Molecular Cloning and Functional Characterization of Nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) Equilibrative Nucleoside Transporter Proteins (rENT1 and rENT2) from Rat Tissues*

(Received for publication, August 5, 1997, and in revised form, September 8, 1997)

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Equilibrative nucleoside transport processes in mammalian cells are either nitrobenzylthioinosine (NBMPR)-sensitive (es) or NBMPR-insensitive (ei). Previously, we isolated a cDNA from human placenta encoding the 456-residue glycoprotein hENT1. When expressed in Xenopus oocytes, hENT1 mediated es-type transport activity and was inhibited by coronary vasoactive drugs (dipyridamole and dilazep) that may compete with nucleosides and NBMPR for binding to the substrate binding site. We now report the molecular cloning and functional expression of es and ei homologs of hENT1 from rat tissues; rENT1 (457 residues) was 78% identical to hENT1 in amino acid sequence, and rENT2 (456 residues) was 49–50% identical to rENT1/hENT1 and corresponded to a full-length form of the delayed-early proliferative response gene product HNP36, a protein of unknown function previously cloned in truncated form. rENT1 was inhibited by NBMPR (IC_{50} = 4.6 nM at 10 μM uridine), whereas rENT2 was NBMPR-insensitive (IC_{50} > 1 μM). Both proteins mediated saturable uridine influx (K_m = 0.15 and 0.30 mM, respectively), were broadly selective for purine and pyrimidine nucleosides, including adenosine, and were relatively insensitive to inhibition by dipyridamole and dilazep (IC_{50} > 1 μM). These observations demonstrate that es and ei nucleoside transport activities are mediated by separate, but homologous, proteins and establish a function for the HNP36 gene product.

In mammalian cells, plasma membrane transport of nucleosides occurs by both Na⁺-dependent and Na⁺-independent mechanisms (1, 2). These processes are essential for nucleotide synthesis by salvage pathways in hemopoietic and other cells that lack de novo pathways and are the route of cellular uptake of many cytotoxic nucleosides used in the treatment of human neoplastic and viral diseases, including leukemias and AIDS (3, 4). Nucleoside transport processes also play an important role in adenosine-mediated regulation of coronary vasodilation, renal vasoconstriction, neurotransmission, platelet aggregation, and lipolysis (5, 6).

Na⁺-dependent mechanisms of nucleoside transport are limited to specialized cells such as intestinal and renal epithelia, choroid plexus, liver, splenocytes, macrophages, and leukemic cells (1, 2). cDNAs for the two major concentrative nucleoside transporter subtypes found in mammalian cells have been isolated from human and rat tissues (7–12). CNT1 and CNT2 are 648–662-residue proteins (M, 71,000) with 14 potential transmembrane segments (TM) and belong to a previously unrecognized gene family that includes the bacterial proton/nucleoside symporter NupC (13). Human and rat CNT1 (hCNT1 and rCNT1) transport pyrimidine nucleosides (7–11), whereas rat CNT2 (rCNT2) transports purine nucleosides and uridine (10, 12). CNT1 also transports the antiviral pyrimidine nucleoside analogs 3’-azido-3’-deoxythymidine and 2’-3’-dideoxyctydine (7, 9, 11).

Na⁺-independent nucleoside transport processes are subdivided into two types on the basis of their sensitivities to inhibition by the nucleoside analog NBMPR (1, 2). NBMPR-sensitive nucleoside transporters bind NBMPR with high affinities (K_b values 0.1–10 nM) and have the functional designation equilibrative sensitive (es), whereas NBMPR-insensitive transporters are unaffected by micromolar concentrations of NBMPR and are designated equilibrative insensitive (ei). Transporters of the es type are widely distributed in different cell types and tissues, bind NBMPR reversibly, and can be covalently radiolabeled with [3H]NBMPR by exposure of the transporter-ligand complex to UV light (14). The es and ei nucleoside transporters are under independent genetic control and are produced either singly or together in different cell types and tissues (15–21). Both have a broad substrate selectivity for purine and pyrimidine nucleosides. Transporters of the es type are pharmacological targets for coronary vasodilator drugs such as dipyridamole and dilazep (5, 6).

* This work was supported in part by the Medical Research Council of Canada, the Alberta Cancer Board, and the Welcome Trust (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF015304 and AF015305.

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†‡ The abbreviations used are: CNT, concentrative nucleoside transporter; TM, transmembrane segment; ENT, equilibrative nucleoside transporter; EST, expressed sequence tag; dbEST, expressed sequence tag data base; MBM, modified Barth’s medium; NBMPR, nitrobenzylthioinosine (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); kb, kilobase(s).

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The prototypic $es$ nucleoside transporter from human erythrocytes, a glycoprotein of apparent $M$, 55,000 (22), is related immunologically to nucleoside transporters in human placenta, pig and rabbit erythrocytes, and rat liver (23, 24). Information derived from amino-terminal sequence analysis of the erythrocyte transporter was used to clone a cDNA from human placenta encoding a 456-residue $es$-type nucleoside transporter protein, hENT1 (25). When expressed in oocytes of *Xenopus laevis*, recombinant hENT1 mediated NBMPR-sensitive, Na$^+$- independent transport of pyrimidine (uridine) and purine (adenosine) nucleosides and was inhibited by anticancer chemotherapy drugs, dipipyramids and dilazep (25). hENT1 is unrelated to other known transporter proteins, including members of the CNT/nrupC gene family (25). However, there is high sequence similarity between the carboxyl-terminal two-thirds of hENT1 and truncated sequences of human and mouse HNP36, two delayed-early proliferative response gene products of unknown function (26).

We report here identification of two ENT proteins from rat tissues by molecular cloning and functional expression of their cDNAs. We used RT-PCR amplification and primers based on hENT1 and HNP36 to isolate cDNAs from jejunum, liver, and lung (rENT1) and from jejunum (rENT2). rENT1 and rENT2 exhibited NBMPR-sensitive (es) and NBMPR-insensitive (ei) nucleoside transport activity, respectively, when expressed in *Xenopus* oocytes. Neither rat transporter was inhibited by dipipyramidole or dilazep. rENT2 had a 2-fold lower affinity for uridine than rENT1 and was less sensitive to inhibition by cytidine. The cloning of cDNAs encoding rENT1 and rENT2 opens the way to identification of structural features within, or adjacent to, the ENT substrate site responsible for the binding of NBMPR and vasoactive drugs.

**MATERIALS AND METHODS**

**cDNA Cloning and Sequencing**—The cDNA encoding rENT1 was obtained by RT-PCR amplification of total RNA extracted from mucosal scrapings of Sprague-Dawley rat jejunum (27) using primers (A1 and A2) flanking the hENT1 open reading frame (25) (GenBank™/EBI accession no. U81757). First-strand cDNA synthesis was performed on 5 μg of RNA as template using the Superscript Preamplification System (Life Technologies, Inc.) and oligo(dT) as primer. The PCR reaction (50 μl) contained 10 ng of template first strand cDNA, 5 units of Taq Deep Vent DNA polymerase (100:1), and 10 pmol each of primers A1 and A2. Primer A1 corresponded to hENT1 nucleotide positions 179–203 (sense, 5′-TGGTCACTCACACACGGGCTC-3′); primer A2 corresponded to hENT1 nucleotide positions 2108–2127 (antisense, 5′-TGGAAACGCTTGGTTCCTGTCTC-3′) and was identical in sequence to nucleotides 39–59 of a 353-bp expressed sequence tag (EST) cloned from a rat PC-12 cell cDNA library (GenBank™/EBI accession no. H31422). Amplification for one cycle at 94 °C for 5 min, 55 °C for 1 min 20 s, and 72 °C for 2 min and 34 cycles at 94 °C for 1 min, 55 °C for 1 min 20 s, and 72 °C for 2 min generated a ~1.8-kb product that was ligated into the PCR vector pGEM-T (Promega, Madison, WI) to generate the plasmid pAN2. The resulting 1766-bp rENT1 cDNA, which was sequenced in both directions by T7 DyeDeoxy terminator cycle sequencing using an automated Model 373A DNA Sequencer (Applied Biosystems, Norwalk, CT), had an open reading frame encoding a 457-residue protein flanked by 4 bp of untranslated 5′-nucleotide sequence and 397 bp of untranslated 3′-sequence. The same RT-PCR procedure was used to amplify rENT1 cDNAs from rat liver and rat lung. The 5′- and 3′-ends of these cDNAs were sequenced (a total of ~400 bp for each cDNA) and shown to be ~99% identical to pAN2.

The cDNA encoding rENT2 was obtained by first amplifying rENT2 partial cDNA from oligo(dT)-primed rat jejunal cDNA using primers (A3 and A4) for conserved regions of mouse-human HNP36 corresponding to human HNP36 nucleotide positions 265–286 (sense, 5′-AAACAATCGTGCCCTAATCGTCT-3′) and 1217–1238 (antisense, 5′-TGTTAGAGACACACATTAGGCC-3′) (26) (GenBank™/EBI accession no. X86681). PCR amplification as described above for rENT1, but with a shorter extension time of 1 min, generated a ~1.0-kb product that was 84% and 99% identical in nucleotide sequence to human and mouse HNP36, respectively. This fragment, labeled with $^{32}$P (T7QuickPrime kit, Pharmacia Biotech, Uppsala, Sweden), was used to screen a directional rat jejunal cDNA library prepared in this laboratory using the Stratagene vector Uni-ZAP™ XR. A clone of ~1.6 kb was obtained that contained ~600 bp of rat HNP36 coding sequence and ~700 bp of unrelated 5′-3′-sequence. Two primers-specific primers A5 and A6 were designed, 5′-TTAGGCGGATGTCACCCACC-3′ and A6 (antisense, 5′-TGCAACCAACTTCATGGAGACAC-3′) and were then constructed to amplify the missing 5′ portion of rat HNP36 by a nested approach. Initial amplification of the Uni-Zap™ XR rat jejunal cDNA library by the protocol described above used A5 as the 3′ primer and a T3 oligonucleotide corresponding to a region of the Uni-Zap™ XR insertion vector upstream of the EcoRI cloning site. The 5′ primer A6 and the second round of amplification using the nested 3′ primer A6 yielded a ~500-bp product that contained 157 bp of 5′-untranslated sequence, as well as the initial 5′ portion of the rat HNP36 coding sequence. A cDNA encoding full-length rat HNP36 was then amplified from oligo(dT)-primed rat jejunal cDNA using a pair of primers (A7 and A8) corresponding to nucleotide positions 157–136 bp upstream of start codon (A7 sense, 5′-TTATCCACTCCCTGCACTCCTGCTC-3′) and positions 138–150 bp downstream of stop codon (A8 antisense, 5′-TACCATATGCAATGTTGGCTAC-3′). The resulting ~1.7-kb product, which contained the complete coding sequence of rat HNP36, designated here as rENT2, was ligated into the pGEM-T vector to generate the plasmid pAN3 and sequenced in both directions. The 1766-bp rENT2 cDNA had an open reading frame encoding a 456-residue protein flanked by 157 bp of untranslated 5′-nucleotide sequence and 150 bp of 3′-untranslated sequence.

Expression in Xenopus Oocytes—Plasmids pAN2 and pAN3 were linearized with SpeI and tranmscribed with SP6 polymerase in the presence of $^{32}$P-gapped cap (Ambion, Austin, TX) using the MEGAscript™ (Ambion) transcription system. Plasmid cDNA encoding hENT1 (25) in the vector pBluescript II KS(+) was linearized with NcoI and transcribed with T3 polymerase. Healthy stage VI oocytes of *Xenopus laevis*, treated with collagenase to remove follicular layers (27), were maintained at 18 °C in modified Barth’s medium (MBM) for 24 h until injection with 10 nl of water or 10 nl of water containing rENT1, rENT2, or hENT1 RNA transcript (1 ng/μl). Injected oocytes were incubated for 3 days at 18 °C with a daily change of MBM before the assay of transport activity.

Transport Assays—Uptake of uridine and adenosine by oocytes was traced with the respective $^{3}H$-labeled nucleoside (Amersham) (1 μCi/ml). Assays were performed at 20 °C on groups of 10–12 oocytes in transport buffer (0.2 ml) containing either 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, pH 7.5 (25). In adenosine uptake and inhibition experiments, the transport buffer also contained 1 μM deoxycoformycin to inhibit adenosine deaminase activity. Unless otherwise indicated, the incubation period was 1 min and the permeant concentration was 10 μM. Competing unlabeled nucleosides and uracil were used at a concentration of 2 mM. For experiments involving NBMPR, dipipyridamole, and dilazep, oocytes were pretreated with inhibitor (1 μM unless otherwise indicated) for 1 h before addition of permeant. At the end of the incubation, extracellular radioactivity was removed by six rapid washes in ice-cold transport buffer. Individual oocytes were dissolved in 5% (w/v) SDS for quantitation of radioactivity by liquid scintillation counting. We have determined previously that oocytes lack endogenous nucleoside transport processes and have a low basal permeability to uridine and adenosine (7, 10, 27). Uridine is only slowly metabolized in oocytes (27), whereas adenosine is efficiently trapped as the 5′-triphosphate ester (10).

Results for uptake experiments are given as means ± standard errors (S.E.) for 10–12 individual oocytes. Kinetic constants (apparent $K_{m}$ and $V_{max}$) and $I_{50}$ values were determined using programs of the ENZFITTER software package (Elsevier-Biosoft, Cambridge, United Kingdom). Each experiment was performed at least twice on different batches of oocytes.

**RESULTS AND DISCUSSION**

Electrophoretic analyses of NBMPR-photolabeled proteins in membrane preparations from various cell types have produced evidence of considerable species and/or tissue-related variations in the size of $es$ transporters (1, 2), raising questions about the extent of structural homology among ENT proteins. In some instances, the apparent heterogeneity has been shown to be due to differences in glycosylation state (18, 28–31). Peptide mapping experiments have established structural conservation among several mammalian $es$ transporter proteins
It has been shown that the locations of NBMPR covalent labeling, carbohydrate attachment, and trypsin cleavage are similar in erythrocyes, liver, and/or lung from human, rat, guinea pig, and pig. The apparent structural similarity between rat and human es transporters is of particular interest because nucleoside transport in rodent species has a reduced sensitivity to inhibition by vasoactive drugs (1, 2). Although nothing was known about the molecular properties of ei-type nucleoside transporter proteins, the functional similarities between es- and ei-mediated processes suggested an underlying structural homology.

**rENT1**—Sequence homology between rodent and human es transporter proteins was suggested by the identification, in the GenBank™/EBI EST data base (dbEST), of ESTs encoding partial sequences of rat and mouse homologs of hENT1 (25). The insert of plasmid pAN2 encoded a rat jejunal protein, designated here as rENT1, that contained one more amino acid than hENT1 (457 residues, $M_r$ 49,984) and was 78% identical (88% similar) to hENT1 in amino acid sequence (Fig. 1). Interestingly, the additional residue was a cysteine, located just...
before TM 8. This residue was not present in an otherwise similar dbEST sequence corresponding to mouse ENT1 (GenBank™/EBI accession no. AA270375). Message for rENT1 was also found in rat liver and lung, tissues used previously in ligand binding (32–34), photoaffinity labeling (30, 32, 33), and immunologic (23) and peptide mapping studies (30) of native rat \( es \)-type nucleoside transporter proteins. Hydropathy analysis (Fig. 2A) predicted a topology for rENT1 similar to that of hENT1 (25). In this model, 11 TMs are connected by short (\( \leq 16 \) residues) hydrophilic regions, with the exception of large loops.

![Hydropathy analyses and topographical model of rENT1 and rENT2](image-url)

**Fig. 2.** Hydropathy analyses and topographical model of rENT1 and rENT2. Hydropathy profiles (A) for the predicted amino acid sequences of rENT1 and rENT2 were determined by the method of Eisenberg et al. (40). Potential membrane-spanning \( \alpha \)-helices in the topographical model (B) are numbered, and putative N-linked glycosylation sites in rENT1 and rENT2 are indicated by solid and open stars, respectively. Residues identical in the two proteins are shown as darkened circles. Residues corresponding to insertions in the sequences of rENT1 or rENT2 are indicated by circles containing “+” and “−” signs, respectively.
linking TMs 1 and 2 (extracellular) and TMs 6 and 7 (intracellular), which contain 41 and 66 residues, respectively (Fig. 2B).

The intracellular amino terminus and TMs 1–8, 10, and 11 were highly conserved between the two proteins. A single putative N-linked glycosylation site at Asn-48 of the extracellular domain between TMs 1 and 2 of hENT1 was present in rENT1, which also contained two additional N-linked glycosylation sites in the same loop at Asn-44 and Asn-54 (Figs. 1 and 2B). Differences in the glycosylation states of rENT1 and hENT1 are consistent with photoaffinity labeling studies of native rat and human transporters from various tissues (18, 30, 32, 33), which have found that rat es migrates on SDS-polyacrylamide gels with an apparent Mr value of 62,000, compared with 55,000 for human es. This difference in apparent molecular weight was abolished by digestion with endoglycosidase-F (18, 30). Since membrane proteins frequently exhibit increased mobilities relative to soluble protein standards on SDS-polyacrylamide gels (35), the value of 47,000 for deglycosylated rat and human es is consistent with the calculated sizes of rENT1 and hENT1 (Mr 49,984 and 50,249, respectively).

Peptide mapping experiments with native human erythrocyte and rat lung/liver es transporters have identified a common trypsin cleavage site situated in an intracellular domain in the approximate center of both proteins (30). The site of N-linked glycosylation was located close to one end of the human protein, and the site of NBMPR photolabeling to within 16 kDa of that site (30). The ENT topographical model presented in Fig. 2B predicts that (i) the site of trypsin cleavage is within the central cytoplasmic loop between TMs 6 and 7, and (ii) the site of NBMPR attachment is within the amino-terminal half of the protein. The deduced amino acid sequences of rENT1 and hENT1 predict a number of potential trypsin cleavage sites within the central cytoplasmic loop.

rENT2—The carboxyl-terminal two-thirds of the hENT1 amino acid sequence showed similarity (44% identity) to the 36-kDa mouse fibroblast and human heart HNP36 proteins (26). The latter are delayed-early proliferative response gene products, predicted to be integral membrane proteins with eight membrane-spanning segments that correspond to TMs 4–11 of hENT1. However, as we have noted previously (25), the 198 nucleotides upstream of the assigned start codon in the

**FIG. 3.** Nucleoside uptake by recombinant rENT1 and rENT2 expressed in Xenopus oocytes. Oocytes injected with either 10 nl of water alone or 10 nl of water containing 10 ng of rENT1 (A) or rENT2 (B) RNA transcript were incubated for 3 days at 18 °C in MBM. Fluxes of uridine and adenosine (10 μM, 20 °C) were determined in transport buffer containing 100 mM NaCl. Each value is the mean ± S.E. of 10–12 oocytes. In A, ● indicates oocytes injected with RNA, and ○ indicates oocytes injected with water.

**FIG. 4.** Nucleoside specificity of rENT1 and rENT2. Uridine influx (10 μM, 20 °C, 1-min flux) in transport buffer containing 100 mM NaCl (open columns) or 100 mM choline chloride (hatched columns) was measured in rENT1 (A) and rENT2 (B) RNA transcript-injected oocytes in the absence (Control) or presence of 2 mM nonradioactive physiological nucleosides (T, thymidine; C, cytidine; G, guanosine; I, inosine; A, adenosine; U, uridine) or uracil. Nonradioactive nucleosides and uracil were added to oocytes at the same time as [14C]uridine. H2O, water-injected oocytes. Each value is the mean ± S.E. of 10–12 oocytes.
mouse HNP36 cDNA are in frame with the rest of the hENT1 coding sequence and could therefore encode an amino acid sequence with 65% identity to the region of hENT1 containing TMs 2 and 3. Similarly, the nucleotide sequence upstream of the assigned start codon of the human HNP36 cDNA includes an open reading frame that is 49% identical in predicted amino acid sequence to the amino-terminal region (TMs 1 and 2) of hENT1. The cellular location of the HNP36 protein, determined by immunocytochemical staining of murine BALB/c fibroblasts, F9 embryonal carcinoma cells, and rat intestinal epithelial cells, was reported as nucleolar (26). Based upon the strong sequence similarity between HNP36 and rENT1/hENT1, we hypothesized that HNP36 might be a plasma membrane nucleoside transporter, perhaps with NBMPR-insensitive (ei-type) transport activity.

pAN3 encoded a rat jejunal protein with the same number of residues as hENT1 (456 amino acids, M, 50,232). The predicted start codon lies in a reasonably good Kozak consensus sequence and is preceded by an in-frame stop codon. There was 88% and 95% identity (93% and 98% similarity) to the incomplete amino acid sequences of human and mouse HNP36 (Fig. 1), establishing that the protein, designated here as rENT2, corresponds to full-length rat HNP36. The sequence of rENT2 was 49% identical (68% similar) to rENT1 and hENT1 (Fig. 1). A search of the GenBank™/EBI dbEST database revealed that the human homolog of rENT2, human HNP36, is expressed in adult human ovary and ovarian tumors, and in fetal heart and brain. Hydropathy analysis of rENT2 (Fig. 2A) predicted the same membrane topology as rENT1/hENT1. Homology between rENT2 and rENT1/hENT1 was greatest...
within the putative transmembrane helices, and there was little homology in either of the two large loops. The predicted extracellular loop of rENT2 between TMs 1 and 2 contained 14 fewer amino acids than rENT1/hENT1, while the corresponding intracellular loop linking TMs 6 and 7 had 14 more residues (Fig. 2B). The putative N-linked glycosylation site at Asn-48 of rENT1 and hENT1 was conserved in rENT2 at Asn-47, allowing for the presence of a 1-residue insertion before this point in the ENT1 sequences, and was similarly present in the human HNP36 sequence (Figs. 1 and 2B). In addition, a second putative glycosylation site was present at Asn-56 in rENT2 and at a corresponding location in human HNP36 (Figs. 1 and 2B).

Functional Expression and NBMPR Sensitivity of Recombinant rENT1 and rENT2—To investigate the functional characteristics of rENT1 and rENT2, the recombinant proteins were expressed in Xenopus oocytes. As shown in Fig. 3A, uptake of the pyrimidine nucleoside [14C]uridine (10 μM, 20 °C) was substantially greater in oocytes injected with rENT1 RNA transcript than in control oocytes injected with water alone. Uptake was essentially linear for the first minute of incubation, and in subsequent experiments this incubation period was used to approximate initial rates of transport. At this time interval, uptake in rENT1 RNA-injected oocytes was 1.13 ± 0.06 pmol/oocyte, compared with 0.004 ± 0.002 pmol/oocyte in water-injected oocytes (Fig. 3A). Similar transport data were obtained in oocytes injected with rENT2 RNA transcript. In the experiment shown in Fig. 3B, uptake of uridine (10 μM, 20 °C, 1 min of flux) by rENT2 RNA-injected oocytes was 0.47 ± 0.009 pmol/oocyte, compared with 0.01 ± 0.003 pmol/oocyte in water-injected cells. Neither protein was dependent upon the presence of sodium ions in the external medium (Fig. 4, A and B). Therefore, rENT1 and rENT2 were both confirmed to be functional equilibrative nucleoside transporters.

To investigate the functional characteristics of rENT1 and rENT2—hENT1 and hENT2, recombinant rENT1 and hENT1 exhibited comparable NBMPR inhibition profiles, with IC50 values of 4.6 and 3.6 μM, respectively. In contrast, rENT2 was unaffected by 1 μM NBMPR (Fig. 5D). rENT1 is therefore NBMPR-sensitive (es-type), while rENT2 is NBMPR-insensitive (ei-type).

Kinetic Properties and Substrate Selectivity of Recombinant rENT1 and rENT2—Mediated influx of uridine by the two transporters, as defined in the difference in uptake between RNA-injected and water-injected oocytes, was saturable (Fig. 6, A and B) and conform to simple Michaelis-Menten kinetics. The calculated apparent Ks value of 0.15 ± 0.02 μM for rENT1 was within the range expected for uridine transport by mammalian es-type nucleoside transporters (1, 2), including hENT1 (25), and was significantly lower than the value of 0.30 ± 0.04 μM determined for rENT2 under the same experimental conditions.

A number of studies have reported differences in affinity between native es-type and ei-type nucleoside transporters (18, 31, 36, 37). Vmax estimates for rENT1 and rENT2 in Fig. 6 (A and B) were 19 ± 1 and 14 ± 1 pmol/oocyte.min⁻¹, respectively.

Transport of uridine by rENT1 was unaffected by the presence of a high concentration of the nucleobase uracil (2 mM), but was strongly inhibited by both pyrimidine (thymidine, cytidine, and uridine) and purine nucleosides (adenosine, guanosine, and inosine) (Fig. 4A). This result is consistent with the broad specificity typical of es-type nucleoside transporters (1, 2) and has also been found for recombinant hENT1 (25). Greatest inhibition was observed with adenosine, suggesting that, like native es transporters (1, 2), heterologously expressed rENT1 has a higher affinity for this purine nucleoside than for other substrates. Direct measurements of [14C]adenosine (10 μM) transport confirmed that adenosine was an rENT1 permeant (Fig. 3A, inset). The rate of mediated influx of adenosine was greater than for uridine.

Similar transport characteristics were obtained for rENT2 (Figs. 3B and 4B), except that cytidine was a relatively poor inhibitor of rENT2-mediated uridine influx. The kinetic characteristics of cytidine transport by native ei-type nucleoside transporters have not been investigated (1, 2).

Interaction of rENT1 and rENT2 with Dipyridamole and Dilazep—Nucleoside transporters of the es type are pharmacologic targets of the coronary vasodilators dipyridamole and dilazep, which compete with permeant (and NBMPR) for the substrate binding site (1, 2, 38, 39). These agents, therefore, block adenosine influx across the plasma membrane, thereby potentiating the interaction of extracellular adenosine with
purinoreceptors (5, 6). In most species, including humans, dipyridamole and dilazep inhibit es transport with potencies in the nanomolar concentration range (1, 2), whereas they inhibit ei transport with micromolar apparent Ki values. In contrast, es and ei transporters of rat origin are generally both resistant to dipyridamole and dilazep inhibition.

Fig. 5 (B and C) compares dose-response curves for dipyridamole and dilazep inhibition of uridine transport by recombinant rENT1 and hENT1. hENT1 was, as expected, highly sensitive to inhibition by dipyridamole and dilazep, with IC50 values of 140 ± 2 and 60 ± 2 nM, respectively. In contrast, concentrations of dipyridamole or dilazep up to 1 μM had no effect on rENT1 transport activity, demonstrating insensitivity of the rat transporter to both compounds. rENT2 was also relatively unaffected by 1 μM dipyridamole or dilazep (Fig. 5D). Since recombinant rENT1, rENT2, and hENT1 proteins were expressed in the same membrane environment, it can be concluded that the observed differences in sensitivity of the three transporters to inhibition by vasoactive drugs and NBMPR reflect differences in their amino acid sequences. Differences in vasoactive drug sensitivities are unlikely to be a consequence of glycosylation state because the interaction of dipyridamole with the native rat and human es transporters is unaffected by carbohydrate removal (30).

Conclusions—We have cloned and expressed cDNAs encoding two nucleoside transporter proteins from rat tissues. rENT1 and rENT2 were NBMPR-sensitive and NBMPR-insensitive, respectively, and thus correspond functionally to the two major equilibrative nucleoside transporter subtypes (es and ei) found in mammalian cells. Neither rat protein was inhibited by the coronary vasoactive drugs dipyridamole or dilazep. Since rENT1 and hENT1 are close structural homologs, it is likely that small changes in the molecular structure within, or adjacent to, the nucleoside substrate binding site lead to differences in vasoactive drug binding without affecting the affinity of the site for either NBMPR or nucleoside substrates. That site may include structural domains located within the amino-terminal half of the transporter. The cloning and functional expression of rENT1 and rENT2, identified previously as the delayed-early proliferative response gene product HNP36, establishes that es and ei transport activities are mediated by separate, but homologous, proteins. Despite close topographical and kinetic similarities, rENT1 and rENT2 are only moderately similar in amino acid sequence. Residues conserved between the two proteins, which may have structural and/or functional significance, were located largely within putative TMs.

The identification of ENT2 (HNP36) as a delayed-early response gene product that is activated by growth factors and implicated as participating in the proliferative response (26) is consistent with the biochemical requirement for nucleic acid precursors during cell division. rENT1 and rENT2 were both expressed in jejunum, where, like their concentrative counterparts rCNT1 and rCNT2, they may have a physiological role in the absorption of dietary nucleosides.