We stably transfected the cloned human equilibrative nucleoside transporters 1 and 2 (hENT1 and hENT2) into nucleoside transporter-deficient PK15NTD cells. Although hENT1 and hENT2 are predicted to be 50-kDa proteins, hENT1 runs as 40 kDa and hENT2 migrates as 50 and 47 kDa on SDS-polyacrylamide gel electrophoresis. Peptide N-glycosidase F and endoglycosidase H deglycosylate hENT1 to 37 kDa and hENT2 to 45 kDa. With hENT1 being more sensitive, there is a 7000-fold and 71-fold difference in sensitivity to nitrobenzylthioinosine (NBMPR) (IC50 0.4 ± 0.1 nM versus 2.8 ± 0.3 μM) and dipyridamole (IC50 5.0 ± 0.9 nM versus 356 ± 13 nM), respectively. [3H]NBMPR binds to ENT1 cells with a high affinity Kd of 0.377 ± 0.098 nM, and each ENT1 cell has 34,000 transporters with a turnover number of 46 molecules/s for uridine. Although both transporters are broadly selective, hENT2 is a generally low affinity nucleoside transporter with 2.6-, 2.8-, 7.7-, and 19.3-fold lower affinity than hENT1 for thymidine, adenosine, cytidine, and guanosine, respectively. In contrast, the affinity of hENT2 for inosine is 4-fold higher than hENT1. The nucleobase hypoxanthine inhibits [3H]uridine uptake by hENT2 but has minimal effect on hENT1. Taken together, these results suggest that hENT2 might be important in transporting adenosine and its metabolites (inosine and hypoxanthine) in tissues such as skeletal muscle where ENT2 is predominantly expressed.

Two classes of mammalian nucleoside transporters have been described (1, 2). The Na+-independent nucleoside transporters mediate equilibrative transport (facilitated diffusion) of nucleosides and are inhibited by nitrobenzylthioinosine (NBMPR).1 The Na+-dependent nucleoside transporters are characterized by their Na+ dependence, resistance to inhibition by NBMPR, and the ability to concentrate nucleosides intracellularly against a concentration gradient.

The Na+-independent equilibrative nucleoside transporters are further classified into two subclasses according to their sensitivity to NBMPR (1, 2). The equilibrative NBMPR-sensitive system (es) is sensitive to nM concentrations of NBMPR, whereas the equilibrative-insensitive system (ei) is resistant to NBMPR concentrations up to 1 μM. Both es and ei systems are broadly selective, transporting both purine and pyrimidine nucleosides, and are inhibited by the vasodilators dipyridamole and dilazep. The es system is ubiquitously expressed, whereas the ei system is found as a minor component in intestine, leukemia cells, skeletal muscles, and cardiovascular tissues/cells (1–3). Both systems appear to be involved in scavenging nucleosides, which is especially important in cells that are unable to synthesize nucleosides de novo, such as those of the intestinal epithelium and lymphocytes (4, 5).

The NBMPR-sensitive Na+-independent nucleoside transporter has recently been cloned and is termed ENT1 (6). This was achieved by library screening using an oligonucleotide probe that was designed from the N-terminal amino acid sequences obtained from the highly purified human erythrocyte nucleoside transporter. The cloned human ENT1 cDNA (hENT1) encodes a protein of 456 amino acids. Although this NBMPR-sensitive nucleoside transporter has several similar molecular properties to the erythrocyte glucose transporter (GLUT1) (7, 8), hENT1 has no homology to the GLUT1 (6). When the cDNA is expressed in oocytes, [3H]uridine transport by hENT1 is inhibited by both purine and pyrimidine nucleosides and by low concentrations of NBMPR, dilazep, and dipyridamole. This confirms that hENT1 is an NBMPR-sensitive nucleoside transporter (6).

Sequence search of the GenBank™ data base showed that hENT1 exhibits 48% identity in amino acids to the 38-kDa mouse and human HNP36 proteins, which are delayed-early proliferative response gene products with unknown function (9). This suggests that HNP36 may belong to the family of Na+-independent nucleoside transporters. Based on this information, a rat homolog of HNP36 was subsequently cloned and is named rENT2 (10). The cloned rENT2 cDNA encodes a protein of 456 amino acids that is 48% identical to hENT1 and rat ENT1 (rENT1) (10). Like hENT1, the [3H]uridine transport by rENT2 is inhibited by both purine and pyrimidine nucleosides. However, rENT2 is resistant to NBMPR inhibition, suggesting that rENT2 is an ei nucleoside transporter (10). The human HNP36, now re-named hENT2, has also been function-
ally characterized in oocytes (11). hENT2 has also been independently cloned by functional complementation in nucleoside transport-deficient CEM human leukemia cells (12).

In intestine, Na+-independent nucleoside transport has been shown to indirectly affect chloride secretion by regulating extracellular concentrations of a potent secretagogue, adenosine (13, 14). Recently, we have demonstrated by function and by message expression that the human colonic secretory epithelial cells, T84, express both ENT1 and ENT2 (3). We then cloned full-length hENT1 and hENT2 from T84 cells (3).

At this time, most of the functional characterization of cloned mammalian ENT1 and ENT2 has been performed in oocytes (6, 10, 11). Although both hENT1 and hENT2 are believed to be broadly selective as demonstrated by competition studies, the kinetics of transport of natural nucleosides, such as adenosine, inosine, thymidine, guanosine, and cytidine, have not yet been characterized. These nucleosides have not yet been established as “permeants” of ENT1 and ENT2, as there has been no direct measurement of the uptake of the radioactive forms of these nucleosides. Physiologically, the $\alpha$ transport system is poorly defined as this process is normally found as a minor component of nucleoside transport in tissues and cells which express multiple nucleoside transport systems including $\alpha$ and other Na-dependent nucleoside transporters (1–3, 15).

Na+-independent nucleoside transport systems are ubiquitously expressed. In the present study, we generated a cell line deficient in all endogenous nucleoside transporters, expressed the cloned hENT1 and hENT2 in this null cell model, and fully characterized the biochemical, pharmacological, and kinetic properties of these transporters. The swine kidney tubular epithelial cell line, PK15, was used because its endogenous nucleoside transport system has been well characterized and consists of only the NBMPR-sensitive Na+-independent system (16). Thus, a transport-deficient mutant can be isolated in a single step by chemical mutagenesis followed by selection with cytotoxic nucleosides (17). In contrast to lymphoma cell lines that have also been used for generating such nucleoside transporter-deficient cells (18), PK15 cells are adherent and are easy to transfect and selection of positively transfected cells that have also been used for generating such nucleoside transporter-deficient cells (18), PK15 cells are adherent and are easy to transfect and selection of positively transfected cells with antibiotics. The success of isolation of PK15 cells deficient in endogenous nucleoside transporters has previously been described by Aran and Plagemann (17).

**Experimental Procedures**

**Cell Lines**—The swine kidney tubular epithelial cell line, PK15, was obtained from ATCC (Manassas, VA). Cells were maintained in Eagle’s minimal essential medium/Earle’s Balanced Salt Solutions (1:1), with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5% fetal bovine serum, penicillin/streptomycin (50,000 units/liter, 50 mg/liter), at 37 °C with 5% CO2 and 95% air. For uptake experiments, cells were grown on plastic 12- or 24-well culture plates (Falcon). Media were changed every 3–4 days, with all cells fed on the day prior to experiments.

**Generation of a Nucleoside Transport-deficient PK15 (PK15NTD) Cell Line**—Active proliferating PK15 cells grown to 50% confluency in a T75 flask were incubated for 20 h in complete media with 0.025% (v/v) ethylmethanesulfonate (19). The cells were then allowed to proliferate for 7 days, allowing sufficient time for the turnover of endogenous Na+-dependent nucleoside transporter and the expression of nucleoside transporter-deficient phenotype in cells that had been mutated successfully (17, 18). After 7 days the nucleosides cytosine arabinoside (AraC) (1 mM) and tubercidin (1 μM) were added to the culture medium. Following 3 weeks of selection with the cytotoxic nucleosides, clones of surviving PK15 cells were expanded and screened for a lack of NBMPR-sensitive [3H]uridine uptake.

**Cloning, Epitope Tagging, and Transfection of hENT1 and hENT2 into Nucleoside Transport-deficient PK15 Cells**—The cloning of hENT1 and hENT2 full-length cDNA from the T84 human colonic epithelial cell line has been described (3). Total RNA was annealed with either oligo(dT)$_{12-18}$ or random hexanucleotides, and first strand cDNA synthesis was carried out with Superscript II RNase H Reverse Transcriptase (SuperScript Preamplification System, Life Technologies, Inc.). Primers for hENT1 were designed from the published sequence (3) (GenBank™ accession number U81375) CCACTGAAACAGGTCAC-CAGC (5′-primer) and CTGGACAATTCGCCCGGAAACG (3′-primer) for ENT1. Primers for hENT2 were CTTCGGACCTTCTACAG (5′-primer) and CTGGACAATTCGCCCGGAAACG (3′-primer). Note that an XhoI site (underlined) was included in the 3′-primer. This XhoI site changes the stop codon into a serine residue and allows the reading frame to be maintained to read through a C-terminal 11-amino acid VSVG tag when the sequence is subcloned into the eukaryotic expression vector PECE/VSVG (20). Reactions were carried out in a PE Applied Biosystems GeneAmp 9700 (Foster City, CA) for 50 cycles (45 s at 94 °C, 45 s at 55 °C, and 1.5 min at 72 °C), followed by 72°C for 10 min. Full-length hENT1 and hENT2 cDNAs were excised, purified from the gel, digested with XhoI, cloned into the PECE/VSVG vector, and subjected to fluorescent sequencing according to the manufacturer’s protocols (PE Applied Biosystems 377 Automated DNA sequencer). hENT1/VSVG and hENT2/VSVG were then excised from the PECE vector and ligated into pcDNA3 (Invitrogen) at the HindIII/XhoI sites, creating the constructs hENT1/VSVG/pcDNA3 and hENT2/VSVG/pcDNA3. These constructs were transfected into PK15NTD cells using LipofectAMINE (Life Technologies, Inc.). Clones were selected by adding 0.5 mg/ml G418 to the culture medium, followed by evaluation for the success of reconstitution of dipridamole (10 μM)-sensitive [3H]uridine uptake in PK15NTD cells. For simplicity, the PK15NTD cells stably transfected with hENT1 and hENT2 were named as ENT1 and ENT2 cells, respectively.

**Membrane Preparation and Western Blot Analysis**—Crude membranes were purified from untransfected PK15NTD cells and from ENT1 and ENT2 cells by lysis and sonication in 5 mM sodium phosphate (pH 7.5) containing protease inhibitors. The cell lysate was then centrifuged for 10 min at 3,000 × g followed by centrifugation of the resulting supernatant for 30 min at 30,000 × g. The final pellet was fine-needle homogenized in 5 mM sodium phosphate buffer (21). SDS-PAGE was performed, after which the proteins were transferred onto nitrocellulose membranes. After blocking in Tris-buffered saline containing 150 mM NaCl, 13 mM Tris-HCl (pH 7.5) (TBS), 5% non-fat dry milk, membranes were incubated in blocking buffer containing mouse P5D4 VSVG monoclonal antibody, washed 5 times with TBS, 0.02% Triton X-100, incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Excess secondary antibody was extensively washed with TBS, 0.02% Triton X-100, and antigen reactivity was detected by enhanced chemiluminescence.

**Enzymatic Deglycosylation**—ENT1 and ENT2 cell membranes (20 μg) were incubated in 0.5% SDS and 1% mercaptoethanol by boiling for 10 min (21). The denatured membranes were then incubated with 500 units of PNGase F (New England Biolabs) in 1% Nonidet P-40 and 50 mM sodium citrate (pH 7.5) or with 500 units of Endo H (New England Biolabs) in 50 mM sodium citrate (pH 5.5) for 3 h at 37 °C (21). The endoglycosidase-treated membranes were then analyzed by Western blotting with VSVG antibody as described above.

**Kinetics of hENT1 and hENT2**

Concentration Dependence of $^{3}$H-Nucleoside Uptake—All experiments were carried out in HEPES-buffered Ringer’s solution containing (in mM) 135 NaCl, 5 KCl, 3.33 NaH$_2$PO$_4$, 0.83 Na$_2$HPO$_4$, 1.0 CaCl$_2$, 1.0 MgCl$_2$, 10 glucose, and 5 HEPES (pH 7.4). Na+-free buffer contained in (pH 7.4) 140 N-methyl-d-glucamine, 5 HEPES, 5 K$_2$PO$_4$, 1.0 CaCl$_2$, 1.0 MgCl$_2$, and 10 glucose (pH 7.4). Confluent monolayers of cells were washed three times in HEPES-buffered solution, followed by 10 min preincubation in the same buffer. HEPES-buffered solution containing varying concentrations of $^{3}$H-nucleosides (2 μCi/ml, ± 1 μM NBMPR (ENT1) or ± 10 μM dipridamole (ENT2)) was then added, followed by 1 min incubation, and plates were washed 3 times rapidly with ice-cold phosphate-buffered saline (pH 7.4). Cells were solubilized overnight in 1 ml of 5% Triton X-100, and radioactivity was measured by a β-scintillation counter. The protein content of representative monolayers was determined spectrophotometrically using a commercial bicinchoninic acid (Pierce).

**High Affinity [3H]NBMPR Binding**—For ENT1 cells, cells grown to confluence in 24-well plates were exposed to 1 ml of varying concentrations of [3H]NBMPR (0.03 to 10 nM) with and without a 10 μM nonsaturative NBMPR, for 20 min at room temperature. Varying concentrations of [3H]NBMPR was then removed from duplicate wells. The cells were then washed rapidly in ice-cold isonicotinic phosphate-buffered saline, solubilized with 0.1 μM NaOH, and counted for radioactivity. For ENT1 cell membranes, membranes (100 μg) were incubated with varying concentrations of [3H]NBMPR (0.03 to 10 nM, ± 10 μM nonradioactive NBMPR) for 20 min at room temperature. Bound and free [3H]NBMPR were then separated from each
other by rapid filtration with Whatman GF/B filters (23). The radioactivity that was retained on filters (bound \(^{3}H\)NBMPR) was counted by a \(\beta\)-scintillation counter after dissolving in Liquiscint (National Diagnostics).

**Data Analysis**—Nucleoside uptake data are expressed as means \(\pm\) S.E. for triplicate estimates of individual experiments \((n = 3–4)\). Apparent \(K_{d}\) and \(V_{max}\) values were calculated by non-linear regression analysis of the \(v\) versus \([S]\) plots and by Hill equation \((v = V_{max}[S]/[S] + [K])\), where \(v\) is the rate of \(^{3}H\)-nucleoside uptake, \([S]\) is substrate concentration, \(n\) is Hill coefficient, and \(K\) is the affinity for substrate) using Origin\(^{\text{\textregistered}}\) software. For uptake inhibition studies, data are expressed as means \(\pm\) S.E. for three experiments. Student’s \(t\) test and analysis of variance were used for paired and multiple variates, respectively. An overall \(p < 0.05\) was considered significant. Equilibrium binding of \(^{3}H\)NBMPR was transformed for Scatchard analysis to calculate \(K_{d}\) and \(B_{max}\). Concentration response curves were fit by a 4-parameter logistic function curve, and IC\(_{50}\) values were determined.

**Materials**—All standard chemicals were purchased from Sigma, Fisher, or Life Technologies, Inc. Dipyridamole and NBMPR were from Research Biochemicals (Natick, MA). Cell culture media and supplements were from Life Technologies, Inc. All \(^{3}H\)-nucleosides were purchased from ICN Pharmaceuticals (Irvine, CA), and \(^{3}H\)NBMPR was from Moravek.

**RESULTS**

**Generation of a Nucleoside Transport-deficient PK15 Cell Line**—Since nucleoside transporters are ubiquitously expressed, we generated a mutant cell line deficient in all endogenous nucleoside transporters. Previous studies showed that PK15 cells contain the NBMPR-sensitive nucleoside transport as its only endogenous nucleoside transport system (16). As shown in Fig. 1, \(^{3}H\)uridine uptake by PK15 cells was completely inhibited by \(1 \mu M\) NBMPR. This \(^{3}H\)uridine uptake was \(Na^{+}\)-independent and was inhibited by \(10 \mu M\) dipyridamole. This \(^{3}H\)uridine uptake was \(Na^{+}\)-independent and was not cross-reactive with untransfected PK15NTD cell membranes. ENT1 had \(V_{max}\) for uracil; however, ENT2 had \(V_{max}\) for guanosine (84%), cytidine (76%), and thymidine (87%).

For ENT2, the order of potency of inhibiting \(^{3}H\)uridine uptake by both cells was linear for up to 10 min and was completely inhibited by \(10 \mu M\) dipyridamole. As shown in Fig. 4, \(^{3}H\)uridine uptake by \(10 \mu M\) for ENT1 was \(Na^{+}\)-independent and was inhibited by non-radioactive nucleosides (2 mM), with a rank order of potency adenosine (97%) > inosine (92%) = uridine (91%) = guanosine (90%) > thymidine (87%) > cytidine (81%). The nucleoside analog drugs AZT (72%), AraC (46%), and 5-FdUrd (61%) were less effective at inhibiting uridine uptake, whereas the nucleosbases hypoxanthine and uracil demonstrated insignificant inhibition (15% and 10%, respectively).

For ENT2, the order of potency of inhibiting \(^{3}H\)uridine uptake was inosine (94%) > uridine (87%) = adenosine (84%) > thymidine (76%) > cytidine (70%) > AZT (53%) = 5-FdUrd > guanosine (35%). In contrast to ENT1, AraC minimally inhibited \(^{3}H\)uridine uptake (12%).

**Stable Expression of hENT1 and hENT2 in PK15NTD**

We transfected hENT1/VSVG/pcDNA3 and hENT2/ VSVG/pcDNA3 into PK15NTD cells. Positively transfected cells were selected by G418 resistance and were screened for dipyridamole-sensitive \(^{3}H\)uridine uptake (10 \(\mu M\)). The time course of \(^{3}H\)uridine uptake in ENT1 and ENT2 cells is shown in Fig. 3. Although \(^{3}H\)uridine uptake by ENT2 is 2.2-fold faster than ENT1, \(^{3}H\)uridine uptake by both cells was linear for up to 10 min and was completely inhibited by \(10 \mu M\) dipyridamole. As shown in Fig. 4, \(^{3}H\)uridine uptake (10 \(\mu M\); 2 \(\mu M\) for ENT1) was \(Na^{+}\)-independent and was inhibited by non-radioactive nucleosides (2 mM), with a rank order of potency adenosine (97%) > inosine (92%) = uridine (91%) = guanosine (90%) > thymidine (87%) > cytidine (81%). The nucleoside analog drugs AZT (72%), AraC (46%), and 5-FdUrd (61%) were less effective at inhibiting uridine uptake, whereas the nucleosbases hypoxanthine and uracil demonstrated insignificant inhibition (15% and 10%, respectively).

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To confirm further that transfected cells express hENT1 or hENT2, Western blot analysis of crude membranes from ENT1 and ENT2 cells was performed using VSVG antibody. As shown in Fig. 5, control experiments showed that VSVG antibody did not cross-react with untransfected PK15NTD cell membranes.
This antibody recognized hENT1 as a protein of 40 kDa and hENT2 as proteins of 50 and 47 kDa. The apparent molecular size of hENT1 is very similar to that reported by Vickers et al. (24) using recombinant hENT1 expressed in yeast. PNGase F and Endo H increased the mobility of hENT1 from 40 to 37 kDa and that of hENT2 from 50 and 47 kDa to 45 kDa. This result confirmed that both hENT1 and hENT2 are glycoproteins, as predicted from the primary sequences (6, 11, 12).

Pharmacological Inhibition of hENT1 and hENT2 by NBMPR and Dipyridamole—Previous expression studies of hENT1 and hENT2 have shown that hENT1 is highly sensitive to NBMPR inhibition, and hENT2 is resistant (6). Although both hENT1 and hENT2 can be inhibited by dipyridamole (6, 11, 12), it is not known whether there is any difference in dipyridamole sensitivity between these two human isoforms of nucleoside transporters. Therefore, we examined the dose response of inhibition of hENT1 and hENT2 by NBMPR and by dipyridamole. As shown in Fig. 6A, [3H]uridine uptake by hENT1 was sensitive to NBMPR in a concentration-dependent fashion with an IC50 of 0.4 ± 0.1 nM, whereas ENT2 was relatively resistant with an IC50 of 2.8 ± 0.3 µM, a difference of 7000-fold. Similarly, dipyridamole sensitivity of [3H]uridine uptake by the two transporters exhibited a 71-fold difference between ENT1 and ENT2, with an IC50 of 5.0 ± 0.9 nM and 356 ± 13 nM, respectively (Fig. 6B).

High Affinity [3H]NBMPR Binding—[3H]NBMPR binding has been used as an assay for the number of functional ENT1 nucleoside transporter units. Therefore, we determined the affinity and the density of NBMPR-binding sites in ENT1 cells. As shown in Fig. 7, high affinity [3H]NBMPR binding to ENT1 cells was saturable with an IC50 of 0.4 ± 0.1 nM, whereas ENT2 was relatively resistant with an IC50 of 2.8 ± 0.3 µM, a difference of 7000-fold. Similarly, dipyridamole sensitivity of [3H]uridine uptake by the two transporters exhibited a 71-fold difference between ENT1 and ENT2, with an IC50 of 5.0 ± 0.9 nM and 356 ± 13 nM, respectively (Fig. 6B).

Kinetic Analysis of Nucleoside Uptake—Based on competition experiments using non-radioactive nucleosides, ENT1 and ENT2 appear to be broadly selective (Fig. 4). Therefore, we...
further probed into the similarities and differences between these two transporters by comparing their affinity for transporting nucleosides. The concentration-dependent uptake of \([3H]\)uridine, \([3H]\)thymidine, \([3H]\)cytidine, \([3H]\)adenosine, \([3H]\)guanosine, and \([3H]\)inosine by ENT1 and ENT2 is shown in Figs. 8 and 9, respectively. The uptake of these nucleosides by ENT1 and ENT2 is saturable and conforms Michaelis-Menten kinetics. Kinetic parameters (apparent \(K_m\) and \(V_{max}\)) were calculated by both non-linear regression analysis of the \(v\) versus \(v/s\) plots (graph insets) and by Hill equation. There is no statistical difference in apparent \(K_m\) and \(V_{max}\) values using either method of computation, and these parameters for each nucleosides transported by ENT1 and ENT2 are summarized in Table I. The Hill coefficient for nucleosides transported by ENT1 and ENT2 ranges from 0.8 to 1.35, indicating one substrate-binding site per transporter. Although the \(V_{max}/K_m\) ratio ranges from 0.85 to 7.34 (nmol/mg/min/mM) for ENT1 and from 1.28 to 8.24 (nmol/mg/min/mM) for ENT2, there is a good correlation with the \(K_m\) and the \(V_{max}\) value of nucleosides transported by either ENT1 and ENT2. This suggests that both ENT1 and ENT2 transport nucleosides with an inverse relationship between substrate affinity and transport capacity, i.e. with high affinity and low capacity or vice versa. Since the high affinity \([3H]\)NBMPR binding assay allows us to determine the density of ENT1, this enables us to calculate the turnover number of nucleosides transported by ENT1 (Table I) with the assumptions that high affinity \([3H]\)NBMPR binding is mediated by cell surface ENT1 and that intracellular ENT1 does not

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**Fig. 7.** High affinity \([3H]\)NBMPR binding to hENT1. Concentration dependence of \([3H]\)NBMPR binding to ENT1 cells (A) and ENT1 crude membranes (B) was determined in the absence and the presence of 10 \(\mu M\) non-radioactive NBMPR; the difference in \([3H]\)NBMPR binding under these conditions was defined as specific binding. The inset shows the Scatchard analysis (B/F versus B) that was used to estimate for the \(B_{max}\) (372 ± 81 fmol/mg protein and 1125 ± 146 fmol/mg protein for ENT1 cells and ENT1 crude membrane, respectively) and \(K_d\) (0.377 ± 0.098 nM and 0.17 ± 0.03 nM for ENT1 cells and ENT1 crude membranes, respectively).

**Fig. 8.** Kinetic analysis of \(^3\)H-nucleoside uptake by ENT1 cells. Concentration dependence of uridine (0.01–5 mM) (A), adenosine (0.01–5 mM) (B), guanosine (0.01–2 mM) (C), thymidine (0.01–5 mM) (D), cytidine (0.01–5 mM) (E), and inosine (0.01–3 mM) (F) uptake by ENT1 cells was determined by measuring initial rate of \(^3\)H-nucleoside uptake (≤ 1 \(\mu M\) NBMPR) at room temperature for 1 min. The insets show the Eadie-Hofstee plots (\(v\) versus \(v/s\)) that were used for estimation of \(K_m\) and \(V_{max}\).

**Fig. 9.** Kinetic analysis of \(^3\)H-nucleoside uptake by ENT2 cells. Concentration dependence of uridine (0.01–5 mM) (A), adenosine (0.01–5 mM) (B), guanosine (0.01–2 mM) (C), thymidine (0.01–5 mM) (D), cytidine (0.01–15 mM) (E), and inosine (0.01–0.5 mM) (F) uptake by ENT2 cells was determined by measuring initial rate of \(^3\)H-nucleoside uptake (≤ 10 \(\mu M\) dipyridamole) at room temperature for 1 min. The insets show the Eadie-Hofstee plots (\(v\) versus \(v/s\)) that were used for estimation of the apparent \(K_m\) and \(V_{max}\) values. \([3H]\)Guanosine uptake by ENT2 cells was not saturated at 2 mM, and \([3H]\)guanosine uptake at higher concentrations was not attempted due to insolubility. Because of this, the apparent \(K_m\) and \(V_{max}\) values of \([3H]\)guanosine uptake by ENT2 cells was not analyzed by linear-regression analysis of the Eadie-Hofstee plot and was estimated by Hill equation as described under “Experimental Procedures.”
the apparent Km values of nucleosides (Vmax of nucleoside uptake/the number of high affinity [3H]NBMPR binding) by ENT1 was determined using the Bmax value of 372 fmol/mg protein (Fig. 7) and Vmax values estimated by Hill equation, assuming that high affinity [3H]NBMPR binding is mediated by cell surface ENT1 and that intracellular ENT1 does not contribute to ligand binding. The Vmax/Km ratio was obtained using the apparent Km and Vmax estimated by Hill equation.

### DISCUSSION

**Generation of a Nucleoside Transporter-deficient PK15NTD Cell Line**—Characterization of the uridine uptake in wild type PK15 cells in the present study confirmed that PK15 cells contain only the NBMPR-sensitive nucleoside transport system (16). These cells were then mutagenized with ethylmethanesulfonate, followed by selection with tubercidin and AraC, which are substrates of the endogenous es transport system. The success of isolation of a nucleoside transport-deficient cell in a single step further confirms that the parental PK15 cell line contains only the es transporter. The PK15NTD cells that we generate in the present study are very stable, and there is no evidence of reversion to the wild type after at least 30 passages. The PK15 cells stably transfected with hENT1 or hENT2 are also very stable. We have characterized the ENT1 and ENT2 cells for up to 25–30 passages after transfection, and there is no change in nucleoside transport activity. Therefore, PK15NTD cells might represent a viable and a valuable system for the stable expression and characterization of the cloned nucleoside transporters.

**Glycosylation of hENT1 and hENT2**—hENT1 and hENT2 exhibit 46% identity in amino acids and are made up of 456 amino acids with similar predicted size of 50 kDa (6, 11, 12). Surprisingly, the apparent molecular sizes of these two proteins, as determined by their mobility on SDS-PAGE, differ from each other by 7–10 kDa, and this difference is not due to glycosylation (Fig. 5). The results suggest that hENT1 migrates in a peculiar manner on the SDS-PAGE, whereas hENT2 runs as predicted. hENT1 and hENT2 are predicted to be glycoproteins (6, 11, 12). Previous studies have shown that both cloned and endogenous ENT1 are glycoproteins (24, 25). However, there is no experimental evidence to support that hENT2 is a glycoprotein.

In the present study, we experimentally demonstrated that both hENT1 and hENT2 are glycoproteins. hENT2 is made up of 50- and 47-kDa proteins and was deglycosylated to 45 kDa by PNGase F and Endo H. This result further suggests that hENT2 is heterogeneously glycosylated at either one or both of the putative glycosylation sites (Asn-48 and Asn-57) (11, 12). Since Endo H deglycosylates hENT1 and hENT2, this suggests that both proteins contain only core glycosylation and do not have complex high mannose carbohydrate moieties when they are stably expressed in PK15NTD cells.

**Kinetic and Substrate Selectivity**—It has been previously determined that PK15 cells have an intracellular volume of 5 μl per 10^6 cells (16). We have determined that there were 6.5 × 10^6 cells/mg of protein. ENT cells therefore have a relatively large intracellular volume of 32.5 μl/mg of protein. In kinetic analysis, the initial rate of [3H]nucleoside uptake was determined over 1 min. Under the experimental conditions used, intracellular accumulation of [3H]nucleoside is far less than the extracellular concentration of [3H]nucleosides at the saturating concentration of extracellular [3H]nucleosides (Fig. 8). Table II shows the calculated intracellular accumulation (expressed as percent of extracellular concentration) of the [3H]nucleosides

### Table I

| Nucleoside | v vs. v/s plot | Hill plot | Turnover number | Vmax/Km | ENT1 | ENT2 |
|------------|----------------|----------|-----------------|--------|------|------|
| ENT1 | Adenosine | 0.04 ± 0.004 | 351 ± 13 | 0.05 ± 0.01 | 367 ± 15 | 0.81 ± 0.14 | 16 | 7.34 |
| Guanosine | 0.14 ± 0.01 | 639 ± 19 | 0.14 ± 0.02 | 657 ± 20 | 0.82 ± 0.09 | 29 | 4.62 |
| Uridine | 0.17 ± 0.02 | 163 ± 8 | 0.20 ± 0.05 | 170 ± 16 | 1.03 ± 0.02 | 8 | 0.85 |
| Thymidine | 0.26 ± 0.02 | 853 ± 56 | 0.48 ± 0.26 | 1024 ± 166 | 0.78 ± 0.21 | 46 | 2.13 |
| Cytidine | 0.30 ± 0.03 | 698 ± 46 | 0.24 ± 0.06 | 646 ± 53 | 1.30 ± 0.37 | 29 | 2.69 |
| ENT2 | Adenosine | 0.14 ± 0.02 | 1147 ± 88 | 0.14 ± 0.04 | 1154 ± 70 | 1.00 ± 0.01 | 8 | 2.24 |
| Guanosine | Not calculated | 2.70 ± 1.64 | 3450 ± 914 | 0.80 ± 0.08 | 1.28 |
| Uridine | 0.05 ± 0.006 | 72 ± 5 | 0.05 ± 0.01 | 73 ± 4 | 1.00 ± 0.02 | 1.46 |
| Thymidine | 0.25 ± 0.04 | 2055 ± 190 | 0.27 ± 0.05 | 2084 ± 138 | 1.30 ± 0.29 | 7.72 |
| Cytidine | 0.71 ± 0.05 | 2486 ± 113 | 0.62 ± 0.09 | 2387 ± 136 | 1.03 ± 0.11 | 3.85 |

### Table II

| Intracellular accumulation of [3H] nucleosides at the Km | ENT1 | ENT2 |
|--------------------------------------------------------|------|------|
| Adenosine                                              | 10  | 12  |
| Guanosine                                              | 7   | 2   |
| Uridine                                                | 1   | 2   |
| Thymidine                                              | 4   | 6   |
| Cytidine                                               | 3   | 3   |
measured for 1 min with the concentration of $^3$H-nucleosides at the apparent $K_m$ value where the nucleoside uptake rate was at half of the $V_{max}$. The intracellular accumulation of $^3$H-nucleosides was <10 and <12% of the extracellular concentrations for ENT1 and ENT2 cells, respectively.

Kinetic analysis in the present study reveals that hENT2 generally has a lower affinity for nucleosides compared with hENT1. It exhibits a 2.6-fold lower in affinity for thymidine, 2.8-fold for adenosine, 7.7-fold for cytidine, and 19.3-fold for guanosine. This finding is consistent with the previous observations by Griffiths et al. (11) and by Crawford et al. (12) that guanosine and cytidine were relatively poor inhibitors of $^{[3}\text{H}]\text{uridine}$ uptake by ENT2. Previous characterization of the endogenous ei transport system in Ehrlich ascites tumor cells also suggested a low affinity for guanosine (1.78 mM) (26), which is very similar to that obtained for hENT2 in the present study (2.7 mM). To our knowledge, there is no information on the affinity of the ei transport system for cytidine in any cell types or tissues. We did not observe any difference in the affinity for uridine between hENT1 and hENT2. This is in contrast to a previous study by Yao et al. (10), which demonstrated a 2.5-fold difference in the affinity for uridine between rat ENT1 and rat ENT2. What contributes to the difference in kinetic properties for ENT1 and ENT2 in the study by Yao and co-workers (10) and in the present studies is not clear, but differences might be due to species variation (human versus rat) and/or the difference in expression system (mammalian PK15NTD cells versus Xenopus oocytes). Although hENT2 is a low affinity nucleoside transporter, it has a 4-fold higher in affinity for inosine (Table I). This suggests that hENT2 might be important physiologically in mediating cellular influx and efflux of inosine. Reports of the tissue distribution of ENT2 message has demonstrated a prominent expression of ENT2 in skeletal muscle (12). It has been suggested by Crawford et al. (12) that ENT2 might be important physiologically in transporting adenosine metabolites, such as inosine and hypoxanthine, across muscle cell plasma membranes during strenuous exercise and during the recovery process. The high affinity of ENT2 for inosine is consistent with this speculation. Furthermore, $^{[3}\text{H}]\text{uridine}$ uptake by hENT2, but not by hENT1, is inhibited by hypoxanthine (Fig. 3), suggesting that hypoxanthine might well be a substrate of hENT2. Previous studies of the endogenous ei transport system in ECV304 human vascular endothelial cells also demonstrated that the ei transporter could mediate the transport of hypoxanthine with an affinity of physiological significance (27). These studies and our present observations support the importance of ENT2 in salvaging of nucleosides and nucleobases. However, because PK15NTD cells contain an endogenous high affinity hypoxanthine (nucleobase) transporter, this did not allow us to test directly whether $^{[3}\text{H}]\text{hypoxanthine}$ is a substrate of hENT2. Nevertheless, the endogenous nucleobase transporter in PK15NTD cells is insensitive to the nucleoside transport inhibitors, dipyridamole and NBMPR, and to the inhibition by nucleosides such as uridine. Thus, the functionally distinct endogenous nucleobase transporter does not interfere with the characterization of the stably expressed hENT1 and hENT2 in PK15NTD cells.

The synthetic nucleoside analog drugs AZT and 5-FlUrd are able to inhibit $^{[3}\text{H}]\text{uridine}$ uptake by hENT1 and hENT2, indicating that these transporters might mediate cellular uptake of these drugs. The ability of AraC to inhibit hENT1 but not hENT2 further discriminates the substrate selectivity between hENT1 and hENT2 (Fig. 4).

$^{[3}\text{H}]\text{NBMPR}$ binds to hENT1 cells with a high affinity $K_d$ of 0.377 ± 0.098 nM and a $B_{max}$ of 372 ± 81 fmol/mg protein (Fig. 7). This translates into 3.4 × 10^4 high affinity NBMPR-binding sites per hENT1 cell. The parental wild type PK15 cells have 5 × 10^3 to 2 × 10^4 sites per cell (16). Since each high affinity NBMPR-binding site represents a nucleoside transporter, this allows the turnover number of nucleosides to be determined (Table I). The turnover number of uridine by hENT1 (46 molecules/s) is similar to that determined for the endogenous es transporter in various mammalian cells (1, 7). On the other hand, we failed to detect any high affinity NBMPR-binding sites in hENT2 cells (data not shown), and thus the turnover number for nucleosides by hENT2 cannot be calculated.

**Conclusion**—We have generated a PK15 cell line devoid of endogenous nucleoside transport activity. This allows us to characterize a single nucleoside transporter in a single cell line without interference by other endogenous nucleoside transport systems. We have demonstrated that both hENT1 and hENT2, when stably expressed in PK15NTD cells, contain only Endo H-sensitive core glycosylation. Although hENT1 and hENT2 are broadly selective for nucleosides, we demonstrated, for the first time, that hENT2 has a relatively lower affinity for all natural nucleosides except inosine, being most markedly lower for cytidine and guanosine. These transporters can also be differentiated by their sensitivity to the vasodilator drugs, NBMPR and dipyridamole. Since the expression of hENT1 and hENT2 cDNAs in PK15NTD cells is sufficient to account for the functional characteristics of the physiologically described es and ei nucleoside transport systems, respectively, we conclude that the expression of nucleoside transporter function by hENT1 and hENT2 does not require co-expression of any exogenous associated proteins or additional subunits.

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