Molecular Identification and Characterization of Novel Human and Mouse Concentrative Na\(^+\)-Nucleoside Cotransporter Proteins (hCNT3 and mCNT3) Broadly Selective for Purine and Pyrimidine Nucleosides (System cib)*

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The human concentrative (Na\(^+\)-linked) plasma membrane transport proteins hCNT1 and hCNT2 are selective for pyrimidine nucleosides (system cit) and purine nucleosides (system cif), respectively. Both have homologs in other mammalian species and belong to a gene family (CNT) that also includes hfCNT, a newly identified broad specificity pyrimidine and purine Na\(^+\)-nucleoside symporter (system cif) from the ancient marine vertebrate, the Pacific hagfish (Eptatretus stouti). We now report the cDNA cloning and characterization of cif homologs of hfCNT from human mammary gland, differentiated human myeloid HL-60 cells, and mouse liver. The 691- and 703-residue human and mouse proteins, designated hCNT3 and mCNT3, respectively, were 79% identical in amino acid sequence and contained 13 putative transmembrane helices. hCNT3 was 48, 47, and 57% identical to hCNT1, hCNT2, and hCNT3, respectively. When produced in Xenopus oocytes, both proteins exhibited Na\(^+\)-dependent cif-type functional activities. hCNT3 was electrogenic, and a sigmoidal dependence of uridine influx on Na\(^+\) concentration indicated a Na:\(^+\) uridine coupling ratio of at least 2:1 for both hCNT3 and mCNT3 (cf 1:1 for hCNT1/2). Phorbol myristate acetate-induced differentiation of HL-60 cells led to the parallel appearance of cif-type activity and hCNT3 mRNA. Tissues containing hCNT3 transcripts included pancreas, bone marrow, trachea, mammary gland, liver, prostate, and regions of intestine, brain, and heart. The hCNT3 gene mapped to chromosome 9q22.2 and included an upstream phorbol myristate acetate response element.

Most nucleosides, including those with antineoplastic and/or antiviral activities (1, 2), are hydrophilic, and specialized plasma membrane nucleoside transporter (NT) proteins are required for uptake into or release from cells (3, 4). NT-mediated transport is therefore a critical determinant of metabolism and, for nucleoside drugs, their pharmacologic actions (5). NTs also regulate adenosine concentrations in the vicinity of cell surface receptors and have profound effects on neurotransmission, vascular tone, and other processes (6, 7).

Seven nucleoside transport processes (2) that differ in their cation dependence, permeant selectivities and inhibitor sensitivities have been observed in human and other mammalian cells and tissues. The major concentrative systems (cit, cif, and cib) are inwardly directed Na\(^+\)-dependent processes and have been primarily described in specialized epithelia such as intestine, kidney, liver, and choroid plexus, in other regions of the brain, and in splenocytes, macrophages, and leukemic cells (3, 4). Concentrative NT transcripts have also been found in heart, skeletal muscle, placenta, and pancreas. The equilibrative (bi-directional) transport processes (es and ei) have generally lower substrate affinities and occur in most, possibly all, cell types (3, 4). Epithelia (e.g. intestine and kidney) and some nonpolarized cells (e.g. leukemic cells) coexpress both concentrative and equilibrative NTs, whereas other nonpolarized cells (e.g. erythrocytes) exhibit only equilibrative NTs (3, 4). Systems cit and cif are generally pyrimidine nucleoside selective and purine nucleoside selective, respectively, whereas systems cib, es, and ei transport both pyrimidine and purine nucleosides. System ei also transports nucleobases.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF305210 and AF305211.

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1 The abbreviations used are: NT, nucleoside transporter; AZT, 3′-azido-3′-deoxythymidine; BAC, bacterial artificial chromosome; CNT, concentrative nucleoside transporter; hp, base pair(s); ENT, equilibrative nucleoside transporter; kb, kilobase(s); NBMPR, nitrobenzylthioinosine 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NMDG, N-methyl-D-glucamine; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; TM, putative transmembrane helix; EST, expressed sequence tag; contig, group of overlapping clones.

2 The abbreviations used in transporter acronyms are: c, concentrative; e, equilibrative; s and i, sensitive and insensitive to inhibition by NBMPR, respectively; f, formycin B (nonmetabolized purine nucleoside); t, thymidine; g, guanosine; b, broad selectivity.
Molecular cloning studies have isolated cDNAs encoding the human and rat proteins responsible for four of these NT processes (cit, cif, es, and ei) (8–17). These proteins and their homologs in other mammalian species comprise two previously unrecognized families of integral membrane proteins (CNT and ENT) with quite different predicted architectural designs (3, 4). The relationships of these NT proteins to the processes defined by functional studies are: CNT1 (cit), CNT2 (cif), ENT1 (es), and ENT2 (ei). Although the NT protein(s) responsible for mammalian cib have remained elusive, we have recently identified a CNT protein with cib-type transport activity from the ancient marine vertebrate, the Pacific hagfish (Eptatretus stouti) (18). The CNT family also includes the Escherichia coli proton/nucleoside symporter NupC (19). Human and rat CNT1 (650 and 648 residues, 71 kDa), designated hCNT1 and rCNT1, respectively, are 83% identical in amino acid sequence (8, 11) and contain 13 putative TM s with an exofacial glycosylated tail at the carboxyl terminus (18).4 hCNT2 (658 residues) (12, 13) is 83% identical to rCNT2 (659 residues) (9, 10) and 72% identical to hCNT1 (11). The hagfish transporter hCNT1 (683 residues) (18) is 50–52% identical to h/rCNT1/2 and has a similar predicted membrane topology. NupC (19), in contrast, is a smaller protein with 27% identity to mammalian CNTs, with the major difference being the absence of the equivalents of TM 1–3 and the amino- and carboxyl-terminal regions of the other proteins.

In structure/function studies, the characteristics of hCNT1/2 chimeras and sequence comparisons between h/rCNTs and hCNT1 have identified two sets of adjacent residues in TM 7 and 8 of hCNT1 that, when converted to the corresponding residues in hCNT2, changed the specificity of the transporter from cit to cif (18). Mutation of the two residues in TM 7 alone produced a protein with intermediate, cif-like activity. In this cit/cif conversion, mutation of hCNT1 Ser319 to Gly was sufficient to enable transport of purine nucleosides, whereas mutation of the adjacent residue Gln320 to Met (which had no effect on its own) augmented this transport. TM 7 and 8 have also been identified as potential determinants of substrate selectivity in rCNT1/2 (21), and mutation of rCNT1 Ser318 (the rat counterpart of hCNT1 Ser319) resulted in a cif-like phenotype similar to that seen with the hCNT1 Ser319 mutation (22).

Although an earlier study had identified a member of the SGLT glucose transporter family, SNST1, as a candidate cib-type transporter (23), its nucleoside-transport activity is very low, and we hypothesized that the missing mammalian concentrative NT was more likely to be a CNT transporter. Following a search for additional mammalian CNT isoforms, we now report the cDNA cloning of new human and mouse members of the CNT transporter family. The encoded proteins, designated hCNT3 and mCNT3, respectively, exhibit strong cib-type functional activity when expressed in Xenopus oocytes and have primary structures that place them together with hCNT in a CNT subfamily separate from h/rCNT1/2.

EXPERIMENTAL PROCEDURES

Molecular Cloning of hCNT3—BLAST searches of CNT sequences in the GenBankTM data base identified overlapping human ESTs from mammary gland (AI905993) and colon adenocarcinoma (AW803022) different from established members of the CNT transporter family. Together, they formed a composite cDNA fragment 807 bp in length with an open reading frame of 245 residues followed by 69 bp of 3′-untranslated sequence. The cDNA was 62% identical in nucleotide sequence to corresponding regions of the hCNT1 (U62968) and hCNT2 (AF036109) cDNAs and 68% identical to the hCNT1 (AF132298) cDNA. The encoded amino acid sequence was 79% identical to the carboxyl terminus of hCNT and 58 and 62% identical, respectively, to hCNT1 and hCNT2.

These indications of a novel human CNT distinct from hCNT1 and hCNT2 were tested by RT-PCR in a panel of total RNA samples from human mammary gland, small intestine, kidney (CLONTECH, Palo Alto, CA), and liver (13). PCR reaction (30 μl) contained 50 ng total RNA and a complete first-strand cDNA, 2.5 units of Taq-DeepVent DNA polymerase (100:1) and 10 pmol each of the 5′- and 3′-oligomeric primers primers 5′-AAAGATGTTTGGACCCACCG-3′ and 5′-GGGACCGTGAAGCATTCTTCAAACGT-3′. Amplification for one cycle at 94 °C for 55 s, 54 °C for 55 s, and 72 °C for 70 s, and 30 cycles at 94 °C for 55 s, 55 °C for 55 s, and 72 °C for 70 s, and 72 °C for 70 s (Robocycler® 60 Temperature Cycler, Stratagene, La Jolla, CA) generated visible PCR products of the predicted size (480 bp) from four of the samples (differentiated HL-60 cells, mammary gland, small intestine, and liver).

We extended the partial EST cDNA sequence by 5′-rapid amplification of cDNA ends amplification of mRNA from differentiated HL-60 cells using the FirstChoice RLM-RACE kit (Ambion, Austin, TX, Poly(A)+)–selected RNA was treated with calf intestinal phosphatase to degrade 5′-truncated transcripts, followed by tobacco acid pyrophosphatase to remove cap from the remaining full-length mRNAs. A synthetic RNA adaptor from the kit was then ligated to the full-length 5′-monophosphate transcript population using T4 RNA ligase, followed by first strand cDNA synthesis with oligo(dT) as primer. For the initial PCR, the 5′-primer was the outer adaptor primer provided by the kit and the gene-specific 3′-primer was 5′-GATATATATGTGCTGCACACGC-TTTTACAA-3′. Amplification by Taq-DeepVent DNA polymerase (100:1) was for 40 cycles at 94 °C for 55 s, 65 °C for 55 s, and 72 °C for 3 min and 1 cycle at 72 °C for 10 min, the reaction mixture being heated to 94 °C for 1 min before addition of the Taq-DeepVent DNA polymerase mixture. The PCR reaction mixture was resolved on a 1% agarose gel, and faint bands between 1.5 and 2.0 kb in size from human liver, mammary gland, and purified (QIAE II Gel Extraction kit; Qiagen, Inc.) PCR products was then reamplified by nested PCR (35 cycles at 94 °C for 55 s, 65 °C for 55 s, and 72 °C for 3 min and 1 cycle at 72 °C for 10 min) using an inner 5′-primer from the kit and the gene-specific 3′-primer 5′-TGTACCTAAAACCTACGCTGGTGAGTGC-3′. A defined band of −1.7 kb was identified and cloned into pGEM-T (Promega, Madison, WI), and sequenced by Taq DyeDeoxyterminator cycle sequencing using an automated model 373A DNA Sequencer (Applied Biosystems, Foster City, CA). The insert was overlapped the 807-bp EST sequence by 114 bp and generated an additional 1633 bp of upstream sequence. The new composite 2440-bp sequence was 66% identical to the hCNT1 cDNA and contained an open reading frame of 691 amino acids. cDNAs containing the complete coding region were then obtained by RT-PCR using total RNA from HL-60 and cells from mouse liver RNA (Jackson Laboratories, Bar Harbor, ME) with 5′-primer 5′-AGATGTTTGGACCCACCG-3′ and 3′-primer 5′-AGATCGCATTTATATAGGATTTCAACAGG-3′. First strand cDNA was synthesized using the Thermoscript

2 S. Y. M. Yao, A. M. L. Ng, S. K. Loewen, C. E. Cass, and J. D. Young, manuscript in preparation.
3 R. Hamilton, S. Y. M. Yao, M. P. Gallagher, P. J. F. Henderson, C. E. Cass, J. D. Young, and S. A. Baldwin, manuscript in preparation.

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were filled with 3M KCl and had resistances that ranged from 1–2.5 MΩ. The microelectrodes were fabricated using a glass pipette and a Wheatstone bridge circuit and had a tip diameter of 3–5 μm. At the end of the incubation period, extracellular label was removed by six rapid washes in ice-cold transport medium, and individual oocytes were dissolved in 5% (w/v) trichloroacetic acid.

**Human and Mouse CNT3**

RT-PCR System (Life Technologies, Inc.), and amplification by Taq-DeepVent DNA polymerase (100:1) was for 2 cycles at 94 °C for 2 min, 64 °C for 1 min, and 72 °C for 2.5 min, 2 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 2.5 min, 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2.5 min, and one final extension cycle for 10 min at 72 °C. The 2°/°C °C °C construct gave greater functional activity and was used in subsequent transport characterization of the mouse protein. pGEM-HE was also used for electrophysiological studies of hCNT3.

**Expression of Recombinant hCNT3 and mCNT3 in Xenopus Oocytes—**hCNT3 and mCNT3 plasmid DNAs were linearized with NotI (pGEM-T) or NheI (pGEM-HE) and transcribed with T7 polymerase mMESSAGE mMACHINE™ (Ambion). Stage VI oocytes of Xenopus laevis (8) were microinjected with 20 nl of water or 20 nl of water containing capped RNA transcripts (20 ng) and incubated in modified Barth’s medium (changed daily) at 18 °C for 72 h prior to the assay of transport activity.

**hCNT3 and mCNT3 Radioisotope Flux Studies—**Transport was traced using the appropriate [3H]-labeled nucleoside, nucleoside drug, or nucleobase (Moravek Biochemicals, Brea, CA or Amersham Biosciences). Each sample of mRNA (0.5 ng) from parent and differentiated HL-60 cells on Bright-Star-Plus nylon transfer membrane (Ambion) was incubated with a cDNA probe corresponding to hCNT3 amino acid residues 359–549 labeled with [3P] using the [3P]QuickPrime kit (Amersham Pharmacia Biotech). Hybridization at high stringency (88 °C) was performed using ExpressHyb hybridization solution (CLONTECH) and 100 μg/ml of sheared herring sperm DNA. Wash conditions were as described in the CLONTECH ExpressHyb user manual. Signals on exposed blots were converted to a high resolution tiff image (Heiwlett Packard ScanJet 4C) and quantified using the public domain NIH Image program, version 1.60. For Northern analysis, 5-μg samples of mRNA from human pancreas, bone marrow, trachea, intestine, liver, brain, heart, and kidney (CLONTECH) were separated on a 0.8% formaldehyde-agarose gel, and blotted to BrightStar-Plus nylon transfer membrane, and hybridized with the same hCNT3 probe (residues 359–549) under identical high stringency conditions.

Possible cross-hybridization between CNT family members was tested on dot blots of dilutions (0.5 μg-5 ng) of hCNT1, hCNT2, and hCNT3 in vitro transcripts. Three identical series of blotts were probed with hCNT3 probe or with equivalent probes for hCNT1 or hCNT2. The hCNT3 probe, which was 63% and 58% identical in nucleotide sequence to the corresponding regions of hCNT1 and hCNT2, respectively, showed no cross-hybridization with hCNT1 or hCNT2 transcripts. Similarly, there was no cross-reactivity between the hCNT1 and hCNT2 probes and hCNT3 RNA. Some cross-hybridization was seen between the hCNT1 and hCNT2 probes (73% nucleotide identity) and respective transcripts at RNA loadings ≥50 ng. Under the conditions of high stringency used in our experiments, the hCNT3 probe was therefore specific.

**Quantitative Real Time RT-PCR—**In TaqMan™ quantitative RT-PCR (Applied Biosystems), an oligonucleotide probe, labeled with a fluorescent tag at the 5'-end and a quenching molecule at the 3'-end, is located between two PCR primers. The 5'-nucleotide cleavage activity of Taq polymerase cleaves the fluorescent dye from the probe during each PCR cycle. The fluorescence signal generated is monitored in real time and is proportional to the amount of starting template in the sample.

RNA from parent or differentiated HL-60 cells was reverse transcribed using the TaqMan™ Gold RT-PCR kit (Applied Biosystems) and subjected to real time PCR using an Applied Biosystems PRISM 7700 Sequence Detection System and TaqMan™ Universal PCR Master Mix kit. Amplification conditions were a single cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C using hCNT3 probe and primers designed using Primer Express software (Applied Biosystems). The hCNT3 probe 5'-6-carboxy-fluorescein-CGGAGCTCATACATCATGTCCTC-3' was purchased from Operon. The 5'-3'-primers were 5'-GGGTTCCCTAGGAATCTGATC-3' and 5'-CGAGGCGGATACCGCTTCTC-3', respectively. GAPDH and 18 S ribosomal RNA probes and primers, used as internal controls, were purchased as a TaqMan™ RNA Control Reagent kit. Relative quantification of hCNT3 message was determined as described previously (27).
have recently established that systems well, a broadly selective transport activity for both pyrimidine
nucleosides and nucleoside drugs is mediated by at least three
distinct mechanisms (3, 4). Systems
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distinct mechanisms (3, 4).

RESULTS AND DISCUSSION

Membrane transport studies in various human and other
mammalian cell and tissue preparations have produced evi-
dence that concentrative (Na\(^{+}\)-linked) cellular uptake of
nucleosides and nucleoside drugs is mediated by at least three
distinct mechanisms (3, 4). Systems
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nucleosides and nucleoside drugs is mediated by at least three
distinct mechanisms (3, 4).

Molecular Identification of hCNT3 and mCNT3—The possi-
bility that cib might be a CNT transporter (18) led to identi-
fication of ESTs encoding partially overlapping regions at the
carboxy terminus of a new, previously unrecognized human
CNT distinct from hCNT1 or hCNT2. The full-length cDNA
obtained by 5′-rapid amplification of cDNA ends/RT-PCR
amplification of phorbol 12-myristate 13-acetate-differentiated
human myeloid HL-60 cells and by RT-PCR of human mam-
mary gland encoded a 691-residue protein (77 kDa), designated
here as hCNT3.\(^5\) Differentiated HL-60 cells represent a func-
tionally defined source of cib transport activity (see below),
whereas human mammary gland was the origin of one of the
carboxy terminus ESTs. The hCNT3 sequence enabled us, in
turn, to identify ESTs from mouse mammary gland encoding
the amino- and carboxy terminus ends of a mouse homolog.
The corresponding full-length mouse cDNA, obtained by RT-
PCR from liver (also a source of hCNT3 transcript), encoded a
703-residue protein designated here as mCNT3.\(^5\)

hCNT3 and mCNT3 Amino Acid Sequences—hCNT3 was
57% identical in amino acid sequence to hCNT1 and 48 and 47%
identical to hCNT1 and hCNT2, respectively (Fig. 1A). Protein
structure algorithms predicted a topology for hCNT3 similar to
that of hCNT1 and hCNT1/2 (20), with relatively large extra-
membrane domains and carboxy termini (carboxy terminus
external) linked by 13 TMs and short hydrophilic se-
quences (≤22 residues), with the exception of larger
extracellular loops between TMs 5–6, 9–10, and 11–12 (Fig.
1B). Residues within TMs 4–13 were particularly highly
conserved between hCNT3 and hCNT1 (67% sequence identity),
whereas TMs 1–3 and the amino and carboxy termini were
much more divergent. The conserved TM 4–13 domains of
hCNT3 and hCNT1 corresponded closely to the predicted mem-
brane architecture of the shorter E. coli CNT proton/nucleoside
cotransporter NupC (19), suggesting that these regions repres-
ent the functionally important core structure of the proteins.

We engineered an amino-terminal truncated form of rCNT1
and established that the TM 1–3 region is not required for
transport activity.\(^6\) mCNT3 contained additional amino acids
at the amino terminus (Fig. 1A) and was 78% identical in

sequence to hCNT3, 57% identical to hCNT1, and 48% identical
to mCNT2 (AF079853), the other known mouse CNT.

Since we first identified rCNT1 from rat jejunum by expres-
sion selection in Xenopus oocytes in 1994, more than 40 mem-
bers of the CNT protein family have been identified from mam-
mals, lower vertebrates, insects, nematodes, pathogenic yeast,
and bacteria. As shown in Fig. 1C, phylogenetic analysis iden-
tified discrete clusters of proteins, including two for bacteria
and one for vertebrate transporters. hCNT3 and mCNT3 were
placed together with hCNT1 in a different vertebrate CNT
subfamily from the human and other mammalian CNT1 and
CNT2 proteins. Characteristically conserved motifs of the CNT
transporter family present in hCNT3 and mCNT3 included
OX(G/A)OX,FXFG between TMs 5 and 6, (G/A)XX(N/T)E(F/Y)-
(Y/F/T)(A/G/S)(Y/M/F) between TMs 11 and 12, and (G/S)(F/A-
S)N(F/P)(S/G)(S/T)(G/A) in TM 12. In common with other
CNTs, hCNT3 and mCNT3 also contained multiple consensus
sites for N-linked glycosylation, grouped at the carboxyl termin-
u (hCNT3 Asn\(^636\) and Asn\(^664\), mCNT3 Asn\(^648\) and Asn\(^676\)).
The extracellular location of this region has been confirmed by
mutagenesis of rCNT1, which is glycosylated at Asn\(^605\) and
Asn\(^643\) (20).

Previously (18), we have identified two adjacent pairs of
residues (Ser\(^319\)/Gln\(^320\) and Ser\(^353\)/Leu\(^354\)) in the TM 7–9 region
of hCNT1 that, when mutated together to the corresponding
residues in hCNT2 (Gly\(^313\)/Met\(^314\) and Thr\(^347\)/Val\(^348\)),
converted hCNT1 (cif) into a transporter with cif-type functional
characteristics. An intermediate broad specificity cif-like
transport activity was produced by mutation of the two TM 7
residues alone; mutation of Ser\(^319\) to Gly allowed for transport
of purine nucleosides, and this was augmented by mutation of
Gln\(^320\) to Met. Mutation of Ser\(^353\) to Thr converted the
CNT proton/nucleoside
transport capability of the TM 7/8 triple mutant, producing a
full cif transport phenotype. On its own, mutation of Ser\(^353\)
converted hCNT1 into a transporter with novel uridine-selective
transport properties. The sequences of hCNT3 and mCNT3 at
these positions were intermediate between hCNT1 and
hCNT2, one member of each pair of residues being identical to
the corresponding residue in hCNT1 and the other to that in
hCNT2. These sequences in hCNT3 and mCNT3 were identical to
hCNT1 (Gly\(^349\)/Gln\(^341\) and Ser\(^375\)/Val\(^375\) in the case of
hCNT3).

Functional Expression and Substrate Specificity of Recombi-
nant hCNT3 and mCNT3—hCNT1 and hCNT2 display cif- and
cif-type Na\(^{+}\)-dependent nucleoside transport activities (11, 13).
Therefore, although both hCNT1 and hCNT2 transport uridine
and certain uridine analogs, they are otherwise selective for
pyrimidine (hCNT1) and purine (hCNT2) nucleosides (except
for modest transport of adenosine by hCNT1). hfCNT, in con-
trast, exhibits cif-type Na\(^{+}\)-dependent nucleoside transport
activity and is broadly selective for both pyrimidine and
purine nucleosides.

Fig. 2A shows a representative transport experiment in Xe-
opus oocytes measuring uptake of uridine and a panel of other
radiolabeled pyrimidine and purine nucleosides (cytidine, thy-
midine, adenosine, guanosine, and inosine) and nucleobases
(uracil and hypoxanthine) in cells injected with water alone
(control) or with water containing hCNT3 transcripts. Uptake
of uridine (20 μM, 30-min flux) by hCNT3-expressing oocytes
was Na\(^{+}\)-dependent (60.7 ± 4.5 and 6.1 ± 0.7 pmol/oocyte in

\(^5\) GenBank\(^\text{™}\) / EBI Data Bank accession numbers AF305210 and
AF305211.

\(^6\) M. W. L. Ritzel, A. M. L. Ng, S. Y. M. Yao, K. Graham, S. K. Loewen,
K. M. Smith, R. G. Ritzel, D. A. Mowles, P. Carpenter, X.-Z. Chen, E.
Karpinski, R. J. Hyde, S. A. Baldwin, C. E. Cass, and J. D. Young,
unpublished observation.
FIG. 1. hCNT3 and mCNT3 are members of the CNT family of nucleoside transporters. A, alignment of the predicted amino acid sequences of hCNT3 (GenBank™ accession number AF305210) and mCNT3 (GenBank™ accession number AF305211) with those of hCNT1 (GenBank™ accession number U62968), hCNT2 (GenBank™ accession AF036109), and hfCNT (GenBank™ accession number AF132298), using the GCG PILEUP program. Potential membrane-spanning α-helices are numbered. Putative glycosylation sites in predicted extracellular domains of hCNT3, mCNT3, hCNT1, hCNT2, and hfCNT are shown in lowercase (n), and their positions are highlighted by an asterisk above the aligned sequences. Residues in hCNT3 identical to one or more of the other transporters are indicated by black boxes. B, topological model of hCNT3 and hfCNT. Potential membrane-spanning α-helices are numbered, and putative glycosylation sites in predicted extracellular domains in hCNT3 and hfCNT are indicated by solid and open stars, respectively. Residues identical in the two proteins are shown as solid circles. Residues corresponding to insertions in the sequence of hCNT3 or hfCNT are indicated by circles containing 1 and 2 signs, respectively. C, phylogenetic tree showing relationships between hCNT3 and mCNT3 and other eukaryotic and prokaryotic members of the CNT transporter family. In addition to those listed in A, these are: rCNT1 (rat CNT1, GenBank™ accession number U10279); pkCNT1 (pig kidney CNT1, GenBank™ accession number AF009673); rCNT2 (rat CNT2, GenBank™ accession number U25055); mCNT2 (mouse CNT2, GenBank™ accession number AF172652); rbCNT2 (rabbit CNT2, GenBank™ accession number AF161716); F27E11.1 (Caenorhabditis elegans, GenBank™ accession number AF016413); CG11778_DROME (Drosophila melanogaster, GenBank™ accession number AA585996); CG8083_DROME (D. melanogaster, GenBank™ accession number AF458997); F27E11.2 (C. elegans, GenBank™ accession number AF016413); YEIM_HAEIN (Hemophilus influenzae, Swisprot accession number P47490); HP1890_HELPR (Helicobacter pylori, GenBank™ accession number AF000622); YEIM_ECOLI (E. coli, Swisprot accession number P36024); YEIL_ECOLI (E. coli, Swisprot accession number P36021); YXJA_BACSU (Bacillus subtilis, Swisprot accession number P42312); NUPC_ECOLI (E. coli, Swisprot accession number P30301); NUPC_BACSU (B. subtilis, Swisprot accession number P39141); HI0519_HAEIN (H. influenzae, GenBank™ accession number U32734); YUTK_BACSU (B. subtilis, GenBank™ accession number Z99120); VC2352_VIBCH (Vibrio cholerae, GenBank™ accession number AA95495); VC1953_VIBCH (V. cholerae, GenBank™ accession number AA95101); VCA0179_VIBCH (V. cholerae, GenBank™ accession number AA96092); UNKNOWN_STREP (Streptococcus pyogenes, open reading frame (284) present in contig0001 from the S. pyogenes genome sequencing project, Oklahoma University); UNKNOWN_YERPE (Yersinia pestis,
Na\(^+\) and choline medium, respectively) and concentrative (60.7 pmol/oocyte corresponds to an in-to-out concentration ratio of \(~3:1\), calculated assuming an oocyte water content of 1 \(\mu\)l). In Na\(^+\) medium, uridine uptake in control water-injected oocytes was only 0.5 ± 0.1 pmol/oocyte, giving a mediated flux (uptake by RNA-injected oocytes minus uptake in water-injected oocytes) of 60.2 pmol/oocyte and a mediated-to-basal flux ratio of 120:1. Consistent with cib-type functional activity, each of the other pyrimidine and purine nucleosides tested (cytidine, thymidine, adenosine, guanosine, and inosine) gave similar mediated fluxes. mCNT3 (Fig. 2B) exhibited a similar pattern of Na\(^+\)-dependent cib-type functional activity, and neither protein transported uracil or hypoxanthine.

Fig. 3 compares the differences in substrate specificity between hCNT3, mCNT3, hCNT1, and hCNT2 by measuring the mediated uptake of three diagnostic nucleoside permeants (uridine, thymidine, and inosine). All five proteins transported uridine. However, hCNT1 (cif) exhibited pyrimidine nucleoside selective characteristics (marked thymidine uptake, low inosine transport), whereas hCNT2 (cif) was purine nucleoside selective (low thymidine uptake, marked inosine transport). hCNT3, mCNT3, and hfCNT exhibited similar cib-type profiles, with marked transport of both thymidine and inosine. Subsequent in depth transport experiments focused on the human transporter hCNT3.

Kinetic Properties and Inhibitor Sensitivity of Recombinant hCNT3—Fig. 4 shows representative concentration dependence curves for uridine, cytidine, thymidine, adenosine, guanosine, and inosine, measured as initial rates of transport (5-min flux) in hCNT3-expressing oocytes and in control water-injected cells. Kinetic constants for the hCNT3-mediated component of influx are presented in Table I. \(K_m\) values varied between 15 and 53 \(\mu\)M (cytidine, adenosine < uridine, thymidine < guanosine, inosine) and were within the range expected for native cib-type transporters (30–32) and for hCNT in oocytes (17–54 \(\mu\)M). They were also similar to \(K_m\) values obtained previously for permeants of recombinant mammalian CNT1/2 transporters. For example, the hCNT3 \(K_m\) for uridine was 22 \(\mu\)M compared with 37–45 \(\mu\)M for hCNT1, rCNT1, and hCNT2 (8, 11, 13). hCNT3 \(K_m\) values for thymidine and inosine were 21 and 53 \(\mu\)M, respectively, compared with 13 \(\mu\)M for thymidine transport by rCNT1 (33) and 20 \(\mu\)M for inosine transport.

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**Fig. 1—continued**

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open reading frame present in contig971 from the *Y. pestis* genome sequencing project, Sanger Center); UNKNOWN_YERPE (*Y. pestis*, open reading frame present in contig976 from the *Y. pestis* genome sequencing project, Sanger Center); UNKNOWN_SALTY (*Salmonella typhi*, open reading frame present in contig18 (CT18) from the *S. typhi* genome sequencing project, Sanger Center); UNKNOWN_BACAN (*Bacillus anthracis*, open reading frame in contig185 from the *B. anthracis* genome sequencing project, TIGR); UNKNOWN_BACAN (*B. anthracis*, open reading frame in contig1745 from the *B. anthracis* genome sequencing project, TIGR); UNKNOWN_CAUCR (*Caulobacter crescentus*, open reading frame present in contig12574 from the *C. crescentus* genome sequencing project, TIGR); UNKNOWN_STAU (*Staphylococcus aureus*, open reading frame present in contig6185 from the *S. aureus* genome sequencing project, TIGR); UNKNOWN_STAU (*S. aureus*, open reading frame present in contig6186 from the *S. aureus* genome sequencing project, TIGR); UNKNOWN_SHEPU (*Shewanella putrefaciens*, open reading frame present in contig6184 from the *S. putrefaciens* genome sequencing project, TIGR); UNKNOWN_SHEPU (*S. putrefaciens*, open reading frame present in contig6410 from the *S. putrefaciens* genome sequencing project, TIGR); UNKNOWN_SHEPU (*S. putrefaciens*, open reading frame present in contig6413 from the *S. putrefaciens* genome sequencing project, TIGR); PM1292_SHEPU (*Pasteurella multocida*, open reading frame gene product PM1292 from the *P. multocida* genome sequencing project, University of Minnesota); UNKNOWN_CANAL (*Candida albicans*, open reading frame present in contig5–2704 from the *C. albicans* genome sequencing project, Stanford); and UNKNOWN_HAEDU (*Hemophilus ducreyi*, open reading frame present in contig730 from the *H. ducreyi* genome sequencing project, University of Washington). The phylogenetic tree was constructed from a multiple alignment of the 43 CNT sequences using ClustalX version 1.81 for Windows (50) and KITSCH, PHYLIP version 3.57c (51) software (20, 53). The CNT3/hfCNT and CNT1/2 subfamilies are highlighted.
transport by rCNT2 (21). hCNT3 V_max values were in the range 24 and 51 pmol/oocyte.min⁻¹ (uridine, thymidine < cytidine, adenosine < guanosine, inosine), giving V_max/K_m ratios of 0.9–2.1 (Table I). These data support the cib-type specificity profile of hCNT3 shown in Fig. 2A and demonstrate that hCNT3 transports different pyrimidine and purine nucleosides with very similar efficiencies. For all of the nucleosides tested (Fig. 4), influx in water-injected oocytes was linear with concentrations approaching the physiological concentration range for the Na⁺/nucleoside coupling ratio of at least 2:1. Similar values of the Hill coefficients (n) were determined from the slopes of Hill plots of the data (Fig. 6, B and D), and in five independent experiments, Hill plot transformations gave a mean hCNT3 Hill coefficient of 2.1 ± 0.3. Because rCNT1 exhibited a Hill coefficient of 1 in similar experiments (10), our data establish, for the first time, that the stoichiometry of Na⁺/nucleoside coupling is different in different CNT family members. In this respect, the CNTs resemble the SGLT glucose transporter family, where examples of proteins with 2:1 and 1:1 Na⁺/sugar coupling ratios (SGLT1 and SGLT2, respectively) have been described (36–39). Similarly, the PepT2 and PepT1 proton-linked peptide transporters have 2:1 and 1:1 H⁺/peptide coupling ratios, respectively (40). There was an interesting difference in K_m values for Na⁺ activation between hCNT3 and mCNT3 (16 ± 1 and 7 ± 1 mM, respectively), although both transporters were fully saturated with Na⁺ at cation concentrations approaching the physiological concentration range (Fig. 6, A and C).

In addition to radioisotope flux studies, we also used the two-electrode voltage clamp technique to investigate the Na⁺ dependence of hCNT3-mediated nucleoside transport. As shown in Fig. 7, external application of uridine, thymidine, and inosine (200 μM) to oocytes expressing recombinant hCNT3 induced inward currents for all three nucleosides that returned to baseline upon removal of permeant. No currents were seen in water-injected oocytes or when Na⁺ in the extracellular medium was replaced by choline, demonstrating that hCNT3 functions as a broad specificity electrogenic Na⁺/nucleoside symporter. In parallel with the radioisotope transport data shown in Fig. 6 (A and C), there was a sigmoid relationship between uridine-evoked current and Na⁺ concentration (data not shown). In preliminary experiments to determine directly the Na⁺/nucleoside coupling ratio by simultaneous measurement of Na⁺ currents and [¹⁴C]uridine influx under voltage clamp conditions, as described previously for the SDCT1 rat kidney dicarboxylate transporter (41), we have confirmed that hCNT3 has a Na⁺/nucleoside coupling ratio ≥ 2:1, whereas for hCNT1 the ratio is 1:1.⁶ A two-Na⁺/one-nucleoside symporter...
will have a greater ability to transport permeant against its concentration gradient than a one-Na\(^+\)/one-nucleoside symporter, and they may have evolved to transport nucleosides under different conditions. Experiments are in progress with hCNT, mCNT3, and other CNTs to determine whether the 2:1 stoichiometry is limited to members of the CNT3/hfCNT subfamily (Fig. 2A) or has a more widespread distribution.

**hCNT3-mediated Transport of Anticancer and Antiviral Nucleoside Drugs**—The difference in substrate specificity between CNT1 and CNT2 for physiological pyrimidine and purine nucleosides is reflected in their complementary roles for transport of pyrimidine and purine antiviral and anticancer nucleoside drugs. Lower, but still significant, uptake was observed for pyrimidine (AZT and 2’,3’-dideoxycytidine) and purine (2’,3’-dideoxyinosine) antiviral nucleoside drugs, the magnitudes of the fluxes being similar to those found previously for hCNT1 (AZT and 2’,3’-dideoxycytidine) and hCNT2 (2’,3’-dideoxyinosine) (11, 13). Only ganciclovir, an antiviral drug with an acyclic ribose moiety, was not transported. Therefore, by virtue of its ability to transport both pyrimidine and purine nucleosides, hCNT3 is capable of transporting a broader range of therapeutic nucleosides than either hCNT1 or hCNT2.

**Tissue and Cell Distribution**—The cib process has been described functionally in rabbit choroid plexus (30), rat MSL-9 microglia cells (31), *Xenopus* oocytes injected with rat jejunal mRNA (32), human leukemic (45) and colorectal carcinoma CaCo cells (46) and, after induction of differentiation, in human HL-60 cells (see below). The human and mouse ESTs that led to the identification of h/mCNT3 were from human/mouse mammary gland and human colon adenocarcinoma, whereas the full-length transporter cDNAs were isolated from differentiated HL-60 cells and mammary gland (hCNT3) and liver (mCNT3). Fig. 9 shows a multiple tissue expression RNA array for 76 human tissues and cells probed with hCNT3 cDNA (a second commercial RNA array from the same supplier gave essentially identical results). As described under “Experimental Procedures,” this analysis was performed under conditions of high stringency where there was no cross-hybridization between hCNT3 and hCNT1 or hCNT2. The distribution pattern of hCNT3 transcripts, although selective, was surprisingly widespread. Highest levels were found in a number of normal tissues, including mammary gland, pancreas, bone marrow, and trachea, with substantial levels in various regions of the intestine (but very much less in kidney) and more modest levels in liver, lung, placenta, prostate, testis, and other tissues, including some regions of the brain and heart. hCNT3 transcripts were generally present in fetal tissues but were low in various cultured cell lines, including K562, HeLa, and undifferentiated HL-60 (see also below). In contrast, h/rCNT1 and h/rCNT2 transcripts are found primarily in specialized epithe-
lia, including small intestine, kidney, and liver (8–13). Other reported sources of h/rCNT1 and h/rCNT2 transcripts include brain, spleen, heart, pancreas, and skeletal muscle (9, 12, 47). A systematic analysis of CNT1/2 transcript distribution similar to that shown in Fig. 9 for hCNT3 would be helpful to more fully characterize the different expression patterns of the three transporters.

In parallel with the multiple tissue expression RNA array, we also investigated the distribution of hCNT3 transcript in selected tissues by Northern blotting. This less sensitive technique detected hCNT3 transcripts in pancreas, bone marrow, and trachea but not in intestine, liver, brain, or heart (Fig. 10). Kidney, as expected from Fig. 9, was also negative. In pancreas, bone marrow, and trachea, three bands were apparent: a major 5.3-kb transcript and secondary bands at 6.5 and 4.8 kb. Although two of the bands were similar in size to the major transcripts of hCNT1 (3.4 kb) and hCNT2 (4.5-kb) (13), the blot was probed at high stringency under conditions where there was no cross-reactivity with hCNT1/2 (see “Experimental Procedures”). It is likely, therefore, that they represent alternate hCNT3 gene transcripts rather than cross-hybridization with other CNT family members. The absence of bands in kidney, which contains transcripts for both hCNT1 and hCNT2 (13), is further evidence of the specificity of the hCNT3 probe. The same tissues (plus mammary gland) were also analyzed by TaqMan™ quantitative RT-PCR using hCNT3-specific primers as described below for HL-60 cells. Relative levels of hCNT3 transcript by this method were pancreas > bone marrow, trachea, intestine > mammary gland >> liver, brain, heart > kidney (data not shown).

**HL-60 Cells (Functional Studies)—**The human promyelocytic leukemia cell line HL-60 can be induced to differentiate into adherent monocyte/macrophage-like cells treatment with phorbol esters (48, 49). Upon differentiation, the cells exhibit a decrease in equilibrative, Na⁺-independent nucleoside transport that is accompanied by an increase in concentrative, Na⁺-dependent transport of both pyrimidine and purine nucleosides (50). To rigorously identify the concentrative transport processes contributing to this uptake, we first determined the uridine transport profile of parent and phorbol 12-myristate 13-acetate-treated HL-60 cells under conditions previously shown to be optimal for induction of the concentrative transport activity (50). Equilibrative transport was measured by replacing Na⁺ in the transport medium with NMDG, and concentrative transport was determined in the presence of Na⁺ but with the addition of dilazep (100 μM) to inhibit equilibrative transport activity. We have shown that this concentration of dilazep has no effect on hCNT3 transport activity (Fig. 5). Although previ-

| Substrate | Apparent $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|-----------|----------------|-----------|---------------|
| Uridine   | 21.6 ± 5.4     | 25.8 ± 1.3| 1.19          |
| Cytidine  | 15.4 ± 2.7     | 32.8 ± 1.0| 2.13          |
| Thymidine | 21.2 ± 6.3     | 24.2 ± 1.4| 1.14          |
| Adenosine | 15.1 ± 1.8     | 30.4 ± 0.7| 2.01          |
| Guanosine | 43.0 ± 6.6     | 51.4 ± 1.8| 1.20          |
| Inosine   | 52.5 ± 12.6    | 44.8 ± 2.5| 0.85          |

*From Fig. 4.*
ous studies have reported a small amount of Na$^{+}$-dependent transport activity in untreated HL-60 cells (50, 51), our assays did not detect any concentrative transport in the parent cell line, which exhibited only equilibrative uptake of 10 μM uridine (Fig. 11A). However, there was a notable increase in Na$^{+}$-dependent uridine transport in differentiated, adherent HL-60 cells (Fig. 11B). Uptake of thymidine and formycin B (a non-metabolized analog of inosine) was then used to define which of the concentrative transport processes (cit, cif, cib) was active in differentiated HL-60 cells (Fig. 11, C and D). Both nucleosides were taken up by the concentrative process(es) of differentiated HL-60 cells. Transport of thymidine was totally inhibited by unlabeled thymidine, inosine, and uridine, whereas formycin B uptake was reciprocally inhibited by thymidine. Thus, cib (rather than cit + cif) was the dominant concentrative transport activity in differentiated HL-60 cells. Consistent with this result, concentrative uridine transport was inhibited by uridine, thymidine, and inosine (data not shown). Dot blot analysis (Fig. 9A) and nonquantitative RT-PCR (Fig. 12A) established that the appearance of cib functional activity correlated with substantially increased levels of hCNT3 transcripts in differentiated versus parent HL-60 cells, the latter exhibiting only small amounts of hCNT3 mRNA. In RT-PCR experiments, parent and differentiated HL-60 cells were negative for hCNT2 mRNA and expressed only very small amounts of hCNT1 mRNA, most likely as a consequence of bleed-through transcription (data not shown). These results provided further evidence that the concentrative nucleoside transport activity seen in differentiated HL-60 cells was mediated by cib and not by (cit + cif).

**HL-60 Cells (TaqMan$^\text{TM}$ Quantitative Real Time RT-PCR)—** The relative levels of hCNT3 transcripts in HL-60 parent and differentiated cells were determined by quantitative real time RT-PCR (Fig. 12B). Glyceraldehyde-3-phosphate dehydrogen-
ase and hCNT3 were demonstrated to amplify with equal efficiency, and glyceraldehyde-3-phosphate dehydrogenase was therefore used as the internal control to normalize levels of expression of hCNT3 mRNA between samples. To compare samples, a threshold line was set at the phase of the PCR reaction during which the fluorescent signal accumulated exponentially. As shown in Fig. 12B, there was a substantial difference between the HL-60 parent and differentiated samples in the PCR cycle numbers at the threshold line, and three independent experiments gave a mean (± S.E.) ratio of 4.08 ± 0.09, indicating (because PCR amplification is an exponential process) that there was 16.9 ± 1.05 more hCNT3 mRNA in differentiated versus parent HL-60 cells. Similar results were obtained when the data were normalized to 18 S gene expression (data not shown). The human tumor cell lines K562 (erythroleukemia) and HeLa (cervical carcinoma) were also tested in this assay (data not shown) and gave signals that were close to background levels (see also Fig. 9A). These results, taken with those of the transport experiments, indicated that the small amount of hCNT3 transcription in the HL-60 parent cells did not result in enough protein to be functionally detected, whereas the differentiated cells that expressed 16-fold more hCNT3 mRNA had a readily measurable cib transport process. Because the analyses were performed on exponentially growing parent and differentiated cells, the difference in transcript levels between parent and differentiated HL-60 cells could not be attributed to cell proliferation.

Chromosomal Localization of the hCNT3 Gene—Although the genes encoding hCNT1 and hCNT2 have both been mapped to chromosome 15 (15q25–26 (11) and 15q15 (13), respectively), fluorescence in situ hybridization analysis localized the hCNT3 gene to 9q22.2. The same chromosomal band location was determined by screening a human BAC library. Searches of the Sanger Center human genomic sequence data base, and the Unfinished High Throughput Genomic Sequence GenBank™ data base identified two chromosome 9 clones (GenBank™ accession numbers AL356134 and AL353787) containing multiple hCNT3 genomic fragments that, when aligned, revealed 27.3 kb of composite hCNT3 gene sequence containing 74% of the hCNT3 coding sequence. The coding sequence that was obtained was an exact match with corresponding regions of the hCNT3 cDNA sequence. Analysis of hCNT3 5′-genomic sequence in the potential upstream promoter region of the gene revealed the presence of a eukaryotic phorbol myristate acetate (ester) response element (5′-TGAGTCA-3′) that may potentially contribute to the transcriptional regulation of hCNT3 seen in HL-60 cells. Studies are in progress to compare the organization of the hCNT3 gene with that for hCNT1 (32 kb), which has been determined to contain 18 exons separated by 17 introns (GenBank™ accession numbers 187967–187978).

Conclusions—The CNT protein family in humans is represented by three members, hCNT1, hCNT2, and the presently described hCNT3. Searches of the Unfinished High Throughput Genomic Sequence GenBank™ data base have so far revealed no other closely related members of this family in humans. hCNT3 is a transcriptionally regulated electrogenic transport protein that, unlike hCNT1 and hCNT2, has a broad permeant selectivity for pyrimidine and purine nucleosides and...
probed with a 32P-labeled cDNA corresponding to hCNT3 amino acid residues 359–549. The inset in A is a dot blot of mRNA (0.5 µg) from suspension parent and adherent differentiated HL-60 cells probed with the same cDNA. The numbered samples are: 1, leukemia (undifferentiated HL-60); 2, HeLa S3; 3, leukemia (K-562); 4, leukemia (MOLT-4); 5, Burkitt’s lymphoma (Raji); 6, Burkitt’s lymphoma (Daudi); 7, colorectal adenocarcinoma (SW480); 8, lung carcinoma (A549); 9, whole brain; 10, cerebral cortex; 11, frontal lobe; 12, parietal lobe; 13, occipital lobe; 14, temporal lobe; 15, paracentral gyrus of cerebral cortex; 16, pons; 17, cerebellum (left); 18, cerebellum (right); 19, corpus callosum; 20, amygdala; 21, caudate nucleus; 22, hippocampus; 23, medulla oblongata; 24, putamen; 25, substantia nigra; 26, acumbens nucleus; 27, thalamus; 28, pituitary gland; 29, spinal cord; 30, esophagus; 31, stomach; 32, duodenum; 33, jejunum; 34, ileum; 35, illocecum; 36, appendix; 37, colon (descending); 38, colon (transverse); 39, colon (ascending); 40, rectum; 41, heart; 42, aorta; 43, atrium (left); 44, atrium (right); 45, ventricle (left); 46, ventricle (right); 47, interventricular septum; 48, apex of the heart; 49, kidney; 50, skeletal muscle; 51, spleen; 52, thymus; 53, peripheral blood leukocyte; 54, lymph node; 55, bone marrow; 56, trachea; 57, lung; 58, placenta; 59, bladder; 60, uterus; 61, prostate; 62, testis; 63, ovary; 64, liver; 65, pancreas; 66, adrenal gland; 67, thyroid gland; 68, salivary gland; 69, mammary gland; 70, fetal brain; 71, fetal heart; 72, fetal kidney; 73, fetal liver; 74, fetal spleen; 75, fetal thymus; 76, fetal lung; 77, human DNA (100 ng); 78, human DNA (500 ng).

FIG. 10. Tissue distribution of hCNT3 mRNA. A and B, a commercial human multiple tissue expression RNA array probed with a 32P-labeled cDNA corresponding to hCNT3 amino acid residues 359–549. The inset in A is a dot blot of mRNA (0.5 µg) from suspension parent and adherent differentiated HL-60 cells probed with the same cDNA. The numbered samples are: 1, leukemia (undifferentiated HL-60); 2, HeLa S3; 3, leukemia (K-562); 4, leukemia (MOLT-4); 5, Burkitt’s lymphoma (Raji); 6, Burkitt’s lymphoma (Daudi); 7, colorectal adenocarcinoma (SW480); 8, lung carcinoma (A549); 9, whole brain; 10, cerebral cortex; 11, frontal lobe; 12, parietal lobe; 13, occipital lobe; 14, temporal lobe; 15, paracentral gyrus of cerebral cortex; 16, pons; 17, cerebellum (left); 18, cerebellum (right); 19, corpus callosum; 20, amygdala; 21, caudate nucleus; 22, hippocampus; 23, medulla oblongata; 24, putamen; 25, substantia nigra; 26, acumbens nucleus; 27, thalamus; 28, pituitary gland; 29, spinal cord; 30, esophagus; 31, stomach; 32, duodenum; 33, jejunum; 34, ileum; 35, illocecum; 36, appendix; 37, colon (ascending); 38, colon (transverse); 39, colon (descending); 40, rectum; 41, heart; 42, aorta; 43, atrium (left); 44, atrium (right); 45, ventricle (left); 46, ventricle (right); 47, interventricular septum; 48, apex of the heart; 49, kidney; 50, skeletal muscle; 51, spleen; 52, thymus; 53, peripheral blood leukocyte; 54, lymph node; 55, bone marrow; 56, trachea; 57, lung; 58, placenta; 59, bladder; 60, uterus; 61, prostate; 62, testis; 63, ovary; 64, liver; 65, pancreas; 66, adrenal gland; 67, thyroid gland; 68, salivary gland; 69, mammary gland; 70, fetal brain; 71, fetal heart; 72, fetal kidney; 73, fetal liver; 74, fetal spleen; 75, fetal thymus; 76, fetal lung; 77, human DNA (100 ng); 78, human DNA (500 ng).

FIG. 9. Tissue distribution of hCNT3 mRNA. A and B, a commercial human multiple tissue expression RNA array probed with a 32P-labeled cDNA corresponding to hCNT3 amino acid residues 359–549. The inset in A is a dot blot of mRNA (0.5 µg) from suspension parent and adherent differentiated HL-60 cells probed with the same cDNA. The numbered samples are: 1, leukemia (undifferentiated HL-60); 2, HeLa S3; 3, leukemia (K-562); 4, leukemia (MOLT-4); 5, Burkitt’s lymphoma (Raji); 6, Burkitt’s lymphoma (Daudi); 7, colorectal adenocarcinoma (SW480); 8, lung carcinoma (A549); 9, whole brain; 10, cerebral cortex; 11, frontal lobe; 12, parietal lobe; 13, occipital lobe; 14, temporal lobe; 15, paracentral gyrus of cerebral cortex; 16, pons; 17, cerebellum (left); 18, cerebellum (right); 19, corpus callosum; 20, amygdala; 21, caudate nucleus; 22, hippocampus; 23, medulla oblongata; 24, putamen; 25, substantia nigra; 26, acumbens nucleus; 27, thalamus; 28, pituitary gland; 29, spinal cord; 30, esophagus; 31, stomach; 32, duodenum; 33, jejunum; 34, ileum; 35, illocecum; 36, appendix; 37, colon (ascending); 38, colon (transverse); 39, colon (descending); 40, rectum; 41, heart; 42, aorta; 43, atrium (left); 44, atrium (right); 45, ventricle (left); 46, ventricle (right); 47, interventricular septum; 48, apex of the heart; 49, kidney; 50, skeletal muscle; 51, spleen; 52, thymus; 53, peripheral blood leukocyte; 54, lymph node; 55, bone marrow; 56, trachea; 57, lung; 58, placenta; 59, bladder; 60, uterus; 61, prostate; 62, testis; 63, ovary; 64, liver; 65, pancreas; 66, adrenal gland; 67, thyroid gland; 68, salivary gland; 69, mammary gland; 70, fetal brain; 71, fetal heart; 72, fetal kidney; 73, fetal liver; 74, fetal spleen; 75, fetal thymus; 76, fetal lung; 77, human DNA (100 ng); 78, human DNA (500 ng).

nucleoside drugs. Hill-type analysis of the relationship between uridine influx and Na+ concentration indicated a Na+=uridine coupling ratio of at least 2:1, compared with 1:1 for hCNT1/2. These characteristics and the induction of hCNT3 mRNA in HL-60 cells following phorbol ester treatment identified hCNT3 as the physiological human cib transporter. A mouse homolog of hCNT3 (mCNT3) was also cloned, suggesting that CNT3 is widely distributed in mammals.

A candidate cib-type transporter SNST1 that is related to the Na+-dependent glucose transporter SGLT1 was identified in 1992 in rabbit kidney (23). There is no sequence similarity between SNST1 and either the CNT or ENT protein families. Although recombinant SNST1, when expressed in oocytes, stimulated low levels of Na+-dependent uptake of uridine that was inhibited by pyrimidine and purine nucleosides (i.e. cib-type pattern), the function of this protein remains unclear because the rate of uridine transport in oocytes was only 2-fold above endogenous (background) levels, whereas a >100-fold stimulation was observed with h/mCNT3. It is likely that the true physiological substrate of SNST1 is a low molecular weight metabolite for which there is overlapping permeant recognition with nucleosides.

hCNT3 and mCNT3 are more closely related to the hagfish transporter hCNT than to mammalian CNT1/2 and thus form a separate CNT subfamily. Hagfish diverged from the main line of vertebrate evolution about 550 million years ago and represent the most ancient class of extant vertebrates. The high degree of amino acid sequence similarity between h/mCNT3 and hCNT in the TM 4–13 region (67% sequence identity) may indicate functional constraints on the primary structure of this domain and suggests that cib-type concentrative NTs fulfill important physiological functions. The tissue distribution of hCNT3 transcripts was more widespread than anticipated from previous studies of cib functional activity and is different from that of either CNT1 or CNT2. Although transcripts for mammalian CNT1 and CNT2 have been described in jejunum, kidney, liver, and brain (CNT1) and jejunum, kidney,
liver, spleen, heart, skeletal muscle, and pancreas (CNT2), the highest levels of hCNT3 transcripts were found in pancreas, bone marrow, trachea, mammary gland, and duodenum. Clinically, hCNT3 may be expected to contribute to the concentrative cellular uptake of both anticancer and antiviral nucleoside drugs. Future studies of the transcriptional regulation of the hCNT3 gene will enhance our understanding of its physiological function(s) and therapeutic potential.

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