

# A Novel Proton-Dependent Nucleoside Transporter, CeCNT3, from *Caenorhabditis elegans*

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## ABSTRACT

In this study, we describe the cloning and characterization of a proton-dependent, broadly selective nucleoside transporter from *Caenorhabditis elegans*. Recently, we constructed a broadly selective nucleoside transporter which accepts both purine and pyrimidine nucleosides. Based on these studies, we hypothesized that CNTs with novel substrate selectivities exist in nature and that a CNT homolog in the *C. elegans* genomic database may function as a broadly selective nucleoside transporter. We cloned the cDNA for this transporter, termed CeCNT3 because of its broad selectivity, using polymerase chain reaction-based methods. CeCNT3 is predicted to have 575 amino acid residues (63.4 kDa) with 11 to 14 putative transmembrane domains and exhibits ~30% identity to members of the mammalian CNT family. This transporter exhibits a novel substrate selectivity, transporting a wide range of purine

and pyrimidine nucleosides (inosine, guanosine, adenosine, uridine, and thymidine) but not cytidine. The apparent  $K_m$  values for inosine and thymidine are  $15.2 \pm 5.3 \mu\text{M}$  and  $11.0 \pm 2.4 \mu\text{M}$ , respectively. Kinetic studies demonstrate that purine and pyrimidine nucleosides share a common recognition site in the transporter. In contrast to all known members of the mammalian CNT family, CeCNT3-mediated transport of nucleosides is proton-, but not sodium-, dependent. Mutation of tyrosine 332 in CeCNT3 decreased both the maximum uptake rate and apparent  $K_m$  of thymidine, suggesting that this residue is in the domain of nucleoside recognition and translocation. The broad nucleoside specificity of CeCNT3 may be explained by this and other residues that restrict purine and pyrimidine nucleoside uptake and that discriminate among pyrimidine nucleosides.

Membrane transporters play a key role in determining exposure of a cell or organism to a variety of solutes, including nutrients, cellular by-products, environmental toxins, drugs, and other xenobiotics. A major challenge in transporter biology is to understand the structural basis for substrate recognition. Knowledge of the structural basis for substrate recognition is not only important in understanding the initial steps of transport but is also critical in designing and using drugs because membrane transporters are major determinants of drug response.

During evolution, as organisms have increased in complexity, transporters have increased in number and diversity of function. Although many studies have compared the sequences of transporters among organisms and highlighted sequence similarities and differences, this phylogenetic analysis has been used primarily to establish evolutionary relationships among transporters. Only a few studies on comparative functional analysis of transporter homologs have identified critical amino acids and structural domains involved in substrate recognition (Barker and Blakely, 1996;

Barker et al., 1998). Functional comparisons of transporter homologs among organisms may also aid in understanding structural elements responsible for other processes such as coupling mechanisms involved in active transport.

Nucleoside transporters are integral membrane proteins that mediate both the uptake and release of nucleosides and many synthetic nucleoside analogs across plasma membranes. A major class of nucleoside transporters, concentrative nucleoside transporters (CNTs), mediate the active intracellular influx of nucleosides by coupling to the sodium or proton gradients across the plasma membrane (Baldwin et al., 1999).

Based on substrate selectivity, the concentrative nucleoside transporters can be divided into two major subtypes (Belt et al., 1993). One type (N1 or CNT2) is generally purine selective; the other type (N2 or CNT1) is generally pyrimidine selective. Genes for both CNT1 and CNT2 (also termed SPNT) have been cloned from rat and human; their protein products are predicted to be approximately 65% identical in amino acid sequence (Crawford et al., 1990; Dagnino et al., 1991). Both types accept uridine and adenosine. Mammalian CNT family members usually transport nucleosides by cou-

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**ABBREVIATIONS:** CNT, concentrative nucleoside transporter; rCNT, rat concentrative nucleoside transporter; PCR, polymerase chain reaction; MES, 2-(*N*-morpholino)-ethanesulfonic acid; bp, base pair(s); TMD, transmembrane domain; hCNT, human concentrative nucleoside transporter; 5'TMP, thymidine monophosphate; NBMPR, nitrobenzylthioinosine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

pling to sodium (Che et al., 1995; Ritzel et al., 1997) whereas nucleoside transport systems in bacteria usually transport nucleosides by coupling to protons (Craig et al., 1994).

There is growing evidence to show that nucleoside transporters with broader substrate selectivities exist in nature. First, in isolated tissues from several mammalian species, a broadly selective nucleoside transporter, N3, which accepts both purine and pyrimidine nucleosides, and N4 and N5, transporters with distinct substrate selectivities, have been characterized (Wu et al., 1992, 1994; Huang et al., 1993). Second, by construction of chimeras, we engineered a broadly selective N3-type nucleoside transporter from rCNT1 and rCNT2 (Wang and Giacomini, 1997, 1999a). An N3-like transporter was also created by changing a single amino acid at position 318 of rCNT1 (Wang and Giacomini, 1999b). Recently, a nucleoside transporter with broad selectivity has been cloned from hagfish (GenBank accession number AF132298).

The free-living nematode *Caenorhabditis elegans* is a simple model system to study a variety of complex biological problems. Based on its genome sequence (Anonymous, 1998), many transporters in *C. elegans* have been cloned and characterized, including transporters for dopamine (Jayanthi et al., 1998), glutamate (Kawano et al., 1996), phosphate (Lee et al., 1999), organic anions and cations (George et al., 1999; Wu et al., 1999), monoamines (Duerr et al., 1999) and oligopeptides (Fei et al., 1998). Functional studies of these transporters show that, in *C. elegans*, some transporters are sodium-dependent (Jayanthi et al., 1998; Lee et al., 1999) whereas some others are proton-dependent (Fei et al., 1998; Duerr et al., 1999; Wu et al., 1999).

Based on the gene F27E11, two CNT transporters were predicted to exist in *C. elegans*. After multiple sequence alignments and examination of critical amino acids involved in substrate recognition, we hypothesized that one of CNT homologs encoded by gene F27E11 may function as a broadly selective nucleoside transporter. We cloned the cDNA for this transporter, termed CeCNT3, and determined its functional characteristics. CeCNT3 exhibited novel characteristics in terms of both its driving force and selectivity. Namely, unlike CNT transporters in mammals, CeCNT3 was sensitive to proton gradients but not to sodium gradients. The transporter was selective for both naturally occurring purine and pyrimidine nucleosides except for cytidine. These unique functional characteristics permit us to pursue a better understanding of the amino acid residues involved in both substrate recognition and driving forces of the transporters in the CNT family and shed light on the evolution of functional domains in this major family of transporters.

## Materials and Methods

**RNA Isolation and cDNA Preparation.** Total RNA was isolated from *C. elegans* (strain N2) with Trizol reagent (Life Technologies) according to the manufacturer's protocol, then was taken as the template to synthesize cDNA by reverse transcribed polymerase chain reaction (reverse transcription-PCR) using oligo(dT) primer (Life Technologies). CeCNT3 was then amplified by nested PCR technique using the cDNA as template. These primers were designed specially to amplify the potential nucleoside transporter coded by the genomic sequence in the F27E11.2 locus (GenBank accession number AF016413). The first pair of primers were 5'-ttagagatccgcgtg-cgcaccaatca-3' (sense), 5'-aattttatatacaataatatatgtac-3' (anti-

sense). The second pair of primers were 5'-cgaccaatcagcatgcgc-aatggcgctgg-3' (sense), 5'-aaaaacttgaatttatccgctaggaatccgg-3' (antisense). PCR was performed with the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 62°C for 2 min, and 72°C for 2 min. The reaction was then extended by incubation at 72°C for an additional 15 min. A PCR product of 1.8 kilobases (encoding CeCNT3) was obtained, subcloned into the pGEM-T vector (Promega, Madison, WI) and sequenced at the Biomolecular Resource Center at the University of California, San Francisco.

**Sequence Analysis.** Multiple alignment and motif prediction were carried out using the Genetics Computer Group (GCG) software package (Wisconsin Package, Version 9, Madison, WI). Hydropathy analysis was performed using DNA Strider (Version 1.2). BLAST network at the National Center for Biotechnology Information was used in database searching.

**Expression and Transport Assay in *Xenopus Laevis* Oocytes.** To obtain higher expression, CeCNT3 was subcloned into vector pOX (a gift from Andrew T. Gray, University of California, San Francisco), which contains the 5' and 3' untranslated regions of the *X. laevis*  $\beta$ -globin gene flanking the insert (Jegla and Salkoff, 1997; Chavez et al., 1999). CeCNT3 cRNA was synthesized using T3 polymerase (Stratagene, La Jolla, CA) following the manufacturer's protocol. Oocytes were harvested and treated as described previously (Wang et al., 1997). The cRNA (50 nl; ~0.4 ng/nl) or water was injected individually into defolliculated oocytes. Oocytes were incubated at 18°C for 30 to 40 h and then the uptake assays were performed in 100  $\mu$ l of transport buffer [2 mM KCl, 1 mM CaCl<sub>2</sub>, 10 mM HEPES, or 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)] containing different concentrations of <sup>3</sup>H-labeled nucleosides (Moravsek Biochemicals, Brea, CA). To test the effect of sodium-gradient on the activity of CeCNT3, uptake assays were carried out in transport buffer containing 0 to 100 mM NaCl with choline chloride to maintain the iso-osmolality. In experiments testing the effect of proton-gradient on the activity of CeCNT3, oocytes were preincubated in choline buffer at pH 7.4 and then transferred to transport buffers with pH ranging from 5.0 to 8.0. To study the effects of FCCP, a proton ionophore, on the activity of CeCNT3, oocytes were preincubated in choline buffer (pH 8.0, no FCCP) for 3 h. Oocytes were then incubated in choline buffer (pH 5.5 or 8.0, with or without 5  $\mu$ M FCCP) for 0, 30, and 60 min, and the uptake of thymidine or inosine was measured in sodium buffer at pH 5.5 and pH 8.0. In the pH range of 5.0 to 6.5, 10 mM MES was used in the uptake buffer; 10 mM HEPES was used in the pH range of 7.0 to 8.0. In inhibition studies, chemicals at tested concentrations were added to the uptake buffer individually. After 30 min of incubation at 25°C, oocytes were washed five times in 3 ml of ice-cold choline buffer and lysed individually in 10% SDS. The amount of radiolabeled nucleoside transported into each oocyte was determined by liquid scintillation counting. Most studies were carried out in transport buffer containing 100 mM NaCl, pH 6.5. A pH of 6.5 was selected because preliminary studies suggest that uptake of nucleosides was high at pH 6.5 and that this pH did not destroy the viability of the oocytes.

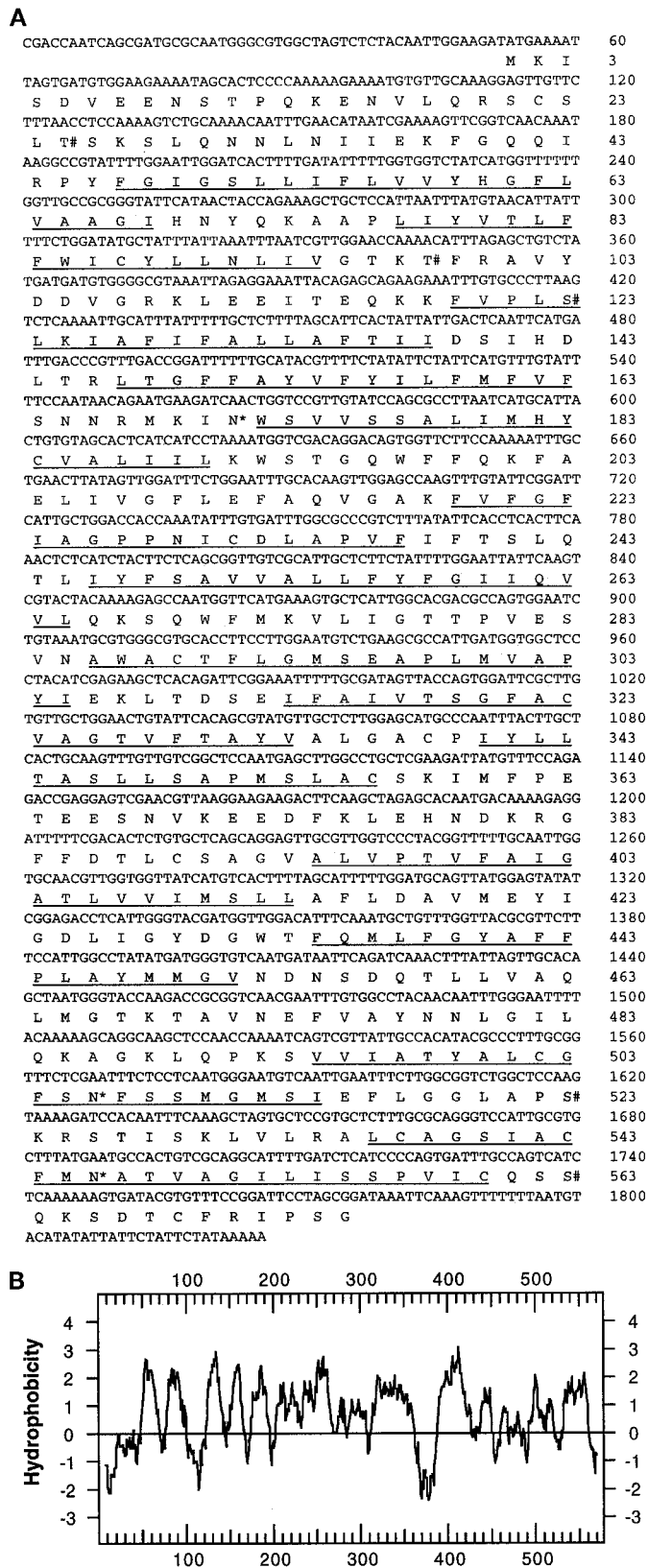
**Site-Directed Mutagenesis.** Single (tyrosine 332 to phenylalanine, Y332F) and double (threonine 327 to serine and valine 328 to leucine, T327S/V328L) mutations were constructed with Quick-Change Site-directed Mutagenesis Kit (Stratagene), using wild-type CeCNT3 cDNA as the template. The sequences of Y332F and T327S/V328L mutants were confirmed by DNA sequencing at the Biomolecular Resource Center at the University of California, San Francisco. Expression and transport assays of Y332F and T327S/V328L mutants were the same as described above for wild-type CeCNT3.

**Statistics and Data Analysis.** Groups of 8 to 10 cRNA-injected or water-injected oocytes were used for each experiment. Uptake values are expressed as mean  $\pm$  S.E. For kinetic studies of CeCNT3, uptake rates (*V*) determined at different substrate concentrations (*S*) were fit to the Michaelis-Menten equation:  $V = V_{\max} \times S / (K_m + S) + K_d \times S$ , where  $V_{\max}$  is the maximal uptake rate,  $K_m$  is the Michaelis-Menten constant (the substrate concentration at  $V_{\max}/2$ ), and  $K_d$  is

the nonsaturable first-order rate constant. Fits were carried out using a nonlinear least-squares regression-fitting program (Kaleidagraph v. 3.0; Abelbeck/Synergy Software, Reading, PA). Kinetic experiments were repeated several times in different batches of oocytes; data for one representative experiment are presented in this study. For inhibition study, statistical analysis was carried out by comparing the uptakes from tested compounds with the uptake from controls in the same experiments using a two-tailed, two-sample equal variance *t* test. Results with the probability of  $p < 0.05$  were considered significantly different.

## Results

**Nucleotide and the Deduced Amino Acid Sequences of CeCNT3.** A BLAST search of the *C. elegans* genome database with the cDNA sequence of rCNT1 revealed only two homologs of the CNT family. These two transporters are encoded by the genes, F27E11.1 and F27E11.2. We cloned the full-length cDNA of both homologs, using primers designed to anneal to the 5' and 3' ends of the predicted exons (GenBank accession number AF016413). Because the activity of the transporter encoded by F27E11.1 was low and we could not make any conclusions about its selectivity, we focused on the other transporter, CeCNT3 (encoded by gene F27E11.2) in this study. The nucleotide sequence and deduced amino acid sequences of CeCNT3 were deposited in GenBank with accession no. AF162674. As shown in Fig. 1A, the cDNA is 1826 bp long with an open reading frame of 1728 bp (including the termination codon). Because there is an in-frame stop codon upstream of the ATG and because the cloned transporter was functional, we concluded that the entire open reading frame was obtained. The sequence predicts a protein of 575 amino acid residues with a molecular mass of 63.4 kDa. Two fragments (158 bp and 48 bp) in the 5' region of the cDNA (GenBank accession number AF016413) predicted from the genomic sequence are absent from our cloned CeCNT3 cDNA, suggesting that either these two fragments were mistakenly predicted as exons or that there is alternative splicing. The initiation codon of CeCNT3 is preceded by a consensus Kozak sequence (A/GXXATG) (Kozak, 1986). Using Kyte-Doolittle (1982) hydropathy analysis, CeCNT3 is predicted to have 11 to 14 transmembrane domains (TMD) (Fig. 1B). The 14 putative TMDs are highlighted (Fig. 1A). Multiple alignments show that, at the amino acid sequence level, this transporter is related to mammalian CNTs (~30% identity) (Fig. 2A) (Huang et al., 1994; Che et al., 1995; Ritzel et al., 1997; Wang et al., 1997) and bacterial CNTs (~14–24% identity) (data not shown) (Craig et al., 1994; Fleischmann et al., 1995; Kunst et al., 1997; Tomb et al., 1997), but is not related to the equilibrative nucleoside transporter family (data not shown). We also compared the identity of the amino acid residues in the regions that have been found to be important in determining the purine and pyrimidine selectivity of hCNT and rCNT (Wang and Giacomini, 1997, 1999b; Loewen et al., 1999). In this particular region, CeCNT3 shows ~44% identity to hCNT2 and rCNT2 and ~33% identity to hCNT1 and rCNT1 (Fig. 2B). The most divergent regions between CeCNT3 and mammalian CNT are in the N-terminal and C-terminal domains. There are three putative N-linked glycosylation sites (Asn-171, Asn-506, and Asn-546) and five potential protein kinase C phosphorylation sites (Thr-25, Thr-98, Ser-123, Ser-523, and Ser-563). The presence of presumptive protein ki-



**Fig. 1.** Nucleotide and deduced amino acid sequences of CeCNT3. A, cDNA of CeCNT3 is 1826 bp. The open reading frame of CeCNT3 is flanked by 52 bp of 5'-untranslated region and 45 bp of 3'-untranslated region. The three potential N-linked glycosylation sites and five potential protein kinase C phosphorylation sites are labeled with \* and #, respectively. The 14 putative TMDs are highlighted. B, Kyte-Doolittle hydropathy plot of CeCNT3 with a window of 11 amino acid residues.



**A**

1

hCNT1 MENDPSRRRE SISLTPVAKG LENMGADFLE .SLEEGQLP RSDLSPAEIR SSWSEAPKPF FSRWRNLQPA  
rCNT1 MADNTQRQRE SISLTPMAHG LENMGAEFLF .SMEEGRLP HSHSLPEGE GLNKAERKA FSRWRSLQFT  
hCNT2 ~MEKASGRQ SISLSTVETG TVNPGLELME KEVEPEGS. .KRTD.AQGH SLGDGLGPST YQR.RSRWPT  
rCNT2 ~MAKSEGRK SASQDTSENG MENPGLELME VGNLEQ GK. .TLEEVTQGH SLKDLGLHSS LWR.RILQPF  
CeCNT3 ~~~~~~MK ISDVEENSTP QKENVLRQSC SLTS....KS LQNNLNLI... 140

71

hCNT1 LRARS CREH MQLRWIGTG LLCTGLSAFL LVCLLDLQRC LALFVLICV VLTFLGHLRL KRLGLPLRL  
rCNT1 VQARS CREH RQLGWICKG LLSTACLGFL MVCLLDLQRC LALFLIICV VLVFLAYDL KRLGLSLRL  
hCNT2 SKARS CKTH ARLKKILGL LLCLAYAAY LACILNFQR LALFVLICL VIFVLVHSP KLLGLKLT  
rCNT2 TKARS YQRH AGLKKILGL LLCLAYAAY LACILNFRR LALFVLICL VIFILACHF KFFAKSTF  
CeCNT3 .TEKGQOI RPYEGIGSL IFLVYHGF VAGIHNYQK BAPIYV...LFFWICYL NLIVGTTF 210

141

hCNT1 FL.....EP QGHPRLLLWF KRGLALAAFL GVLWLSLT SRPEQVSE AGICVFIAL ACCKHHCVA  
rCNT1 CV.....EP QGHSCLSLWL KRGLALAAGV GILWLSLT AQRPQVSE AGICVFLVL EAGCKHHRV  
hCNT2 CL.....EP FENLRRLWT KWFVAGVSL GLWLWLSLT AQRPQVSE AGICMFILIL EACKHHSVA  
rCNT2 CL.....EP KNTLRLRLW KRVFMGAADV GILWLALST AQRPQVSE AGICMFILIL EACKHHSVA  
CeCNT3 AVYDDVGR LEBITEQKKFV PLSLKIAFIF ALAFTIIS IHDLTRTG FAYVFYILFM VFNNRMKI 280

211

hCNT1 SFRAS SWG G LQFVLGLLVI RTEPFIAE WLGEQIRIE SYTKASSS EREAL....VKDFA  
rCNT1 SFRAS SWG G LQFVLGLFVI RTEPFIAE WLGDQIQV SYTEASSS EREAL....VKDFA  
hCNT2 SFRAS FSG G LQFVFGILVI RTDLTYTV WLGEQVQIE NYTVAASS ERTL....VKDFA  
rCNT2 SFRAS FSG G LQFVFGILVI RTEPFNAE WLGDQIQI AYTVASSS ERTL....VQSFA  
CeCNT3 NSVSASSA I MHYCVALLIL KWSTQWFF KFAELIVGS EFAQVAKS EPIAGPPNI CDLAPIT 350

281

hCNT1 VPIIIVFSC MSIVYHVL MWMILIA LMQVTMSTA TTLSVAGNI VSQTAPIL IRVGLADM  
rCNT1 VPIIIVFSC MSIVYYL MWMILIA LMQVTMSTA TTLSVAGNI VSQTAPIL IRVGLADM  
hCNT2 APIIIVFSC VSIYYL VWMVQV FLQITMSTA TTTLAVAGNI VGMTEAPIL IRVGLADM  
rCNT2 SPIIIVFSC MSIYYL VWMVQV FLQITMSTA ATTLAVAGNI VGMTEAPIL IRVGLADM  
CeCNT3 SQTLLIYSA VALFYFI IIVLQNSQ FMKVLITTP VSVNAWACT ELGMSAPIM VAPTEKID 420

351

hCNT1 SVHVVMG YTIASLLG YISFIDAT SIAAVMA SCALSLKL YPEVESKFR REEGVITYG  
rCNT1 SVHVVMG YTIASLLG YISFIDAT SIAAVMA SCALSLKL YPEVESKFR SENGVITYG  
hCNT2 SVHAVMG YTIASVLG YISFIDAS SIAAVMA SCALSKLA YPEVESKFK SEEGVIPRG  
rCNT2 SVHAVMG YTIASVLG YISFIDAS SIAAVMA SCALSLKL YPEVESKFK SKEGVIPRG  
CeCNT3 SVIFAIVS FICVATVFT YVALACPI YLTSLLS SMSLNCIM FETESNVK .EEDFTEHN 490

421

hCNT1 DAQNLIEAAS TGAASVKV ANIAINIAF LAVADINAA LSWLDMVDI QLSLGLICS ILRVAFIL  
rCNT1 DAQNLIEAAS AGAASVKV ANIAINIAF LAVAVNAA LSWLDMVDI QLSLGLICS VLRVAFIL  
hCNT2 KERNVLEAAS NGAVDAIGL TNVAINIAF LAVAINAA LSWLELVDI QLSLGLICS LLRMVFM  
rCNT2 EERNVLEAAS NGATDAIGL ANVAINIAF LAVAINST LSWLEMDI HLTSLGLICS VLRMVFM  
CeCNT3 DKRGFFDTLC SAGVALPTV FAIGTVVI MSLALDAV MEYIDLIGY DWTEMLFG AFFLALYM 560

491

hCNT1 .AWEDCP VIELLIL FLAEVQD SKYQRRRL AEAEWGNRK QWISVRAEVL TFAE  
rCNT1 .AWEDCP VIELLIF FLAEVQE SQYQRRRL AEAEWLGDK QWISVRAEIL TFAE  
hCNT2 .EWTDCP MLEMVIF FIEVQQ SQYKNRRL MEIEWEGEK QWISVRAEII TFAE  
rCNT2 .QWADCP LLEIVVF FIEVQQ SQYKNRRL VEEWINGEK QWISVRAEII AFSE  
CeCNT3 NDNSDQTL LQLMTT AVGVNN GILQK...A K.....LQPKSVVI AYAY 630

561

hCNT1 FSIIMLGG TSMVQRK DFSQIL FTACVSLVN CMGYVP RGAEDMNL LNTLSSSSF  
rCNT1 FSIIMLGG TSLVQRK DFSQIL ITAFVSLLN CMGYVP RGEVDVNL LNQTSSSSF  
hCNT2 LSIIMLGG TSIVHRK DLSKV FTACVSLIS CMGYVP RGAEDVNF PNTSFTNRY  
rCNT2 LTIIMLGG TSMVQRK DLCKLV FTACVSFIS CMGYVP RGAEDVNF LNTNFTNRY  
CeCNT3 FSIIMSIEF GGLASKR TISKLL CAISIACFMN TVSGI...SSPVIQS QKSDTCFRIP 680

631

hCNT1 EIQCCREAF QSV.....NP EFSPEALDNC CRFYNHTICA Q  
rCNT1 EVYLCCRQVF QST.....SS EFSQVALDNC CRFYNHTVCT ~  
hCNT2 ETYMCCRGLF QSTSLNGTNP PSFSGPWEDK EFSAMALTNC CGFYNNTVCA ~  
rCNT2 ETVYCCREL F QSTLLNGTNM PSFSGPWQDK ESSLRNLAKC CDLYTSTVCA ~  
CeCNT3 SG.....

**B**

hCNT1 TMGTTATETLSVAGNIFV**SQ**TEAPLLIRPYLADMTLSEVHVMTGGYATIAG**SL**LGAISF  
 rCNT1 TMGTSATETLSVAGNIFV**SQ**TEAPLLIRPYLADMTLSEVHVMTGGYATIAG**SL**LGAISF  
 CeCNT3 LIGTTPVESVNAWACTFL**SE**APLMVAPYIEKLTDS**EF**IVTSGACVAGVFTAFVAL  
 hCNT2 TMGTTATETL**AV**AGNIFV**Q**TEAPLLIRPYLGDMTLSEHVMTGGATISAG**SL**LGAIFAF  
 rCNT2 TMGTTAETL**AV**AGNIFV**Q**TEAPLLIRPYLADMTLSEHVMTGGATISAG**SL**LGAIFAF

**Fig. 2.** Sequence analysis. A, multiple alignment of CeCNT3, hCNT1, rCNT1, hCNT2, and rCNT2. Amino acid residues with 100% identity among the transporters are highlighted. Periods indicate the gaps to create the alignment. B, putative transmembrane domain 8 and 9 of hCNT1, rCNT1, hCNT2, and rCNT2 and the corresponding region of CeCNT3 (amino acids 275–335) were compared. In this particular region, there are nine conserved amino acid differences between CNT1 and CNT2. Of these nine residues, CeCNT3 differs from both CNT1 and CNT2 at one position; CeCNT3 is identical to CNT2 at seven positions and to CNT1 at one position. Shaded positions indicate conserved residues that are identical to CeCNT3; bold positions indicate conserved residues that differ from CeCNT3.

nase C sites suggests that CeCNT3 may be regulated by phosphorylation.

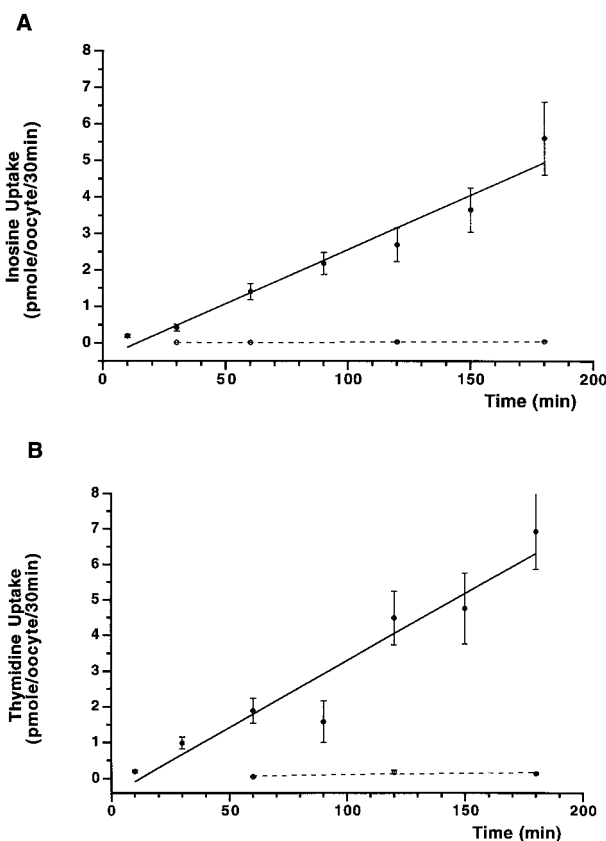
**Substrate Selectivity of CeCNT3.** Functional studies of CeCNT3 were carried out using naturally occurring nucleosides. Oocytes injected with CeCNT3 cRNA were incubated in transport buffer containing 10  $\mu$ M  $^3$ H-labeled nucleosides for 30 min. Initial studies established that the uptake of both thymidine and inosine was linear with time up to 3 h (Fig. 3). A 30-min time point was selected for further kinetic studies. Compared with control oocytes (water-injected), the uptake of inosine, thymidine, uridine, guanosine, and adenosine was significantly increased (20 to 30 fold) (Fig. 4). In contrast, the uptake of cytidine was not increased significantly. These data indicate that CeCNT3 exhibits a unique substrate selectivity, transporting inosine, thymidine, uridine, adenosine and guanosine, but not cytidine.

**Inhibition Profile of Nucleoside Transport.** To further determine the characteristics of CeCNT3, we examined the inhibitory effects of naturally occurring nucleosides (inosine, thymidine, uridine, guanosine, adenosine, and cytidine), nucleobases (hypoxanthine, thymine), ribose, thymidine monophosphate (5'TMP) and nitrobenzylthioinosine (NBMPR) on CeCNT3-mediated transport of inosine and thymidine. We observed that all of the naturally occurring nucleosides, except cytidine, significantly inhibited the transport of both inosine and thymidine (Fig. 5A, 5B). Although cytidine significantly inhibited the transport of thymidine ( $p < 0.05$ ), the inhibitory effect was much lower than that of other naturally occurring nucleosides.

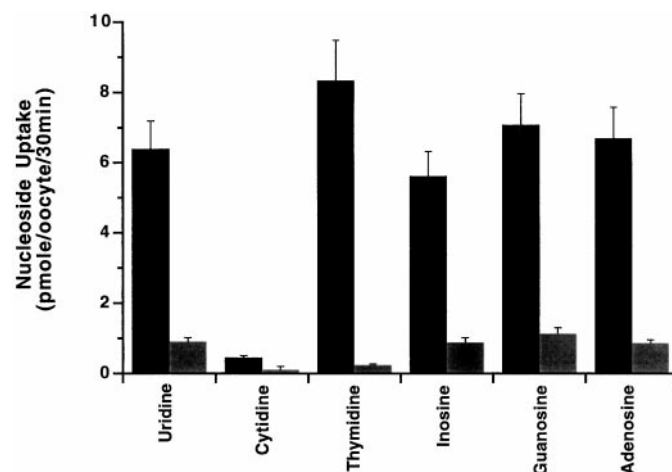
The inhibitory effects of NBMPR and other compounds were also examined (Fig. 5). We observed that hypoxanthine, thymine, and ribose did not significantly inhibit CeCNT3-mediated nucleoside flux. 5'TMP did not inhibit significantly the transport of inosine, whereas it significantly inhibited the transport of thymidine ( $p < 0.05$ ). The potent inhibitor of equilibrative nucleoside transport, NBMPR (100  $\mu$ M), had a significant inhibitory effect on CeCNT3-mediated transport of both inosine and thymidine. However, NBMPR had a much lower inhibitory potency on CeCNT3 than on mammalian equilibrative nucleoside transporters (Ward et al., 2000).

**Kinetic Studies of CeCNT3.** CeCNT3-mediated transport of inosine and thymidine was saturable, with apparent  $K_m$  values of  $15.2 \pm 5.3 \mu$ M and  $11.0 \pm 2.4 \mu$ M, respectively. The apparent  $V_{max}$  values of inosine and thymidine were  $3.20 \pm 0.50$  pmol/oocyte/30 min and  $3.85 \pm 2.88$  pmol/oocyte/30 min, respectively (Table 1). Although differences in the kinetics of inosine and thymidine transport were observed between batches of oocytes, within the same batch of oocytes, the apparent  $K_m$  value of thymidine was always lower than that of inosine; at the same (nonsaturating) concentration, thymidine was always transported at greater rate than inosine.

We next determined whether purine and pyrimidine nucleosides share the same translocation pathway in CeCNT3. To address this question, we examined the  $V_{max}$  and  $K_m$  values of inosine in the presence of thymidine, and the  $V_{max}$  and  $K_m$  of thymidine in the presence of inosine. As shown in Fig. 6 and Table 1, in the presence of 40  $\mu$ M thymidine, the  $K_m$  of CeCNT3 for inosine increased significantly from  $15.2 \pm 5.3 \mu$ M to  $35.7 \pm 11.5 \mu$ M, whereas the  $V_{max}$  did not change (Table 1). In the presence of 40  $\mu$ M inosine, the apparent  $K_m$  of thymidine increased significantly



**Fig. 3.** Time course of transport of inosine and thymidine by CeCNT3. A and B, uptake of [ $^3$ H]inosine (10  $\mu$ M) and [ $^3$ H]thymidine (10  $\mu$ M) into CeCNT3 cRNA-injected (solid line) or water-injected (dotted line) oocytes as the function of time was measured. Transport buffer containing 100 mM NaCl at pH 6.5 was used.



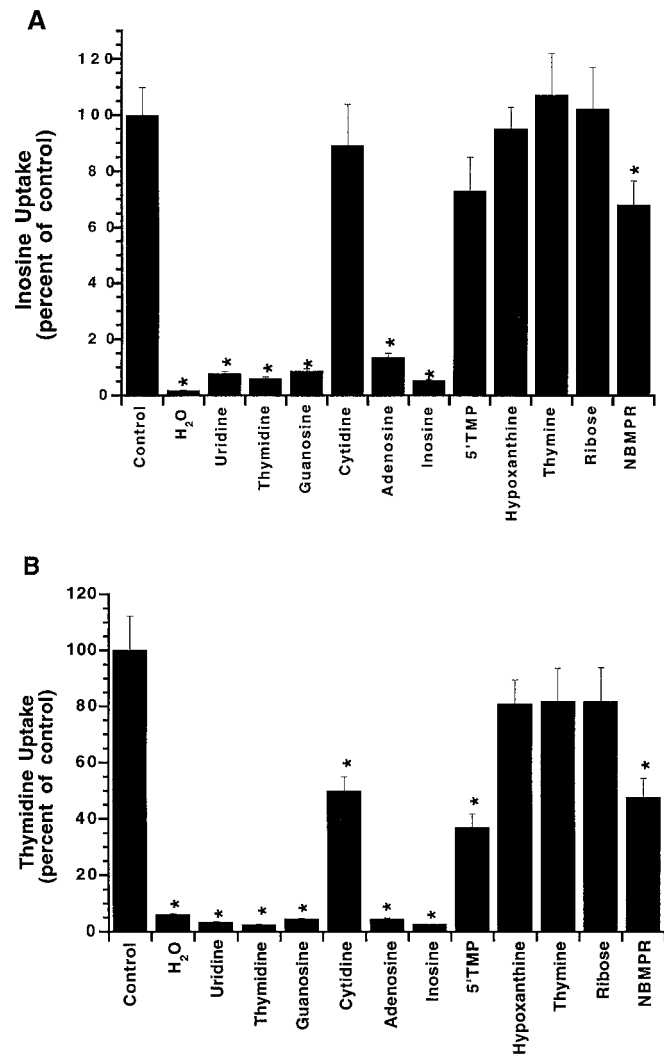
**Fig. 4.** Nucleoside selectivity of CeCNT3. Uptake assays of [ $^3$ H]inosine (10  $\mu$ M), [ $^3$ H]thymidine (10  $\mu$ M), [ $^3$ H]uridine (10  $\mu$ M), [ $^3$ H]cytidine (10  $\mu$ M), [ $^3$ H]adenosine (10  $\mu$ M) and [ $^3$ H]guanosine (10  $\mu$ M) into CeCNT3 cRNA-injected (solid bars, left of pairs) or water-injected (gray bars, right of pairs) oocytes were carried out in transport buffer containing 100 mM NaCl, pH 6.5. Values are expressed as mean  $\pm$  S.E.

from  $11.0 \pm 2.4 \mu$ M to  $61.6 \pm 9.6 \mu$ M, whereas the  $V_{max}$  did not change significantly (Table 1). These data demonstrate that, for CeCNT3, inosine is a competitive inhibitor of thymidine and that thymidine is a competitive inhibitor of inosine.

**Effects of Sodium-Gradient and Proton-Gradient on the Activity of CeCNT3.** In initial studies, we observed that CeCNT3-mediated transport of inosine into oocytes functioned well in media at pH 7.4 containing either 100 mM NaCl or 100 mM choline chloride, with uptake rates of  $6.74 \pm$

$0.56$  and  $6.33 \pm 0.69$  pmol/oocyte/30 min, respectively, indicating that transport was not dependent on sodium. No stimulatory effect of sodium up to concentrations of 100 mM on the activity of CeCNT3 was observed (Fig. 7A).

The effects of a proton-gradient, as a possible driving force, on the activity of CeCNT3 were examined. First, the CeCNT3-mediated transport of thymidine into oocytes was measured under different pH conditions. As shown in Fig. 7B, the transport of thymidine was dependent on pH, having optimal uptake at pH 5.5 to 6.0. This pH dependence was further confirmed by examining the effects of FCCP, a proton ionophore that disrupts the transmembrane proton gradient, on the CeCNT3-mediated transport of nucleosides. As shown in Fig. 8, at pH 5.5, pretreatment with 5  $\mu$ M FCCP significantly reduced the uptake of thymidine; this reduction was more significant with longer FCCP pretreatment. In contrast, at pH 8.0, FCCP pretreatment did not reduce the uptake of thymidine. Furthermore, the uptake of thymidine measured at pH 8.0 was significantly lower than that measured at pH 5.5. As a control, we examined the effects of FCCP on rat CNT1, a sodium-dependent nucleoside transporter, under the same conditions. For rCNT1, treatment with FCCP did not affect the uptake of thymidine at either pH 5.5 or pH 8.0, and the uptakes of thymidine at pH 5.5 and



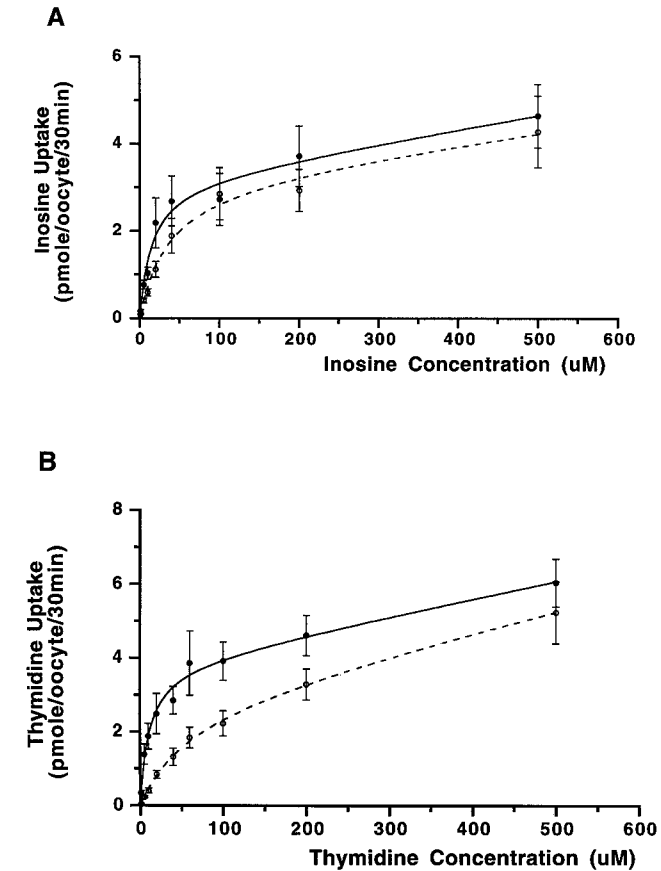
**Fig. 5.** Inhibition profile of CeCNT3. A and B, uptakes of [<sup>3</sup>H]inosine (10  $\mu$ M) and [<sup>3</sup>H]thymidine (10  $\mu$ M) into CeCNT3 cRNA-injected oocytes or water-injected (H<sub>2</sub>O) were measured in the absence of inhibitors (control) or in the presence of naturally occurring nucleosides (inosine, thymidine, uridine, cytidine, adenosine, and guanosine), 5'TMP, hypoxanthine, thymine, ribose, and NBMPR. Final inhibitor concentrations were 1 mM, except NBMPR (100  $\mu$ M). \*, significantly inhibited the CeCNT3-mediated inosine or thymidine uptakes ( $p < 0.05$ ).

**TABLE 1**

Apparent  $K_m$  and  $V_{max}$  values of inosine and thymidine

CeCNT3-mediated uptake of inosine and thymidine were measured in the absence and in the presence of inhibitors as described under *Materials and Methods*. Uptake values are expressed as mean  $\pm$  S.E. For inosine, thymidine was used as inhibitor; and for thymidine, inosine was used as inhibitor. This experiment was repeated several times and  $K_m$  and  $V_{max}$  values from one representative experiment are given.

Inhibitor		$K_m$	$V_{max}$
		$\mu M$	pmol/oocyte/30 min
Inosine	No inhibitor	$15.2 \pm 5.3$	$3.19 \pm 0.42$
	40 $\mu M$ thymidine	$35.7 \pm 11.5$	$3.20 \pm 0.50$
Thymidine	No inhibitor	$11.0 \pm 2.4$	$3.85 \pm 0.26$
	40 $\mu M$ inosine	$61.4 \pm 9.6$	$2.88 \pm 0.27$



**Fig. 6.** Kinetic Study. A and B, in the absence of inhibitor (solid line) or in the presence of inhibitor (dotted line), CeCNT3-mediated uptakes of [<sup>3</sup>H]inosine (10  $\mu$ M) and [<sup>3</sup>H]thymidine (10  $\mu$ M) as a function of the substrate concentration were carried out in transport buffer containing 100 mM NaCl, pH 6.5. For inosine (A), 40  $\mu$ M thymidine was used as inhibitor; for thymidine (B), 40  $\mu$ M inosine was used as inhibitor. Under the same experimental conditions, uptake of [<sup>3</sup>H]inosine and [<sup>3</sup>H]thymidine into water-injected oocytes was less than one-tenth of the uptake into CeCNT3 cRNA-injected oocytes (data not shown).



pH 8.0 were not significantly different (data not shown). The effects of the sodium gradient, proton gradient, and FCCP on the CeCNT3-mediated transport of inosine were also performed and the results were similar to those of thymidine (data not shown). FCCP may have exerted its effects by creation of a proton-diffusion potential or directly, by dissipating proton ions that may be coupled to nucleoside transport via CeCNT3. The finding that rCNT1-mediated nucleoside transport was not changed by treatment with FCCP suggests that CeCNT3 has a driving force (a proton gradient) that is different from that of rCNT1 (a sodium gradient). These data suggest that the activity of CeCNT3 is dependent on a proton gradient and the CeCNT3-mediated transport of nucleosides is coupled to protons.

**Site-Directed Mutagenesis.** Multiple alignments of the amino acid sequence of CeCNT3 with hCNT and rCNT in the region (between amino acids 275 and 335 of CeCNT3) shown previously to be critical in substrate recognition and binding (Wang and Giacomini, 1997, 1999b; Loewen et al., 1999) were performed. These alignments show that, of the nine con-

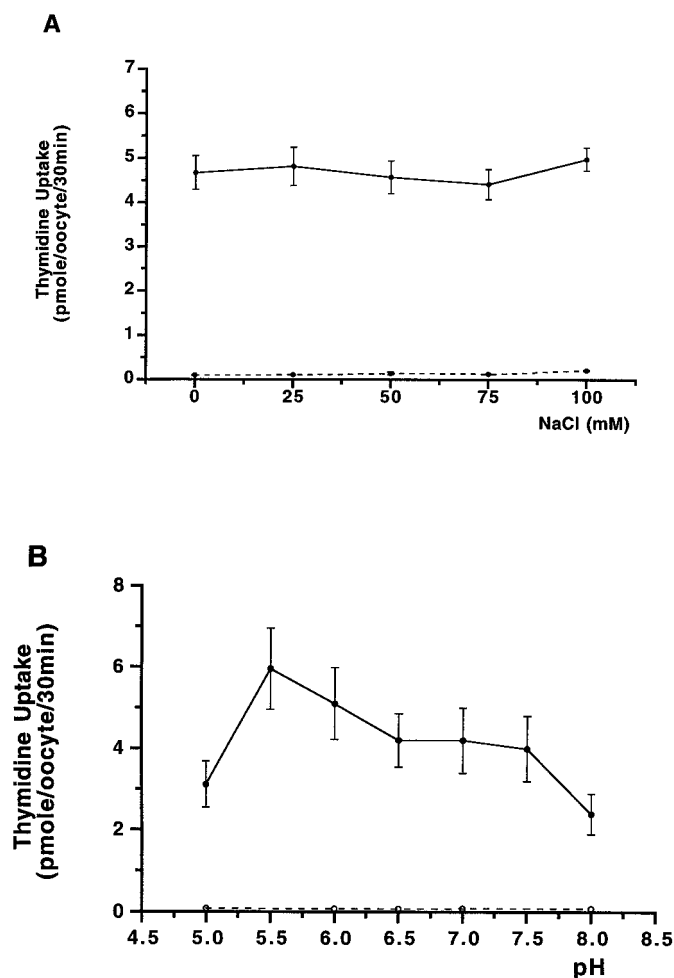
served residues in this particular region, CeCNT3 has seven residues identical with those of hCNT2 and rCNT2 (except the tyrosine at 332, which is identical with hCNT1 and rCNT1) (Fig. 2B). Because CeCNT3 transports not only purine nucleosides but also thymidine, we hypothesized that the tyrosine at position 332 of CeCNT3 may facilitate the recognition and subsequent transport of thymidine as well as other nucleosides. We mutated this residue to the equivalent residue, phenylalanine, in CNT2. Compared with that of the wild-type transporter, the uptake of thymidine mediated by Y332F mutant was decreased (Fig. 9A). At equivalent concentrations, thymidine is the nucleoside transported most rapidly by the wild-type transporter (Fig. 4); however, it is the nucleoside transported most slowly by the Y332F mutant. Kinetic studies revealed that the  $K_m$  value of thymidine is increased from  $11.4 \pm 2.1 \mu\text{M}$  to  $31.7 \pm 5.5 \mu\text{M}$  (Fig. 9B). Further studies demonstrated that the  $K_m$  value of inosine was also increased (Table 2), suggesting that the tyrosine in this position is indeed involved in nucleoside recognition.

Recently, it was reported that, in hCNT1, residues S353 and L354 are involved in the transport of pyrimidines (Loewen et al., 1999). Therefore, we examined the importance of these residues on the selectivity of CeCNT3. Despite its low activity (about 2–3-fold over water control, Table 2), the T327S/V328L mutant maintained the substrate selectivity of the wild-type transporter, transporting all of the naturally occurring nucleosides but not cytidine.

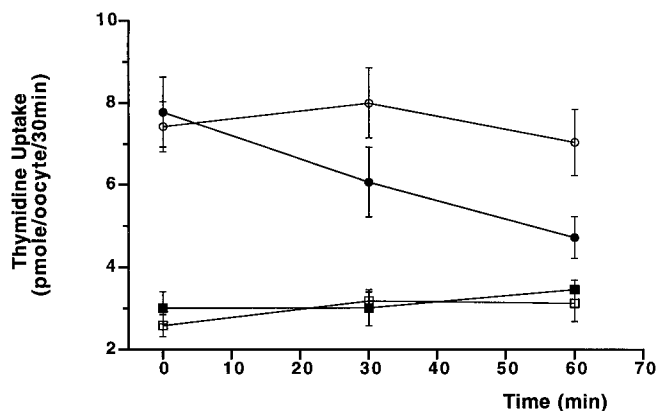
## Discussion

We report here the cloning of a cDNA from *C. elegans* coding for a novel member, CeCNT3, of the concentrative nucleoside transporter family. We demonstrate that CeCNT3 has unique functional characteristics.

CeCNT3 has a unique substrate selectivity compared with other concentrative nucleoside transporters. In particular, in contrast to the cloned purine (CNT2 or SPNT) and pyrimidine (CNT1) selective transporters, CeCNT3 transports all naturally occurring nucleosides tested with the exception of cytidine (Fig. 4). Based upon the competitive interaction between thymidine and inosine (Fig. 6), our data suggest that



**Fig. 7.** Effects of sodium and pH on the activity of CeCNT3. A, sodium dependence of CeCNT3. Uptake of [ $^3\text{H}$ ]thymidine ( $10 \mu\text{M}$ ) in CeCNT3 cRNA-injected (solid line) or water-injected (dotted line) oocytes were measured in transport buffer containing 0 to 100 mM NaCl with choline chloride to maintain the iso-osmolality. B, pH dependence of CeCNT3. Uptake of [ $^3\text{H}$ ]thymidine ( $10 \mu\text{M}$ ) in CeCNT3 cRNA-injected (solid line) or water-injected (dotted line) oocytes were measured in transport buffer containing 100 mM NaCl with pH ranging from 5.0 to 8.0. In both A and B, values are expressed as mean  $\pm$  S.E.



**Fig. 8.** Effects of FCCP on the uptake of thymidine. Oocytes were incubated in choline buffer at pH 5.5 with 5  $\mu\text{M}$  FCCP (●), pH 5.5 without 5  $\mu\text{M}$  FCCP (○), pH 8.0 with 5  $\mu\text{M}$  FCCP (■) or pH 8.0 without 5  $\mu\text{M}$  FCCP (□) for 0, 30, and 60 min after having been preincubation in choline buffer at pH 8.0 for 3 h. Uptake of thymidine (30 min) was then performed in sodium buffer at pH 5.5 and 8.0. Under the same experimental conditions, uptake of [ $^3\text{H}$ ]thymidine into water-injected oocytes was less than one tenth of the uptake into cRNA-injected oocytes (data not shown).

there is a single recognition site in CeCNT3 for both purine and pyrimidine nucleosides. Thus, the molecular basis for broad selectivity resides in a single permeation pathway rather than multiple pathways.

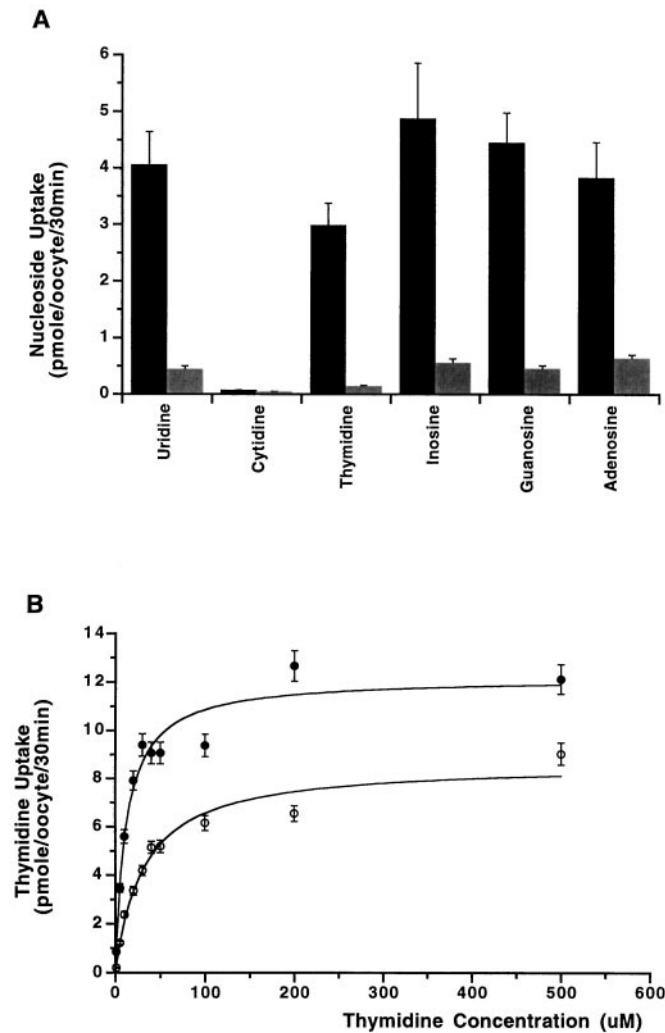
**Molecular Determinants of Substrate Discrimination.** CeCNT3 is ~30% identical with mammalian CNTs (hCNT and rCNT) (Fig. 2A). Like hCNT and rCNT, it also has 11 to 14 putative transmembrane domains (Fig. 1). CeCNT3 thus has the overall structural features of a concentrative nucleoside transporter.

To understand the structural basis for the unique substrate selectivity of CeCNT3, it is of interest to compare its amino acid sequence with that of the CNT1 (pyrimidine-selective) and CNT2 (purine-selective) transporters. Of particular interest are residues that differ between the CNT1 and CNT2 transporters, which may therefore be responsible

for substrate selectivity. Twenty-two amino acid residues of CeCNT3 are identical with the two purine-selective transporters, rCNT2 and hCNT2, but not with the two pyrimidine-selective transporters, rCNT1 and hCNT1. Similarly, 23 amino acid residues of CeCNT3 are identical with the pyrimidine-selective transporters but not the purine-selective transporters. Thus, CeCNT3 is not strikingly more similar to either type of transporter. A bias toward CNT2 is seen in one region (positions 275–335; see below).

Previously, we and others identified amino acid residues of the concentrative nucleoside transporters that are involved in substrate discrimination (Wang and Giacomini, 1997, 1999b; Loewen et al., 1999). In this region, corresponding to amino acids 275–335 of CeCNT3, CNT1 and CNT2 differ at nine positions (Fig. 2B). Of these nine positions, CeCNT3 differs from both CNT1 and CNT2 at one position; CeCNT3 is identical with CNT2 at seven positions and to CNT1 at one position. Our previous work has shown that positions 318 and 319 of rCNT1 are involved in purine discrimination (Wang and Giacomini, 1999a,b). In the wild-type rCNT1, which has serine at position 318 and glutamine at position 319, purines are restricted and the transporter is pyrimidine selective. If the residues are changed to glycine and methionine, respectively, then purines are accepted and the transporter is purine selective or broadly selective (accepting both purine and pyrimidine nucleosides) (Wang and Giacomini, 1999b). The presence of glycine and methionine at the corresponding positions of CeCNT3 (i.e., at positions 293 and 294) leads to the expectation that the transporter would accept purines. This prediction is confirmed in our experiments [i.e., CeCNT3 accepted guanosine, inosine and adenosine (Fig. 4)].

Positions 353 and 354 of hCNT1 were recently shown to be involved in pyrimidine selectivity. In hCNT1, the mutant, S353T/L354V, does not accept pyrimidines (Loewen et al., 1999). Because CeCNT3 has a threonine and a valine residue at the corresponding positions, one might anticipate that similarly, it would not accept pyrimidines. In fact, CeCNT3 accepts thymidine but not cytidine (Fig. 4). Our results showed that merely changing these two residues (T327S/V328L) did not alter the general substrate selectivity [i.e., thymidine was accepted and cytidine was not (Table 2)]; however, the kinetics could not be evaluated because of the



**Fig. 9.** Nucleoside selectivity and kinetic study of Y332F mutant. A, uptake assays of [<sup>3</sup>H]inosine (10  $\mu$ M), [<sup>3</sup>H]thymidine (10  $\mu$ M), [<sup>3</sup>H]uridine (10  $\mu$ M), [<sup>3</sup>H]cytidine (10  $\mu$ M), [<sup>3</sup>H]adenosine (10  $\mu$ M) and [<sup>3</sup>H]guanosine (10  $\mu$ M) into Y332F mutant cRNA-injected (solid bars) or water-injected (gray bars) oocytes were carried out in transport buffer containing 100 mM NaCl, pH 6.5. Values are expressed as mean  $\pm$  S.E. B, wild-type CeCNT3-mediated (solid circles) and Y332F mutant mediated ( $\circ$ ) uptakes of [<sup>3</sup>H]thymidine as a function of substrate concentration were carried out in transport buffer containing 100 mM NaCl, pH 6.5. Under the same experimental conditions, uptake of [<sup>3</sup>H]thymidine into water-injected oocytes was less than one tenth of the uptake into cRNA-injected oocytes (data not shown).

**TABLE 2**  
Nucleoside uptake of wild-type, Y332F, and T327S/V328L

Wild-type, Y332F, and T327S/V328L mediated uptakes of nucleosides were measured as described under *Materials and Methods*. Uptake values are expressed as mean  $\pm$  S.E. These experiments were repeated several times and values for uptake  $K_m$ , and  $V_{max}$  from one representative experiment are given.

Nucleosides	Uptake		
	Wild-type	Y332F	T327S/V328L
pmol/oocyte/30 min			
Uridine	6.39 $\pm$ 0.81	4.06 $\pm$ 0.58	0.72 $\pm$ 0.08
Cytidine	0.46 $\pm$ 0.06	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01
Thymidine	8.33 $\pm$ 1.15	2.98 $\pm$ 0.39	0.39 $\pm$ 0.05
Inosine	5.62 $\pm$ 0.70	4.87 $\pm$ 0.97	0.83 $\pm$ 0.09
Guanosine	7.08 $\pm$ 0.88	4.44 $\pm$ 0.53	0.84 $\pm$ 0.10
Adenosine	6.69 $\pm$ 0.89	3.82 $\pm$ 0.63	0.99 $\pm$ 0.12
Inosine			
$K_m$ ( $\mu$ M)	27.5 $\pm$ 3.7	102.6 $\pm$ 16.5	
$V_{max}$ (pmol/oocyte/30 min)	39.2 $\pm$ 1.7	60.6 $\pm$ 4.0	
Thymidine			
$K_m$ ( $\mu$ M)	10.6 $\pm$ 2.0	31.6 $\pm$ 5.6	
$V_{max}$ (pmol/oocyte/30 min)	21.5 $\pm$ 0.9	19.1 $\pm$ 1.1	



low function of this mutant transporter. These data indicate that the presence of threonine and valine at these positions is insufficient to block the transport of thymidine. Furthermore, the data suggest that other residues are involved in discrimination among pyrimidines. In particular, we noted that CeCNT3 is identical to hCNT1 at position 332. We hypothesize that the phenylalanine at this position in hCNT2 and rCNT2 may block thymidine uptake and that residue at this position may play a role in discriminating among pyrimidines. Indeed, by incorporating a phenylalanine at this position (mutant Y332F), the uptake of thymidine was decreased (Fig. 9A, Table 2) and the apparent  $K_m$  of thymidine was increased (Fig. 9B, Table 2). However the  $K_m$  value of the purine nucleoside inosine also increased (Table 2). These data suggest that residues of position 332 are indeed involved in substrate recognition and translocation, and modulate nucleoside transporter's activity. A phenylalanine at position 332 of CeCNT3 is not sufficient to block thymidine uptake.

**Proton Dependence.** Another unique characteristic of CeCNT3 is that in contrast to mammalian transporters in the CNT family, nucleoside transport is dependent on the proton gradient, but not on the sodium gradient (Figs. 7 and 8). Concentrative transporters use ion-coupled electrochemical energy stored in a transmembrane gradient to actively transport substrates into or out of cells. In bacteria as well as in plants, a proton is the preferred coupling ion and the proton gradient is maintained by  $H^+/K^+$ -ATPase. In vertebrates, sodium is the preferred coupling ion of many coupled transporters because of the evolutionary substitution of  $H^+/K^+$ -ATPase by  $Na^+/K^+$ -ATPase in animal cells (Hediger, 1994; Nelson, 1994). To date, only a few mammalian plasma membrane transporters (e.g., the oligopeptide transporters, PepT1 and PepT2) have been described that use protons as the coupling ion (Fei et al., 1994; Leibach and Ganapathy, 1996). In the case of ion-coupled (also known as concentrative) nucleoside transporters, the NupC family, in bacteria such as *Escherichia coli*, *Bacillus subtilis*, and *Helicobacter pylori*, consists of several proton-coupled nucleoside transporters (Craig et al., 1994). In contrast, all documented concentrative nucleoside transporters in vertebrates are sodium-dependent (Crawford et al., 1990; Dagnino et al., 1991; Wu et al., 1992, 1994; Huang et al., 1993, 1994; Che et al., 1995; Wang et al., 1997).

In *C. elegans*, many kinds of transporters have been cloned. Some of these transporters are sodium-dependent (Jayanthi et al., 1998; Lee et al., 1999), whereas others are pH-dependent (Fei et al., 1998; Duerr et al., 1999; Wu et al., 1999). However, in general, the ion-dependence of transporters in mammalian cells and in *C. elegans* is consistent. For example, in both mammalian cells and *C. elegans*, dopamine transporters (Wall et al., 1993; Jayanthi et al., 1998; Jones et al., 1999) and phosphate transporters (Feild et al., 1999; Lee et al., 1999; Jobbagy et al., 2000) are sodium-dependent, whereas peptide transporters (Fei et al., 1998, 1999; Chen et al., 1999) are pH-dependent. To our knowledge, CeCNT3 represents the only exception to this general paradigm; that is, the ion-dependence of CeCNT3 is not the same as that of its orthologs in mammalian cells. The changing of ion-dependence of nucleoside transporters from proton in *C. elegans* to sodium in mammalian cells is consistent with the evolutionary trend of substitution of  $H^+/K^+$ -ATPase by  $Na^+/K^+$ -ATPase. However, it is notable that many *C. elegans* transport-

ers function well with a sodium gradient coupling mechanism. Studies of the cellular localization and sorting of CeCNT3 and its physiologic role will provide some clues as to why this transporter exhibits a unique driving force and substrate selectivity in comparison to its mammalian orthologs. The  $Na^+$ /nucleoside transporter stoichiometry has been determined to be 1:1 for CNT1 and CNT2 (Griffith and Jarvis, 1996; Yao et al., 1996; Wang and Giacomini, 1999a) and 2:1 for N3 (Wu et al., 1992). Determination of the stoichiometry of a proton-dependent protein is generally made using electrophysiological methods (Chen et al., 1999). Because the substrate-induced proton currents were too small, we were unable to measure a proton/nucleoside stoichiometry for CeCNT3.

In summary, we report here the cloning and characterization of a novel nucleoside transporter from *C. elegans*, CeCNT3. This transporter can transport all naturally occurring purine and pyrimidine nucleosides except cytidine. In contrast to mammalian CNT, transport via CeCNT3 is dependent on protons. Site-directed mutagenesis studies indicate that the tyrosine at position 332 supports the recognition and transport of nucleosides; this residue may contribute to the unique selectivity of CeCNT3. Further studies of CeCNT3 will provide for an enhanced understanding of the physiologic and pharmacologic roles of concentrative nucleoside transporters.

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