An ancient prevertebrate Na\(^+\)-nucleoside cotransporter (hfCNT) from the Pacific hagfish (Eptatretus stouti)

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Yao, Sylvia Y., Amy M. Ng, Shaun K. Loewen, Carol E. Cass, Stephen A. Baldwin, and James D. Young. An ancient prevertebrate Na\(^+\)-nucleoside cotransporter (hfCNT) from the Pacific hagfish (Eptatretus stouti). Am J Physiol Cell Physiol 283: C155–C168, 2002. First published February 20, 2002; 10.1152/ajpcell.00587.2001. The human concentrative (Na\(^+\)-linked) plasma membrane transport proteins hCNT1, hCNT2, and hCNT3 are pyrimidine nucleoside-selective (system cit), purine nucleoside-selective (system cif), or broadly selective for both pyrimidine and purine nucleosides (system cib), respectively. All have orthologs in other mammalian species and belong to a gene family (CNT) that has members in insects, nematodes, pathogenic yeast, and bacteria. Here, we report the cDNA cloning and functional characterization of a CNT family member from an ancient marine prevertebrate, the Pacific hagfish (Eptatretus stouti). This Na\(^+\)-nucleoside symporter, designated hfCNT, is the first transport protein to be characterized in detail in hagfish and is a 683-amino acid residue protein with 13 predicted transmembrane helical segments (TMSs). hfCNT was 52, 50, and 57% identical in sequence to hCNT1, hCNT2, and hCNT3, respectively. Similarity to hCNT3 was particularly marked in the TM 4–13 region. When produced in oocytes, hfCNT exhibited the transport properties of system cib, with uridine, thymidine, andinosine apparent \(K_m\) values of 10–45 \(\mu\)M. The antiviral nucleoside drugs 3’-azido-3’-deoxythymidine, 2’,3’-dideoxyctydine, and 2’,3’-dideoxynosine were also transported. Simultaneous measurement of uridine-evoked currents and radioabeled uridine uptake under voltage-clamp conditions gave a Na\(^+\)-to-uridine coupling ratio of 2:1 (cf. 2:1 for hCNT3 and 1:1 for hCNT1/2). The apparent \(K_{so}\) value for Na\(^+\) activation was >100 mM. A 50:50 chimera between hfCNT and hCNT1 (TMS 7–13 of hfCNT replaced by those of hCNT1) exhibited hCNT1-like cation interactions, suggesting that the structural determinants of cation stoichiometry and binding affinity were located within the cytoxy-terminal half of the protein. The high degree of sequence similarity between hfCNT and hCNT3 may indicate functional constraints on the primary structure of the transporter and suggests that cib-type CNTs fulfill important physiological functions.

Craniata; Xenopus oocyte; 3’-azido-3’-deoxythymidine; 2’,3’-dideoxyctydine; 2’,3’-dideoxynosine

MOST NATURAL AND synthetic nucleosides are hydrophilic and require specialized nucleoside transport (NT) proteins for passage across cell membranes (6, 15). NT-mediated transport is therefore a critical determinant of intracellular nucleoside metabolism and the pharmacological actions of antineoplastic and antiviral nucleoside drugs (1, 31). By modulating the concentration of adenosine in the vicinity of cell surface receptors, NTs also have important physiological effects on neurotransmission, vascular tone, and other processes (10, 38). Five major nucleoside transport processes that differ in their cation dependence, permeant selectivities, and inhibitor sensitivities have been observed in human and other mammalian cells and tissues (6, 15). Three are concentrative (Na\(^+\)-dependent) (systems cit, cif, and cib) and two are equilibrative (Na\(^+\)-independent) (systems es and ei). The former are found primarily in specialized epithelia such as intestine, kidney, liver, choroid plexus, and leukemic cells, whereas the latter are present in most, possibly all, cell types (1, 6, 15). Systems cit and cif transport adenosine and uridine but are otherwise pyrimidime or purine nucleoside selective, respectively. Systems cib, es, and ei are broadly selective for both pyrimidine and purine nucleosides. The ei system also transports nucleobases (51).

Molecular cloning studies have isolated cDNAs encoding the human proteins responsible for each of these NT processes (cit, cif, cib, es, ei) (8, 16, 17, 35–37, 44). These proteins and their orthologs in other mammalian species (5, 26, 35, 48, 49) comprise two new, previously unrecognized families of integral membrane proteins designated CNT (concentrative nucleoside transporter family) and ENT (equilibrative nucleoside transporter family). The relationships of these NT proteins to the processes defined by functional studies are as follows: CNT1 (cit), CNT2 (cif), CNT3 (cib), ENT1

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Abbreviations used in transporter acronyms are as follows: c, concentrative; e, equilibrative; s and i, sensitive and insensitive to inhibition by nitrobenzylthioninosine, respectively; f, formycin B (non-metabolized purine nucleoside); t, thymidine; g, guanosine; b, broad selectivity.

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HAGFISH NUCLEOSIDE TRANSPORTER

SGLT2, a previous candidate for CNT1 has been shown to sequentially change the amino acid residues in TMs 7 and 8 of the transport protein from rabbit kidney (34), and murine (35).2 These CNTs are unrelated to SNST1 (now NupC), a CNT family member from Escherichia coli, has a similar membrane topology to mammalian CNTs but lacks TMs 1–3 (22).

Human (h) CNT1 contains 650 amino acid residues and is 83% identical in sequence to rat (r) CNT1 (648 residues) (26, 36). hCNT2 (658 residues) is 83% identical to rCNT2 (659 residues) and 72% identical to hCNT1 (5, 37, 49). hCNT3 (691 residues) is 78% identical to rCNT3 and mouse (m) CNT3 (both 703 residues) (26, 36). hCNT2 (658 residues) is 83% identical in sequence to rat (r) CNT1 (648 residues) (25) using the universal pUC/M13 forward and reverse primers (Promega), and sequenced by Taq dyedideoxy-terminator cycle sequencing using an automated model 373A DNA Sequencer (Applied Biosystems). This fragment, which showed 61, 62, and 39% sequence identity to the corresponding regions of hCNT1, rCNT1, and NupC, respectively, was radiolabeled with 32P (T7QuickPrime kit; Amersham Pharmacia Biotech) and used as a hybridization probe to screen the hagfish intestinal cDNA library. Ten positive clones were identified, three of which contained full-length hfcNT cDNA. One of these clones in Uni-ZAP XR vector was excised to generate a subclone in the pBluescript SK+ vector. Twelve clones were selected at random and sequenced.

Construction of chimeric hfcNT and hCNT1 transporters. cDNAs of hfcNT and hCNT1 were subcloned into the vector pGem-T vector. Twelve clones were selected at random and sequenced.

EXPERIMENTAL PROCEDURES

Molecular cloning of hfcNT. The cDNA encoding hfcNT was obtained by first amplifying a partial hfcNT cDNA. The template was a directional Stratagene lambda vector Uni-ZAP XR cDNA library prepared in this laboratory using mRNA isolated from hagfish intestinal tissue (mucosal scrapings). Two rounds of nested PCR were employed using a pair of internal primers against regions of conserved sequence among mammalian and bacterial CNTs: Q1 (antisense; rCNT1 nucleotide sequence 5'-TTGCGCACTTACAGTCTCAGGG-3' corresponding to motif FANFSSIG in TM12) and Q2 (sense; hCNT1 nucleotide sequence 5'-AACATGCGTGCACACCTTGGC-3' corresponding to motif NIAANLIA in TM10). Initial amplification of diluted (1,000-fold) hagfish phage cDNA library with Q2 as the sense primer and T7 oligonucleotide sequence corresponding to a region of the Uni-ZAP XR insert was performed. The PCR product was then subjected to a second round of amplification with Q1 and Q2 for 28 cycles under the same conditions. A 366-bp product was identified, cloned into pGEM-T vector (Promega), and sequenced. 2 GenBank/EBI Data Bank accession numbers: AF132298 (hfcNT), AY059413 (hGLUT), and AY059414 (rCNT3).
Chimeric constructs containing the restriction site KpnI downstream of the M13 forward primer and the restriction site SpaI upstream of the M13 reverse primer were subcloned into the respective restriction sites of the pGEM-HE vector. The chimeras were sequenced in both directions to verify the splice sites and ensure that no mutations had been introduced.

In vitro transcription and expression in Xenopus oocytes. hfCNT and chimeric cDNAs were expressed in Xenopus laevis oocytes according to standard protocols (47). Healthy defolliculated stage VI oocytes of X. laevis were microinjected with 20 nl water or 20 nl water containing RNA transcripts (1 ng/nl) and incubated in modified Barth’s medium at 18°C for 72 h before the assay of transport activity.

Radioisotope flux studies. Transport was traced using the appropriate [14C]/[3H]-labeled nucleoside, nucleoside drug, or nucleobase (Moravek Biochemicals or Amersham Pharmacia Biotech) at either 1 or 2 μCi/ml for [14C]-labeled and [3H]-labeled compounds, respectively. Flux measurements were performed at room temperature (20°C) as described previously (39, 47) on groups of 12 oocytes in 200 μl transport medium containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5. Unless otherwise indicated, permeant concentrations were 10 μM. At the end of incubation periods, extracellular label was removed by six rapid washes in ice-cold transport medium, and individual oocytes were dissolved in 5% (wt/vol) SDS for quantitation of oocyte-associated radioactivity by liquid scintillation counting (LS 6000 IC; Beckman). Initial rates of transport (influx) were determined using an incubation period of 1 min (26). Choline replaced sodium in Na⁺-dependence experiments, and the transport medium for adenosine uptake contained 1 μM deoxycoformycin to inhibit adenosine deaminase activity. The flux values shown are means ± SE of 10–12 oocytes, and each experiment was performed at least twice on different batches of cells. Kinetic parameters were determined using programs of the ENZFITTER software package (Elsevier-Biosoft, Cambridge, UK).

Measurement of hfCNT-induced sodium currents. Membrane currents were measured in voltage-clamped oocytes at room temperature using the two-electrode voltage clamp (CA-1B oocyte clamp; Dagan). The microelectrodes were filled with 3 M KCl and had resistances that ranged from 0.5 to 1.5 MΩ. The CA-1B was interfaced to a computer via a Digidata 1200B analog-to-digital converter and controlled by Axoscope software (Axon Instruments). Current signals were filtered at 20 Hz (four-pole Bessel filter) and sampled at intervals of 10 ms. For data presentation, the signals were further filtered at 0.5 Hz by use of pCLAMP software (Axon Instruments). After microelectrode penetration, resting membrane potential was measured over a 15-min period before the start of the experiment. Cells were not used if the resting membrane potential was unstable or less than −30 mV. For measurements of hfCNT-generated currents, the oocyte membrane potential was clamped at −50 mV. Oocytes were perfused with the same medium used for radioisotope flux studies, and transport was initiated by changing substrate-free solution to one containing nucleoside (200 μM). In experiments examining Na⁺ and H⁺ dependence, sodium in the medium was replaced by choline, and pH was varied from 5.5 to 8.5. For the determination of the charge-to-uridine uptake ratio, currents were monitored and recorded after an oocyte was clamped at −50 mV in substrate-free transport medium for a 10-min period. The solution was then exchanged with transport medium of the same composition containing radiolabeled uridine. Current was measured for 3 min, followed immediately by reperfusion with substrate-free transport medium until current returned to baseline. The oocyte was recovered from the chamber and solubilized with 5% SDS for liquid scintillation counting. The total movement of charge across the plasma membrane was calculated by integrating the uridine-evoked current over the uptake period. Charge was converted into picomoles to compare with radiolabeled uridine uptake. Uptake of [14C]-labeled uridine in control H₂O-injected oocytes was used to correct for endogenous basal uptake of uridine over the same incubation period. The coupling ratio of hfCNT was calculated from data collected from 10 individual oocytes.

RESULTS AND DISCUSSION

Previous studies of nucleoside transport in hagfish have been limited to red blood cells, which possess an equilibrative nitrobenzylthioinosine (NBMPR)-insensitive (ei-type) nucleoside transport process (13). The goal of the present study was to use recombinant DNA technology in combination with heterologous expression in Xenopus oocytes to attempt the cDNA cloning and functional characterization of a hagfish concentrative nucleoside transport protein.

Molecular identification of hfCNT. The first step of our cDNA cloning strategy exploited regions of amino acid sequence similarity between CNT family members to isolate a partial-length hagfish CNT cDNA from intestinal epithelium, a tissue in mammals known to express multiple CNT proteins (6). The template for this PCR amplification was a cDNA library prepared from hagfish intestinal mucosa. The resulting 366-bp fragment generated by PCR amplification (see EXPERIMENTAL PROCEDURES) was used as a probe to screen our hagfish intestinal cDNA library. High-stringency hybridization screening yielded a 2,516-bp cDNA, with an open reading frame of 2,049 bp flanked by 21 bp of 5’-untranslated region and 446 bp of 3’-untranslated region containing a poly(A)⁺ tail. The encoded 683-amino acid residue protein, designated hfCNT (Fig. 1A), with 13 predicted TMs, had a putative molecular weight of 76 kDa and was 52% identical (62% similar) to hfCNT1, 50% identical (59% similar) to hfCNT2, and 57% identical (67% similar) to hfCNT3. In addition to having multiple consensus sites for N-linked glycosylation at the carboxy terminus (Asn625, Asn630, Asn652, Asn660, Asn670). hfCNT also contained an additional potential site of glycosylation on the putative extracellular loop between TMs 9 and 10 (Asn411) (Fig. 1A). The extracellular location of the carboxy terminus has been confirmed by mutagenesis of rCNT1, which is glycosylated at Asn605 and Asn643 (22).

The greater sequence similarity of hfCNT with hfCNT3 than with hfCNT1/2 extended to mCNT3 (35) and rCNT3 and was most pronounced in TMs 4–13 (Fig. 1A). For example, hfCNT and hfCNT3 exhibited an average sequence identity of 77% within these transmembrane helices, and in the whole region of 413 residues (including loops), there were only 32 nonconservative substitutions among the human, mouse, rat, and hagfish sequences. Seven motifs of eight or more consecutive identical residues were common to all four proteins: FFSTVMSM and YYLGLMQW in TM 6,
Fig. 1. hfCNT is a member of the CNT family of nucleoside transporters. A: alignment of the predicted amino acid sequences of hfCNT (GenBank accession number AF036109), hCNT3 (GenBank accession number AF305210), mCNT3 (GenBank accession number AF305211), and rCNT3 (GenBank accession number AY059414) using the GCG PILEUP program. Potential membrane-spanning α-helices, identified as described previously (20), are numbered. Putative glycosylation sites in predicted extracellular domains of hfCNT, hCNT3, mCNT3, and rCNT3 are shown in lowercase, and their positions are highlighted by an asterisk above the aligned sequences. Residues identical in hfCNT and one or more of the other transporters are indicated by black boxes. Continued

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GQTESPLL in TM 7, TIAGSVLGAYIS in TM 8, HLL-TASVMSAPA in TM 9, KTFFNEFVAY in the loop between TM 11 and TM 12, and IATYALCGFAN in TM 12. These motifs are likely to have structural and/or functional significance. The importance of the TM 4–13 region as a core structure to the CNT family in general is indicated by the 10 TM membrane architecture of NupC and by the functionality of a truncation construct of hCNT1 with TMs 1–3 removed (22).

Since we first identified rCNT1 from rat jejunum by expression cloning in Xenopus oocytes (36), more than 40 members of the CNT protein family have been identified by functional expression, sequence homology, and genome sequencing projects. At present, there are 14 CNT proteins that have been characterized functionally, and their phylogenetic relationships are illustrated in Fig. 1B. The hCNT and mammalian CNT3 proteins cluster in a discrete CNT subfamily different from that formed by mammalian CNT1 and CNT2. Also shown in Fig. 1B is the relationship between these proteins and C. elegans CeCNT3 (46). In oocytes, CeCNT3 transports both pyrimidine and purine nucleosides (except cytidine). Although not truly broadly selective, CeCNT3 was designated “CNT3” in anticipation that it would prove to be an ortholog of mammalian cib (46). However, as would be expected from an invertebrate sequence, CeCNT3 was not closely related to either hfCNT/CNT3 or CNT1/2.

Other hagfish CNTs. Human and rat intestine contain transcripts for all three mammalian concentrative nucleoside transporters (CNT1–3). We therefore searched for other possible CNT isoforms in hagfish intestine using hfCNT primers corresponding to regions of amino acid sequence in TMs 10 and 12 identical in hfCNT and human and rat CNT1–3. Twelve randomly selected RT-PCR clones were sequenced. Each contained a 363-bp insert identical in nucleotide sequence to the corresponding region of hfCNT, establishing hfCNT as the major CNT transcript present in hagfish intestine.

Production of recombinant hfCNT in Xenopus oocytes. Mammalian CNT1 and CNT2 display pyrimidine nucleoside-selective cif-type and purine nucleoside-selective cif-type transport activities, respectively. hCNT, in contrast, was found to be similar to human, mouse, and rat CNT3 and to mediate cib-type transport of both pyrimidine and purine nucleosides. Figure 2 shows representative time courses for uptake of uri-
dine (a universal CNT1/2/3 permeant), thymidine (a diagnostic CNT1 permeant), and inosine (a diagnostic CNT2 permeant) in oocytes injected with either hfCNT RNA transcript or water. After 30 min, the uptake values of uridine, thymidine, and inosine in hfCNT-producing oocytes were 37 pmol/oocyte, 58 pmol/oocyte, and 62 pmol/oocyte, respectively, 95- to 380-fold higher than those in water-injected oocytes (0.2–0.4 pmol/oocyte).

Substitution of Na⁺ in the incubation medium by choline reduced the fluxes in RNA-injected oocytes by 98%. In the subsequent kinetic experiments (presented in Figs. 4 and 6), we used a 1-min incubation period to define initial rates of uridine, thymidine, and inosine uptake.

Substrate selectivity and antiviral drug transport of recombinant hfCNT. Figure 3 shows a representative transport experiment in *Xenopus* oocytes that measured uptake of a panel of radiolabeled pyrimidine and purine nucleosides in cells injected with water alone (control) or water containing hfCNT transcripts. Consistent with cit-type functional activity, hfCNT-producing oocytes transported all the pyrimidine and purine nucleosides tested (cytidine, thymidine, uridine, adenosine, deoxyadenosine, guanosine, and inosine) and gave similar mediated fluxes (uptake in RNA-injected oocytes minus uptake in water-injected oocytes) for each nucleoside tested (18 ± 3, 22 ± 2, 17 ± 3, 24 ± 5, 22 ± 3, 21 ± 3, and 14 ± 2 pmol oocyte⁻¹·30 min⁻¹, respectively). In contrast, no mediated transport of uracil was detected, establishing the transporter's specificity for nucleosides over nucleobases.

Previously, we have used *Xenopus* oocyte expression to establish that the mammalian CNT1/2/3 proteins transport antiviral dideoxynucleosides: hCNT1 and rCNT1 transported 3'-azido-3'-deoxythymidine (AZT).
and 2',3'-dideoxycytidine (ddC) but not 2',3'-dideoxyinosine (ddI), hCNT2 transported only ddI, and hCNT3 and mCNT3 transported all three compounds (26, 35–37, 50). As shown in Fig. 3 by differences in radiolabeled drug uptake between oocytes injected with water alone (control) or water containing RNA transcript, hfCNT accepted both pyrimidine (AZT, ddC) and purine (ddI) dideoxynucleoside drugs as permeants. The magnitudes of the fluxes were smaller than for physiological nucleosides but similar to those reported previously for hCNT1 (AZT, ddC), hCNT2 (ddI), and hCNT3 (AZT, ddC, ddI). Consistent with transporter-mediated pyrimidine and purine dideoxynucleoside drug uptake, control experiments confirmed that influx of AZT and ddI in hfCNT-producing oocytes was reduced to basal (water-injected oocyte) levels in the presence of excess unlabeled uridine (10 mM) (data not shown). These results indicated that hfCNT and other CNTs are relatively tolerant of the lack of the 3'-OH group on the sugar moiety of nucleosides, a feature also characteristic of the mammalian equilibrative ENT2 protein isoform (50).

**Kinetic properties.** Figure 4 presents representative concentration dependence curves for uridine, inosine, and thymidine transport, measured as initial rates of uptake (1-min fluxes) in hfCNT-producing oocytes and in control water-injected oocytes. Kinetic constants for the hfCNT-mediated component of uptake are presented in Table 1. Apparent Michaelis constant ($K_m$) values for uridine, inosine, and thymidine transport were similar (10, 35, and 45 μM, respectively) and in the same range as values obtained previously for recombinant mammalian CNT1/2/3 proteins (5, 8, 26, 36, 37, 44, 49). Maximum velocity ($V_{max}$) values for the three nucleosides were similar. Influx of uridine, inosine, and thymidine in control, water-injected oocytes was linear with concentration, consistent with nonmediated simple diffusion across the lipid layer.

**hfCNT Na+ nucleoside cotransport.** In mammalian cells, most plasma membrane transporters use the sodium electrochemical gradient to actively transport substrates into or out of cells, whereas in bacteria, $H^+$ is the preferred ion of many coupled transporters (23). A few mammalian transporters have been described that use $H^+$ as the coupling ion, including oligopeptide transporters (32), iron transporters (18), monocarboxylate transporters (19), and a myo-inositol transporter (43). The three mammalian CNTs function as Na$^+$-dependent nucleoside transporters, although recent electrophysiological studies in Xenopus oocytes have found that $H^+$ and Li$^+$ can substitute for Na$^+$ for CNT3, but not for CNT1 or CNT2 (unpublished data). In contrast, Na$^+$ replacement and pH-dependence experiments suggest that C. albicans CaCNT (unpublished data), C. elegans CeCNT3 (46), and E. coli NupC (47) are exclusively $H^+$ dependent. In the case of CaCNT, this has been confirmed by electrophysiology (unpublished data).

As shown in Figure 5, external application of adenosine, cytidine, guanosine, inosine, thymidine, or uridine (200 μM) to hfCNT-producing oocytes generated...
quantitatively similar inward currents that returned to baseline upon removal of permeant. No currents were seen in water-injected oocytes or when Na\textsuperscript{+} in the extracellular medium was replaced by choline, confirming that hfCNT3 functions as an electrogenic Na\textsuperscript{+}/nucleoside symporter. In addition, no uridine-mediated activation that hfCNT3 functions as an electrogenic Na\textsuperscript{+}/nucleoside symporter. In prokaryotes, the melibiose transporter (21). Because hfCNT and hCNT3 are very similar (hfCNT, CNT1, CNT2), and Na\textsuperscript{+}/H\textsuperscript{+} independent (CaCNT, CeCNT3, NupC), Na\textsuperscript{+} dependent (hfCNT, CNT1, CNT2), and Na\textsuperscript{+}/H\textsuperscript{+} (and Li\textsuperscript{+}) dependent (CNT3). In prokaryotes, the melibiose transporter of *E. coli* can also use either H\textsuperscript{+} or Na\textsuperscript{+} as the coupling ion, depending on which sugar is being transported (3), while that of *Klebsiella pneumoniae* couples sugar transport to H\textsuperscript{+} and Li\textsuperscript{+} (20). On the basis of sequence comparisons between the *E. coli* and *K. pneumoniae* proteins, site-directed mutagenesis identified a single residue in TM 2 that was important for Na\textsuperscript{+} recognition (21). Because hfCNT and hCNT3 are very similar in amino acid sequence, particularly in the region from TM4 to TM13 (Fig. 1A), it is likely that introduction of point mutations into hfCNT by site-directed mutagenesis will identify individual amino acid residues that contribute to CNT cation specificity.

A Na\textsuperscript{+}/nucleoside coupling ratio of 2:1 has been reported for system *cib* in choroid plexus and microglia (45, 24), whereas coupling ratios of 1:1 have been described for various *cit* and *cif* transport activities in different mammalian cells and tissues (4). Similarly, Hill coefficients for Na\textsuperscript{+} activation of nucleoside transport by recombinant hCNT3 and mCNT3 were ~2, compared with ~1 for rCNT1 (35, 49). *K*\textsubscript{50} values for Na\textsuperscript{+} activation were in the range 7–16 mM. In the present study, we undertook similar Na\textsuperscript{+}-activation experiments with hfCNT. Consistent with the high Na\textsuperscript{+} concentrations normally experienced by hagfish tissues (~500 mM NaCl), we found an almost linear relationship between nucleoside influx (uridine, inosine, and thymidine) and Na\textsuperscript{+} concentration, even up to 100 mM Na\textsuperscript{+}, the maximum extracellular Na\textsuperscript{+} concentration that is possible for *Xenopus* oocytes (Fig. 6). Thus the *K*\textsubscript{50} value for Na\textsuperscript{+} activation of hfCNT was >100 mM. We have recently described a similar very high *K*\textsubscript{50} value for a Na\textsuperscript{+}-dependent pyruvate transport system in hagfish red blood cells, suggesting that this may be a general characteristic of hagfish Na\textsuperscript{+}-dependent transporters (41).

Although it was not possible to determine a Hill coefficient for Na\textsuperscript{+} activation of hfCNT, we used the two-microelectrode voltage-clamp technique to directly determine the Na\textsuperscript{+}/nucleoside coupling ratio of hfCNT by simultaneous measurement of Na\textsuperscript{+} currents and

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### Table 1. Kinetic Properties of hfCNT

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<th>Substrate</th>
<th>Apparent <em>K</em>\textsubscript{m} (μM)</th>
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<th><em>V</em>\textsubscript{max}/<em>K</em>\textsubscript{m}</th>
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<td>Thymidine</td>
<td>45.0 ± 7.0*</td>
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<td>Inosine</td>
<td>35.0 ± 6.0*</td>
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Values are means ± SE. *K*\textsubscript{m}, Michaelis constant; *V*\textsubscript{max}, maximum velocity. *Data from Fig. 4.

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**Fig. 5.** Sodium currents induced by exposure of recombinant hfCNT to nucleoside permeants. *A*: inward currents caused by perfusing an hfCNT-producing oocyte at room temperature with NaCl transport medium containing different pyrimidine and purine nucleosides at a concentration of 200 μM. *B*: the same oocyte perfused with nucleosides (200 μM) in choline chloride transport medium. No inward currents were generated. Similarly, no inward currents were generated when control water-injected oocytes were perfused with nucleosides either in the presence or in the absence of Na\textsuperscript{+} (traces not shown).
[14C]uridine influx under voltage-clamp conditions, as described previously for the SDCT1 rat kidney dicarboxylate transporter (7). The results of these experiments presented in Fig. 7 demonstrated that hCNT has a Na+/nucleoside coupling ratio of 2:1 (the slope of the regression line (±SE) is 2.1 ± 0.1) similar to that of hCNT3 and mCNT3, but different from the 1:1 coupling ratio of hCNT1 (35, 49; unpublished data). In this respect, the CNTs resemble the SGLT glucose transporter family which has members with Na+/sugar coupling ratios of 2:1 (SGLT1 and SGLT3) and 1:1 (SGLT2) (9). Similarly, the proton-linked oligopeptide transporters PepT1 and PepT2 have 1:1 and 2:1 H+/peptide coupling ratios, respectively (32).

Characterization of hfCNT/hCNT1 chimeras. Previously, we have identified two adjacent pairs of residues (Ser319/Gln320 and Ser353/Leu354) in the TM 7–9 region of hCNT1 that, when mutated together to the corresponding residues in hCNT2 (Gly313/Met314 and Thr347/Val348), converted hCNT1 (cit type) into a transporter with cif-type functional characteristics (30). An intermediate broad specificity cig-like transport activity was produced by mutation of the two TM 7 residues alone. The amino acid residues of hfCNT at these four positions are Gly335/Gln336 in TM7 and Ser369/Val370 in TM8, which represents the intermediate state between hCNT1 and hCNT2 to allow transport of both purine and pyrimidine nucleosides. In addition to differing in substrate specificity, we have shown in the previous section that hfCNT and hCNT1 also exhibit differences in interactions with Na+, the hagfish transporter having a Na+/nucleoside coupling ratio of 2:1 (vs. 1:1 for hCNT1) and a high K50 value for Na+ activation of >100 mM.

The predicted amino acid sequences of hfCNT and hCNT1 are 52% identical and 62% similar, with strongest residue similarity within TMs of the carboxy-terminal halves of the proteins. The major differences lie in the putative amino- and carboxy-terminal tails of the proteins and in the first three TMs (Fig. 8A). To localize domains involved in cation stoichiometry and binding affinity, a chimera (HF/H) in which the carboxy-terminal half of hfCNT (incorporating TMs 7–13) was replaced with that of hCNT1 was constructed. The splice site between the two proteins following hfCNT residue Gly311 was engineered at the beginning of the putative extramembranous loop before TM 7 to divide the proteins into two approximately equal halves as predicted by the topology model in Fig. 8A, and to minimize disruption of native TMs and loops. The resulting chimera (HF/H) transported uridine when

![Fig. 6. Sodium dependence of uridine, thymidine, and inosine of influx by recombinant hfCNT. Initial rates of hfCNT-mediated uridine (A), thymidine (B), and inosine uptake (C) (10 μM, 20° C, 1 min) were measured in transport medium containing 0–100 mM NaCl using choline chloride to maintain isomolarity. Mediated uptake was calculated as uptake in hfCNT-producing oocytes minus uptake in control water-injected oocytes. Each value is the mean ± SE of 10–12 oocytes, and error bars are not shown where SE values were smaller than that represented by the symbols.](http://ajpcell.physiology.org/)}
produced in Xenopus oocytes (Fig. 8B) but displayed lower levels of functional activity than hfCNT and hCNT1 (most likely the result of reduced plasma membrane targeting) and required a longer incubation period (30 min vs. 1 min) to obtain comparable levels of total uptake. A reciprocal chimera to HF/H (H/HF, a 50:50 construct incorporating the amino-terminal half of hCNT1 and the carboxy-terminal half of hfCNT) was nonfunctional and was not studied further.

As predicted by the earlier mutagenesis studies (30), chimera HF/H exhibited hCNT1-like substrate specificity (36). This is illustrated in Fig. 8B, which shows the transportability of a panel of physiological purine and pyrimidine nucleosides (adenosine, cytidine, guanosine, inosine, thymidine, and uridine). Fluxes were similar in profile to those exhibited by wild-type hCNT1 (uridine, thymidine, cytidine > adenosine and no detectable transport of guanosine or inosine). Furthermore, ddi (a substrate of hfCNT but not hCNT1) was not transported by HF/H (Fig. 8B).

In addition to substrate specificity, we also tested HF/H interactions with Na⁺. In Fig. 9, we show that the relationship between cytidine, thymidine, uridine influx, and Na⁺ concentration was saturable and hyperbolic, with Hill coefficients (± SE) of 0.8 ± 0.1 (cytidine), 1.2 ± 0.2 (thymidine), and 1.0 ± 0.1 (uridine), indicating a Na⁺/nucleoside coupling ratio of 1:1 (i.e., hCNT1-like). These results indicated that the residues determining the coupling ratio also reside in the carboxy-terminal half of the transporter. K₅₀ values for Na⁺ activation of 4.0 ± 1.0 (cytidine), 8.7 ± 2.2 (thymidine), and 10.0 ± 1.7 (uridine) were also hCNT1-like, demonstrating that the structural features determining Na⁺-binding affinity are likewise in this half of the protein. Cysteine-scanning mutagenesis studies of E. coli lactose permease have found that coupling between substrate and H⁺ translocation involved six irreplaceable residues located at five different helices from TM 4 to TM 10 (28). It is likely that the three-dimensional conformations of the cation (and substrate) binding sites of hfCNT are also composed of residues from multiple helices. Future site-directed and cysteine-scanning mutagenesis studies in the carboxy-terminal half of hfCNT will therefore not only identify amino acid residues involved in cation stoichiometry and binding affinity, but also provide information on helix packing within the translocation pore of the transporter.

Conclusions. Nucleosides are important precursors of nucleic acids and energy-rich cellular metabolites, and one (adenosine) has functions as a local hormone in a variety of tissues, including the gastrointestinal system (1, 6, 15). Cells obtain nucleosides from breakdown of dietary and endogenous nucleotides. The former are important nutrients and are absorbed as nucleosides by enterocytes of the intestinal mucosa. In mammals, enterocytes have a limited capacity for de novo nucleotide synthesis and require both dietary and endogenous nucleosides for their own metabolism and differentiation (6). hfCNT is a CNT nucleoside transporter from hagfish intestinal epithelium that belongs to the CNT3 subfamily. hfCNT was electrogenic, Na⁺ dependent, H⁺ and Li⁺ independent, and exhibited a broad permeant selectivity for both pyrimidine and purine nucleosides. hfCNT had a 2:1 Na⁺/nucleoside coupling stoichiometry, identifying this characteristic, in addition to cib-type substrate selectivity, as a general functional feature of the hfCNT/ CNT3 subfamily. A two-Na⁺/one-nucleoside symporter such as hfCNT will have greater ability to transport permeant against its concentration gradient than a one-Na⁺/one-nucleoside symporter, particularly when considered in the context of the very high concentration of Na⁺ present in hagfish extracellular fluids or intestinal lumen and the high K₅₀ value for hfCNT Na⁺ activation. hfCNT differed from its mammalian orthologs in that it was unable to substitute H⁺ (and Li⁺) for Na⁺.

The differences in cation stoichiometry, binding affinity, and specificity between hfCNT and mammalian...
CNTs will provide a basis for future site-directed mutagenesis studies to identify the amino acid residues involved. Although there is greater sequence divergence between hfCNT and CNT1/2 than between hfCNT and CNT3, our functionally active hfCNT/hCNT1 chimera HF/H has narrowed down the region of interest to the carboxy-terminal halves of the proteins. Within TMs 7–13, there are only 51 residue differences between hfCNT and hCNT1 that could potentially account for the observed differences in the Na⁺/nucleoside coupling ratio and binding affinity. Many of these residue differences occur in clusters, making it feasible to undertake multiple simultaneous mutations between the two proteins to rapidly identify the amino acid residues involved.

Hagfish (Hyperotreti) are prevertebrates that diverged from the main line of vertebrate evolution about 550 million years ago and represent the most ancient extant member of the craniate subphylum. The fossil record indicates that hagfish have undergone little evolutionary change in body structures (39). In the phylogenetic analysis of functionally characterized CNT family members shown in Fig. 1B, hfCNT clustered with mammalian CNT3 proteins. Since the period around the Hyperotreti-Vertebrata split was a time of very active gene duplication (33), it will be informative from an evolutionary perspective to establish in future studies whether or not hagfish also contain members of the CNT1/2 subfamily. The present finding by RT-PCR that hfCNT is the predominant CNT in hagfish intestine may indicate the absence of other concentrative nucleoside transporter isoforms and contrasts with mammalian intestine which contains transcripts for CNT1, CNT2, and CNT3 (35–37). Even in the absence of other CNTs, the functional characteristics described here for hfCNT would enable the efficient intestinal absorption of both pyrimidine and purine dietary nucleosides required by their scavenging carnivorous life-style and periodic feeding behavior. The high degree of amino acid sequence similarity between hfCNT and mammalian CNT3 proteins, particularly in the TM 4–13 region, may indicate functional constraints on the primary structure of this region and provides structural evidence that cib-type nucleoside transporters fulfill important physiological functions.

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Fig. 9. Sodium dependence of uridine, thymidine, and cytidine influx by hfCNT/hCNT1 chimera HF/H. Initial rates of transporter-mediated influx of uridine (A), thymidine (B), or cytidine (C) (10 μM, 20°C, 30 min) were measured in transport media containing 0–100 mM NaCl, using choline chloride to maintain isosmolality. Mediated uptake was calculated as uptake in hfCNT-producing oocytes minus uptake in control water-injected oocytes. Each value is the mean ± SE of 10–12 oocytes, and error bars are not shown where SE values were smaller than that represented by the symbols.
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