hENT1d mRNA expressions were highly abundant (Fig. 2C), and no significant hENT1a1 or hENT1b mRNA expressions were detected (data not shown). However, differences were found in the ratio between hENT1c and hENT1d mRNA levels between individual lines and the hENT1d mRNA levels were more variable [1.21±0.85 ($\times 10^2$), with a 9.2-fold maximum difference] than hENT1c mRNA levels [1.09±0.39 ($\times 10^2$), with a 3.1-fold maximum difference].

These results clearly showed that hENT1c and hENT1d mRNA isoforms were primarily expressed in human hepatocytes as well as human hepatoma cells.

Differential transactivation property of hENT1 alternative promoters in human hepatic cells

To understand the mechanisms underlying differential expression levels among hENT1 mRNA isoforms, the transactivation properties of alternative promoters (P1, P2 and P3, which are responsible for a1, c, and d mRNAs transcription, respectively) were examined in hepatoma cells (Fig. 3A). We showed that the P2 and P3 promoters were more active than the P1 promoter in HepG2 cells, which is consistent with our previous results.[19] In addition, the P2 and P3 promoters were found to be strongly activated in both Huh-7 cells and FLC-7 cells, while the P1 promoter was inert in these cells. These results showed that the P2 and P3 promoters exerted transactivation ability in human hepatic cells, while the P1 promoter did not.

The above results prompted us to investigate whether different sequence characteristics could be found among the promoter regions. DNA sequence analysis results showed that the vicinity of the transcription start site in the P2 and P3 promoters was enriched with CpG, and that the normalized CpG contents were 0.83 and 0.57, respectively (Fig. 3B). In contrast, the P1 promoter lacked CpG despite the fact that the GC content (61%) was not significantly different from those of the P2 and P3 promoters (70% and 64%, respectively). Another difference was that a TATA-box motif (ATAAAAATA, -40/-32) was predicted in the P1 promoter, but not in the P2 and P3 promoters. Collectively, the sequence analyses clarified that the P2 and P3 promoters were CpG-rich TATA-less promoters, and that the P1 promoter was a CpG-depleted- TATA-containing promoter.

Histone H3 lysine 9 acetylation status in the promoter region

To obtain further evidence that P2 and P3 promoters are activated in hepatic cells, the extent of histone H3 lysine 9 acetylation (H3K9ac), which is one of the well-known markers associated with active transcription in genomic promoter regions,[23,24] was examined by ChIP assays (Fig. 4). The results showed that H3K9ac was more enriched in the P2 and P3 promoters than in the 3'-intergenic region, while enrichment amounts between the P1 promoter and the 3'-intergenic region in HepG2 cells were comparable. Similar results were also obtained using FLC7 cells. These results showed that the genomic region of the P2 and P3 promoters was transcriptionally-active state in HepG2 cells and FLC7 cells, which was consistent with the results of promoter activity analyses.

Identification of primary hENT1 mRNA isoforms in human hepatocytes and hepatoma cells

hENT1c and d mRNA groups consist of three (c1, c2, and c3) and four (d1, d2, d3, and d4) mRNA isoforms (Fig. 1), respectively, and our qPCR methods were unable to discriminate among their mRNA expressions. To determine if the predominant hENT1d mRNA isoforms are either d1/2

or d3/4, 5'-RACE PCR was performed using HH187 and HepG2 cells. In the results, it was found that the band indicative of hENT1d3/4 mRNAs expression was predominantly amplified over the longer one, which contained a previously unidentified transcription start site (asterisk), and the band indicative of hENT1d1/2 mRNAs expression was barely detectable (Fig. 5A), thus showing that the hENT1d3/4 mRNA levels were much higher than the others. We also confirmed that hENT1c mRNAs are exclusively generated from the previously identified transcription start site (Fig. 5A).

Next, RT-PCR analysis was performed to distinguish among c1, c2, and c3 mRNA isoforms and between the d3 and d4 mRNA isoforms. The results showed that the hENT1c1 and (to a lesser extent) c2 mRNA isoforms were expressed in all hepatic cells examined (Fig. 5B), while hENT1c3 mRNA expression was not observed. As for the hENT1d mRNAs, the hENT1d3 mRNA levels were apparently predominant over d4 mRNA levels. Therefore, the results showed that hENT1c1 and d3 were the primary mRNA isoforms in human hepatocytes, and that hENT1c2 mRNA also somewhat contributed to total hENT1 mRNA expression.

hENT1c1, c2, and d3 mRNAs have distinct 5'-UTRs, as shown in Figs. 1 and 5C, which may provide targets for translational regulation.[25] To obtain insights into those regulatory mechanisms, sequence analysis was performed. The results identified two uORFs (with amino acid lengths of 6 and 46, respectively) in the 5'-UTR of d3 mRNA, while none could be found in the 5'-UTRs of c1 and c2. No in-frame ATG embedded in the Kozak sequence was identified in any of the 5'-UTRs. Another element that is potentially involved with translation regulation is the secondary structures of the 5'-UTRs. The results of *in silico* prediction showed that all three 5'-UTRs including exons 1 and 2, appeared to have their own structural properties, and that the extent of their complexity was predicted to be c2 (99 kcal/mol) > d3 (75 kcal/mol) > c1 (65 kcal/mol), in that order (Fig. s1).

Discussion

Our results clearly demonstrate that, among the hENT1 mRNA isoforms, hENT1c1, d3 and (to a lesser extent) c2 mRNA are predominantly expressed in human hepatocytes and hepatoma cells. Therefore, it is considered likely that these mRNA isoforms play important roles in controlling the hENT1 expression levels in human hepatocytes. This is relevant to our previous results showing that the differential hENT1d mRNA expression levels in HH268, HH283, and HH291 (also confirmed in this study) are associated with their different hENT1 transport activities.[19] However, we should disclose that, despite the lowered total hENT1 mRNA level, the dKD-HepG2 cells showed levels of ENT1 activity (NBMPR-sensitive adenosine uptake) that were comparable with the control-HepG2 cells (data not shown). This observation suggests that the hENT1c and 1d mRNA levels may not always translate directly into functional levels due to as yet unidentified compensatory post-transcriptional regulation mechanisms. Considering the complex structure of the hENT1 gene, along with its physiological roles in cellular nucleotide homeostasis, it can be assumed that both cases may be true depending on the cellular circumstances. Identification of the precise functions of each promoter and mRNA isoform in control of the hENT1 functional level will definitely need to await further studies, but several possibilities are given in this discussion.

The findings obtained from our present study provide clear evidence for manipulated utilization of the alternative promoters in the hENT1 gene. This may not be surprising when the data from genome-wide analysis claiming that 30-60% of the human genes have multiple alternative promoters is taken into consideration.[26,27] One possible mechanism behind the strong activation of the P2 and P3 promoters in hepatocytes is the involvement of hepatocyte-enriched TFs. However, no binding elements of such hepatocyte-enriched factors have been identified in their promoters (unpublished observation), and hENT1c and d mRNA levels are approximately comparable between HepG2 cells and FLC7 cells, despite the fact that the hepatocyte nuclear factor 4α are expressed differentially in these cells.[28] Therefore, another possibility, involvement of a ubiquitously activated system, could more plausibly explain the strong P2 and P3 promoter activations. We found that these promoters show a high rate of CpG occurrence (normalized CpG scores > 0.5), and that they are marked by H3K9 acetylation, which is a recognized hallmark of transcriptionally-activated CpG promoters.[23,24] Currently, it has become widely accepted that a promoter possessing normalized CpG > 0.5 is to be regarded as a high CpG promoter (HCP), and that HCPs are overrepresented in ubiquitously expressed genes.[21,29,30] Consistent with this notion, we have observed abundant expression of hENT1c and 1d mRNAs in human lung, pancreas, intestine, and kidney-derived cells (unpublished observation). Based on these findings, it is very likely that the P2 and P3 promoters are activated in not only hepatocytes, but in various other cells, thus resulting in the ubiquitous expression profile of hENT1c and 1d mRNAs. It may also be worthwhile to mention that CpG

promoters are often accompanied by broad transcription initiation sites,[31] which may draw further attention to the possibility that low levels of hENT1b and d1/2 mRNA isoforms result from leaky transcription from the P2 and P3 promoters, respectively.

On the other hand, we found that hENT1a1 and b mRNAs played only a minimal and negligible role in hENT1 expression in human hepatocytes. hENT1b mRNA aside, the significantly low hENT1a1 mRNA expression level in human hepatocytes is somewhat surprising since several reports have focused on the P1 promoter when identifying regulatory elements and the functional SNP (rs747199). The results of these reports accentuate that the P1 is a *bona fide* promoter, but it should also be noted that the cells examined in these studies are not hepatocytes.

The critical differences of the P1 promoter, in comparison with the P2 and P3 promoters, are focused on the presence of a TATA-box and the remarkably low CpGs number, despite the fact that all three promoters have comparable GC content levels. Considering the report showing that CpG-less, TATA-box containing promoters are prevalent amongst tissue-specific genes,[21,31] it is possible that hepatocytes are not the target cells of the P1 promoter. In addition, it is possible that the P1 is an inducible-type promoter, based on the report showing that CpG-less promoters are overrepresented in the gene responses to biotic or external stimulus.[30] While it is currently unclear how the P1 promoter is regulated, we assume that specific mechanisms prevent its activation exist in human hepatocytes.

In the genes generating alternatively transcribed mRNA isoforms that share the identical protein coding regions, such as RUNX1, OTX2 and SHOX genes, it is believed that the individual mRNA have distinctive roles related to controlling eventual protein mass in a tissue-, development-, or signal-dependent manner.[26] Furthermore, it is believed that those roles are not solely played by promoters, but that the 5'-UTRs of the mRNA are also involved.[32] Therefore, it is possible that hENT1c1 (c2) and hENT1d3 mRNA provide an isoform-dependent regulatory mechanism in which the corresponding promoter and 5'-UTR are utilized in an orchestrated manner.

One clue implying their functional difference can be found in Fig. 2C, in which the hENT1d mRNA level shows higher variability than that of hENT1c mRNA. Although it is premature to state so categorically, the differences in CpG contents between the P2 and P3 promoters (0.83 vs. 0.57) might be related to such differential behaviors, because the results of recent reports show that CpG content and CpG island length is likely to define the regulatory properties of the promoter by determining nucleosome density.[33] On the other hand, it should be noted that the two uORFs are exclusively found in the 5'-UTR of hENT1d3 mRNA. Because it has been known that uORFs often have repressive effects on protein translation,[34] the two uORFs may play a similar repressive role in translation efficacy under certain conditions. Moreover, it may also be worthwhile to mention that the secondary 5'-UTR structure of d3 mRNA is expected to be more complicated than that of c1 mRNA, because it has been suggested that the higher degree of secondary structure somehow

decreases translation efficiency.[25] Taken together, although we understand it may be a speculative statement, it is possible that hENT1d3 mRNA provides a crucial target for flexible control of the total hENT1 mRNA level, while hENT1c1 mRNA ensures its basic level, at least in human hepatocytes. In future studies, it would be an intriguing issue to clarify the distinctive roles played by each hENT1 mRNA isoform, and how they coordinate their efforts to control the hENT1 protein level in human hepatocytes, as well as other cell types.

In conclusion, our results show that hENT1c1, d3, and (to a lesser extent) c2, but not a1, are the primary mRNA isoforms in human hepatocytes, that the P2 and P3 promoter activities are apparently regulated in an independent manner, and that the 5'-UTRs of three hENT1 mRNAs might provide differential impacts on the post-transcriptional regulation of gene expression, which highlights the existence of alternative promoter-driven complex mechanisms underlying hENT1 expression in human hepatocytes. In addition, it should be emphasized that such alternative promoter usage is likely to occur in other cell types as well. Therefore, our present findings establish basic information that is applicable to the clarification of tissue-specific regulatory mechanisms for hENT1 expression in various cells including hepatocytes. Furthermore, it is possible that the SNPs found in non-coding region may affect promoter activity, mRNA stability, and/or translational efficiency in a promoter/mRNA isoform-specific and tissue-dependent manner. Therefore, it is important to determine what mRNA isoforms are primarily expressed in the cells of interest, and a step-by-step clarification of the transcriptional- and post-transcriptional mechanisms will be necessary for fully understanding how hENT1 expression level is determined in the cells. We hope that such clarifications ultimately contribute to providing useful information for efficacy improvements of nucleoside analogue-based therapy.

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Table

Table 1. Copy numbers of hENT1 mRNA isoforms in human hepatocytes and hepatoma cells

Cells	hENT1a1 [a]	hENT1c1-3 [a]	hENT1d1-4 [a]
Human hepatocytes [b]	0.4±0.1	43.2±8.4	37.5±6.4
HepG2 cells	1.4±0.2	141.8±11.2	105.1±11.6
Huh-7 cells	1.4±0.1	87.0±7.9	88.9±9.3
FLC7 cells	1.2±0.1	207.1±5.6	95.4±8.6

[a], each value (copies/μg total RNA) represents copies ± S.D. ($\times 10^4$).

[b], Pooled (50-donor) human hepatocytes.

Figure legends

Fig. 1. Schematic illustration of the 5'-side of the *SLC29A1* gene structure and the hENT1 mRNA isoform.

The genomic DNA representing the 5'-side of the *SLC29A1* gene is shown at the top. Open boxes indicate the exonic regions, while the shadowed arrows indicate alternative promoters. Open arrowheads indicate primer positions for ChIP-qPCR assays. The ¶ mark indicates the translation start site. The structures of hENT1 mRNA isoforms are illustrated below the genomic DNA with each name on the left side. The closed boxes indicate mRNA components, while the horizontal lines indicate the regions to be spliced out. Closed arrowheads, thick arrows, and thin arrows indicate primer positions used in RT-PCR, qPCR to analyze mRNA isoforms expression, and RACE analysis, respectively. The pair of primers positioned at exon 1 and exon 2 was used for total hENT1 mRNA detection. The line indicates the position of the target sequence for hENT1d mRNA isoforms-specific knockdown. Exon 12 is the last exon.

Fig. 2. Expression profile of hENT1 mRNA isoforms in human hepatocyte and hepatoma cells.

A, qPCR was performed to characterize the hENT1 mRNA isoform expression profile in pooled (50-donor) human hepatocytes and hepatoma cells (HepG2 cells, Huh-7 cells, and FLC7 cells) using specific primer sets for hENT1a1, b, c, and d mRNA. The hENT1 mRNA level was normalized using the GAPDH mRNA level. The asterisks indicate that hENT1b mRNA expression was detectable, but that the levels were too low to be shown in the same figure as the others. Experiments were performed in duplicate, and each value is the mean ± S.D. obtained from three separate assays. **B**, hENT1d mRNA isoforms-specific knockdown was performed using the shRNA that targeted to the 5'-UTR of hENT1d mRNA (dKD-shRNA). HepG2 cells that stably expressed dKD-shRNA (dKD-HepG2 cells), and HepG2 cells stably expressing the scramble shRNA (control-HepG2 cells) were prepared using a retroviral expression system. The expression levels of the total hENT1 mRNA, hENT1c mRNA, and hENT1d mRNA were independently determined by real-time PCR. The results were presented as percentages of the relative expression levels of that of each mRNA in the control-HepG2 cells, which was set at 100%. Each value was the mean ± S.D. of the values obtained from three separate HepG2 cell preparations. **C**, qPCR was performed to examine hENT1c and hENT1d mRNA expression variability among individual human hepatocyte lines. The hENT1c mRNA levels are plotted on the x-axis, while the hENT1d mRNA levels are plotted on the y-axis. The hENT1 mRNA level was normalized using the GAPDH mRNA level. Experiments were performed in duplicate, and each value is the mean ± S.D. from three separate assays.

Fig. 3. Characterization of the *SLC29A1* gene alternative promoter regions.

A, The transactivation properties of the P1, P2 and P3 promoter regions were examined by luciferase assays. The firefly luciferase plasmids containing one of the promoters were transfected into HepG2 cells, Huh-7 cells, or FLC7 cells. The *renilla* luciferase plasmids were used as transfection control and promoter-less plasmids (empty) were used as background control. The transcriptional activity of each promoter was determined by calculating the relative ratio of luciferase activity levels (firefly/*renilla*) as the ratio obtained from empty plasmids was set to 1. Experiments were performed in duplicate, and each value is the mean ± S.D. from three separate assays. **B**, CpG sequence analyses were conducted focusing on the region +1500/-500 as the transcription start site located at the most 5'-side was set to +1. The shadowed regions indicate exons, and vertical lines indicate positions of CpG sequence. Detailed sequence analyses were performed while narrowing down the region to +500/-500 (dashed lines). The GC content was counted and normalized CpG was calculated using the formula shown in the materials and methods section.

Fig. 4. Histone 3 lysine 9 acetylation status in the *SLC29A1* gene alternative promoter regions in HepG2 cells and FLC7 cells.

ChIP assays were performed to examine the H3K9ac status in each of the *SLC29A1* gene promoters and the 3'-downstream region of the gene, which was used as a comparison. Sheared chromatin prepared from HepG2 cells or FLC7 cells was used for immunoprecipitation with either anti-histone H3 antibodies, anti-histone H3K9 acetylation antibodies, or normal rabbit IgG. Immunoprecipitated DNA was quantified using qPCR and values are represented as fold enrichment calculated with the following formula: $2^{\wedge\Delta Ct(H)}$-$2^{\wedge\Delta Ct(IgG)}$, where $\Delta Ct_{(H)}$ = Ct(input) – Ct (Histone antibodies) and $\Delta Ct_{(IgG)}$ = Ct(input) – Ct (normal IgG). The values of H3K9ac were then normalized for the value obtained from unmodified histone H3 antibodies (H3K9ac/H3). Experiments were performed with independently prepared chromatin more than three times for all cells, and the mean ± S.D. value obtained from three separate qPCR assays are shown as the representative results.

Fig. 5. Predominant isoforms in hENT1c and hENT1d mRNAs expressing in human hepatic cells and their 5'-UTR sequence analyses.

A, RLM-5'-RACE was performed to detect hENT1d1/2 and d3/4 mRNA expression independently and to confirm the hENT1c1/2/3 mRNA start site in HH187 and HepG2 cells. The figures show the results of the nested-PCR using the first RACE PCR product as a template. The asterisk indicates the band containing a previously unidentified transcription start site that should be located between those of d1/2 mRNA and d3/4 mRNA. **B**, RT-PCR was performed to separately detect the hENT1c1 and c2 mRNA expression and the hENT1d3 and d4 mRNA expression. Please note that small amounts of hENT1d1 and d2 mRNA might be included in d3 and d4 mRNA, respectively. NTC indicates non-template control. **C**, The 5'-UTR sequences of hENT1c1, c2, and d3 mRNA are shown.

The first nucleotide of each sequence is the transcriptional start site that was obtained from the RACE analysis. A single space is inserted at the junction position between the exons. The uORF was searched based on the assumption that it starts with ATG having high homology to the Kozak sequence and ends with TGA/TAG/TAA, which are indicated by the underlines. Deduced amino acids length for the first and the second uORFs in the hENT1d3 mRNA are 6 and 46, respectively. The shadowed ATG indicate the translational start codon for mature hENT1 protein.

Figures

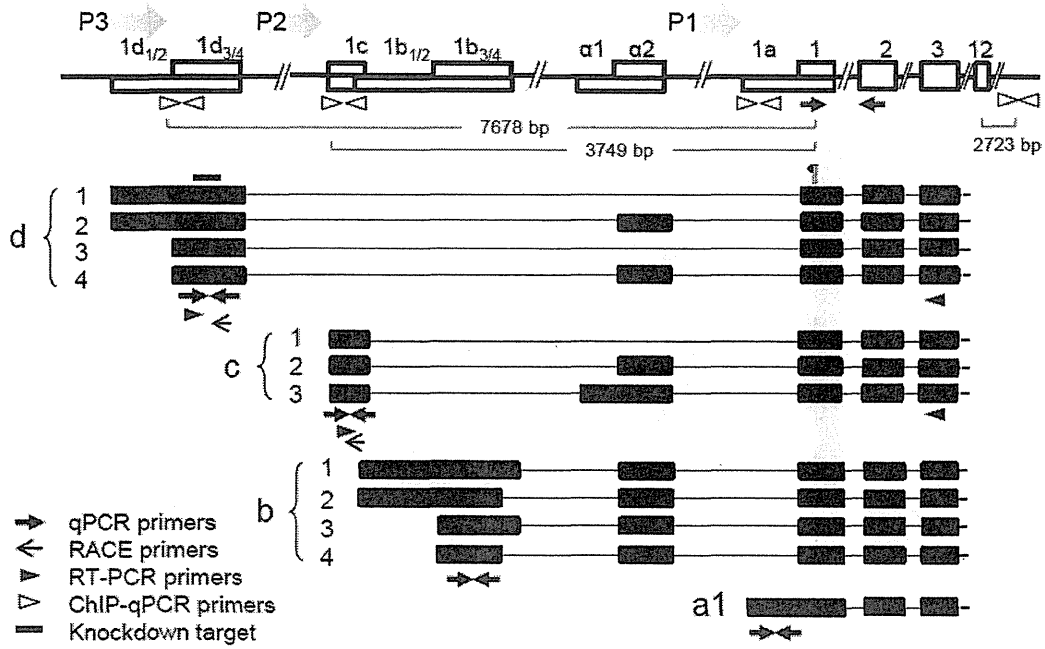


Fig. 1

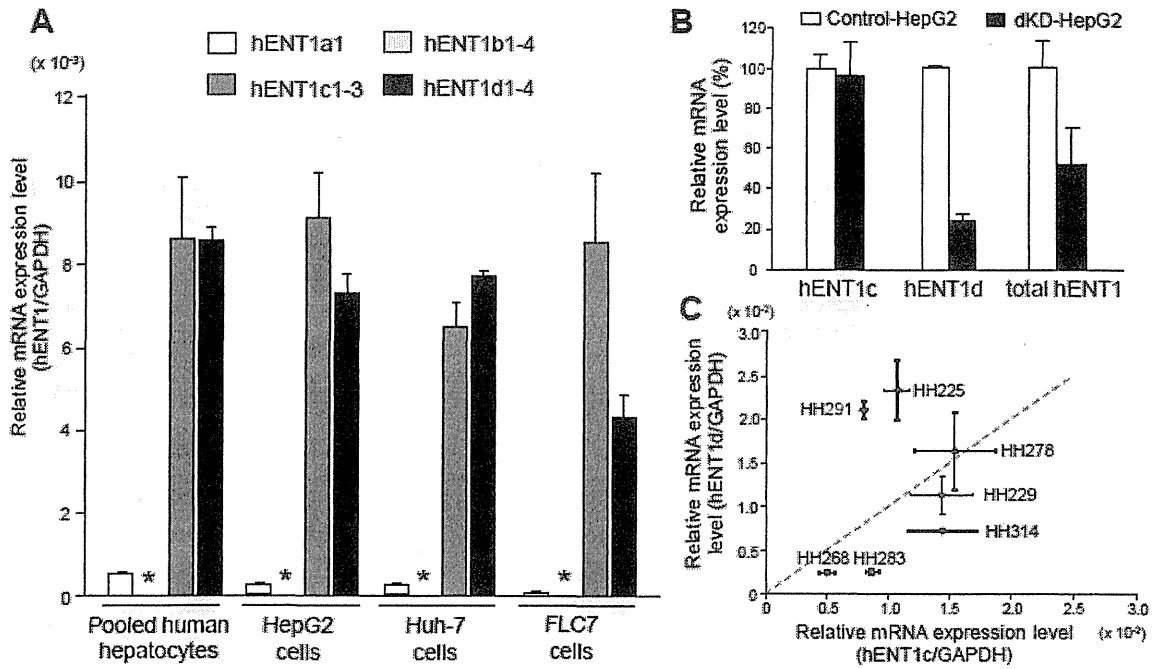

A
hENT1a1
hENT1b1-4
hENT1c1-3
hENT1d1-4
(x 10⁻³)
Relative mRNA expression level (hENT1/GAPDH)
Pooled human hepatocytes
HepG2 cells
Huh-7 cells
FLC7 cells
B
Control-HepG2
dKD-HepG2
Relative mRNA expression level (%)
hENT1c
hENT1d
total hENT1
C
(x 10⁻²)
Relative mRNA expression level (hENT1d/GAPDH)
HH225
HH291
HH278
HH229
HH314
HH268
HH283
Relative mRNA expression level (hENT1c/GAPDH)

Fig. 2

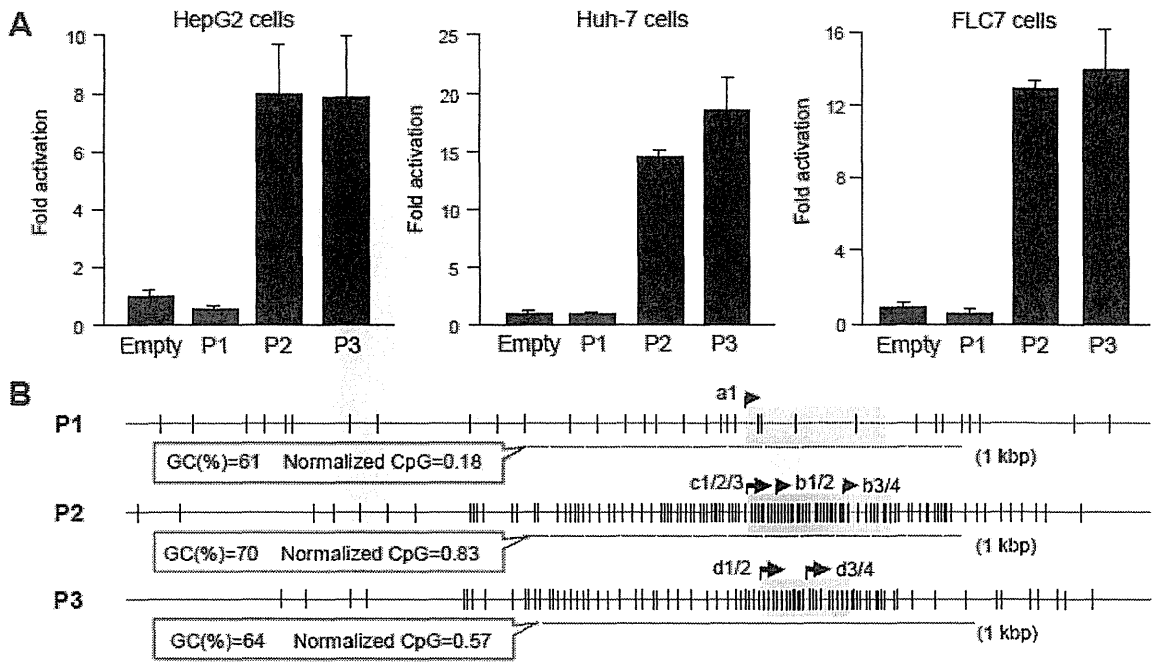

A
HepG2 cells
Huh-7 cells
FLC7 cells
Fold activation
Empty
P1
P2
P3
B
P1
P2
P3
a1
GC(%)=61 Normalized CpG=0.18
c1/2/3
b1/2
b3/4
GC(%)=70 Normalized CpG=0.83
d1/2
d3/4
GC(%)=64 Normalized CpG=0.57
(1 kbp)

Fig. 3

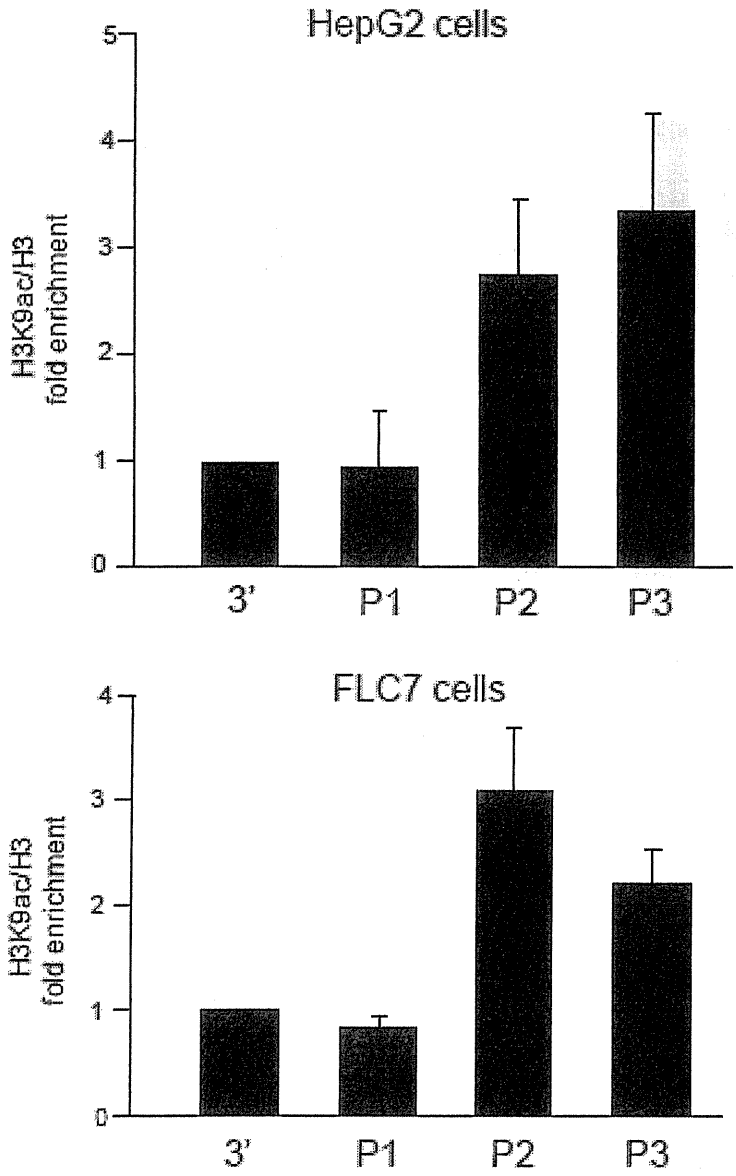
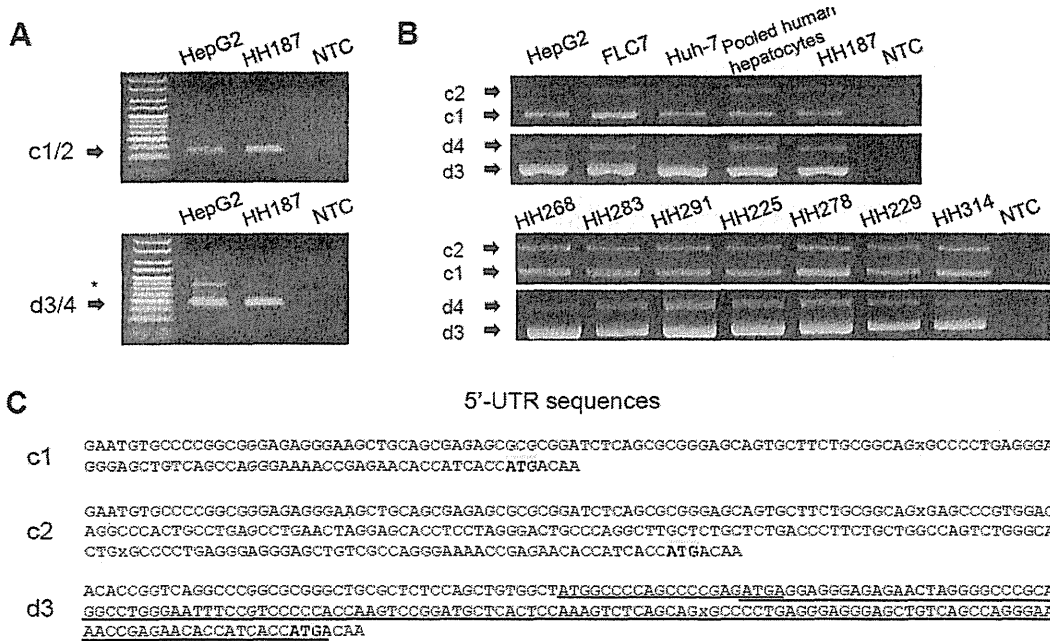

HepG2 cells
H3K9ac/H3
fold enrichment
0
1
2
3
4
5
3'
P1
P2
P3
FLC7 cells
H3K9ac/H3
fold enrichment
0
1
2
3
4
3'
P1
P2
P3

Fig. 4



C

5'-UTR sequences

c1 GAATGTGCCCCGGCGGGAGAGGGAAGCTGCAGCGAGAGCGCGCGGATCTCAGCGCGGGAGCAGTGCTTCTGCGGCAGxGCCCCTGAGGGA
GGGAGCTGTCAGCCAGGGAAAACCGAGAACACCATCACCATGACAA

c2 GAATGTGCCCCGGCGGGAGAGGGAAGCTGCAGCGAGAGCGCGCGGATCTCAGCGCGGGAGCAGTGCTTCTGCGGCAGxGAGCCCGTGGACA
AGGCCCACTGCCTGAGCCTGAACTAGGAGCACCTCCTAGGGACTGCCCAGGCTTGCTCTGCTCTGACCCTTCTGCTGGCCAGTCTGGGCAC
CTGxGCCCCTGAGGGAGGGAGCTGTCGCCAGGGAAAACCGAGAACACCATCACCATGACAA

d3 ACACCGGTCAGGCCCGGCGCGGGCTGCGCTCTCCAGCTGTGGCTATGGCCCCAGCCCCGAGATGAGGAGGGAGAGAACTAGGGGCCCGCA
GGCCTGGGAATTTCCGTCCCCCACCAAGTCCGGATGCTCACTCCAAAGTCTCAGCAGxGCCCCTGAGGGAGGGAGCTGTCAGCCAGGGAA
AACCGAGAACACCATCACCATGACAA

Fig. 5

Supplemental materials for:

Drug Metabolism and Pharmacokinetics

Identification of primary equilibrative nucleoside transporter 1 mRNA isoforms resulting from alternative promoter usage in human hepatocytes

Tomomi Furihata, Misa Mizuguchi, Yuki Suzuki, Shogo Matsumoto, Kaoru Kobayashi, Kan Chiba.

Supplemental materials and methods

Cells and cell culture

Eight human hepatocyte lines (HH187, HH225, HH229, HH268, HH278, HH283, HH291, and HH314) were purchased from Tissue Transformation Technologies (Edison, NJ) or Biopredic international (Rennes, France). The donor characteristics of these lines are shown in Table s1. Pooled (50-donor) human hepatocytes were obtained from Celsis (Baltimore, MD). Pooled hepatocytes were used based on the expectation that the individual variability of mRNA expression levels would be masked. The Ethics Committee of the Graduate School of Pharmaceutical Sciences, Chiba University, approved the use of human samples in this study.

Three human hepatoma-derived cells (HepG2 cells, Huh-7 cells, and FLC7 cells) and 293FT cells were used in this study. The HepG2 cells were obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (D-MEM) (Life Technologies, Carlsbad, CA). The Huh-7 cells were obtained from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and grown in RPMI1640 medium (Life Technologies). The FLC7 cells were provided by Dr. Seishi Nagamori (Kyorin University, Tokyo, Japan) and were cultured in D-MEM/F-12 (Life Technologies). The 293FT cells were obtained from Life Technologies and grown in D-MEM supplemented with Minimum Essential Medium Non-Essential Amino Acids (Life Technologies), GlutaMAX (Life Technologies), and G418 disulfate salt (500 μg/mL, Sigma-Aldrich, St. Louis, MO). Each medium was supplemented with 10% (v/v) fetal bovine serum, penicillin and streptomycin, and all cells were maintained at 37°C with 5% CO_2.

cDNA cloning and *in vitro* transcription

hENT1a1, hENT1c1 and hENT1d3 cDNA were obtained from human liver cDNA[19] by polymerase chain reaction (PCR) with specific primers (Table s2). Each hENT1 cDNA product was separately cloned into pcDNA3.1(-)Neo vector (Life Technologies). The DNA sequence of each insert was confirmed by the method described previously.[19]

hENT1a1, hENT1c1 and hENT1d3 mRNA were produced from the above-prepared plasmids using an *in vitro* transcription T7 kit (Takara Bio, Shiga, Japan). Template plasmids were eliminated by DNase I digestion.

Total RNA isolation and cDNA synthesis