We have recently isolated cDNAs from human placenta and rat jejunum encoding the prototypic human and rat equilibrative nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporters hENT1 and rENT1. The two proteins (456 and 457 residues, Mr 50,000) are 78% identical in amino acid sequence and contain 11 potential transmembrane segments (TMs) with a large putative extracellular loop between TMs 1 and 2 and a large cytoplasmic loop between TMs 6 and 7. When expressed in Xenopus oocytes, recombinant hENT1 and rENT1 transport both purine and pyrimidine nucleosides, including adenosine, and are inhibited by nanomolar concentrations of NBMPR. hENT1 is also potently inhibited by coronary vasodilator drugs (dipyridamole, dilazep, and drafazine), whereas rENT1 is insensitive to inhibition by these compounds (dipyridamole IC50 values 190 nM (hENT1) and ≥10 μM (rENT1) at 10 μM uridine). In the present study, we have generated reciprocal chimeras between hENT1 and rENT1, using splice sites at residues 99 (end of TM 2) and 231 (end of TM 6), to identify structural domains of hENT1 responsible for transport inhibition by vasoactive compounds. Transplanting the amino-terminal half of hENT1 into rENT1 converted rENT1 into a dipyridamole/dilazep-sensitive transporter, whereas the amino-terminal half of rENT1 rendered hENT1 dipyridamole/dilazep-insensitive. Domain swaps within the amino-terminal halves of hENT1 and rENT1 identified residues 100–231 (incorporating TMs 3–6) of hENT1 as the major site of vasodilator interaction. Since these drugs function as competitive inhibitors of nucleoside transport and NBMPR binding, TMs 3–6 are likely to form part of the substrate-binding site.

In mammalian cells, plasma membrane transport of nucleosides occurs by both Na+-dependent, concentrative, and Na+-independent, equilibrative mechanisms (1, 2). These processes are essential for nucleotide synthesis by salvage pathways in enterocytes and hemopoietic and other cells that lack de novo biosynthetic pathways. Protein-mediated transport is also the route of cellular uptake for many synthetic nucleoside analogs used in the treatment of human neoplastic and viral diseases, including leukemias and AIDS (3, 4). Through its effect on adenosine concentration at the cell surface, nucleoside transport plays a key role in many physiological processes, including 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, 7–13). Na+-independent nucleoside transport processes, in contrast, are widely distributed in different cell types and tissues and are classified into two subtypes on the basis of their sensitivities to inhibition by NBMPR1 (1, 2). NBMPR-sensitive nucleoside transporters bind NBMPR with high affinity (Kd 1–10 nM) and have the functional designation equilibrative sensitive (es). NBMPR-insensitive transporters are unaffected by micromolar concentrations of NBMPR and are designated equilibrative insensitive (ei). Both display a broad substrate specificity for purine and pyrimidine nucleosides. Transporters of the es type are pharmacological targets for coronary vasodilator compounds such as dipyridamole, dilazep, and drafazine (1, 2), which compete with permeant (and NBMPR) for the substrate-binding site (1, 2, 16, 17). These agents inhibit adenosine influx across the plasma membrane, thereby potentiating the interaction of extracellular adenosine with purinoreceptors (5, 6). In most species, including humans, dipyridamole, dilazep, and drafazine inhibit es (but not ei) transport with potencies in the nanomolar concentration range (1, 2). In contrast, es transporters of rat origin are generally resistant to vasoactive drug inhibition.

cDNAs have been isolated recently encoding both human and rat es and ei transporters (18–21). These proteins (456–457 residues, Mr 50,000), designated hENT1 and rENT1 (es) and hENT2 and rENT2 (ei), are structurally unrelated to concentrative nucleoside transporters (7–13) and contain 11 potential TMs with a large putative extracellular loop between TMs 1 and 2 and a large cytoplasmic loop between TMs 6 and 7. When expressed in Xenopus oocytes, hENT1 and its close homolog rENT1 had the expected broad substrate selectivity for purine and pyrimidine nucleosides, including adenosine, and were

1 The abbreviations used are: NBMPR, nitrobenzylthioinosine (6-(4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine; AIDS, acquired immunodeficiency syndrome; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; h, human; r, rat; PCR, polymerase chain reaction; TM, transmembrane region.
NBMPR-sensitive (18, 19). hENT2 and rENT2 were also broadly selective for purine and pyrimidine nucleosides but were NBMPR-insensitive (19–21). In agreement with studies of classical es- and ei-type transport in intact cells (1, 2), hENT1 was inhibited by nanomolar concentrations of dipyridamole and dilazep (18, 19). Draflazine was also inhibitory (20). hENT2, in contrast, was only moderately sensitive to vasodilator inhibition (20, 21), whereas rENT1 and rENT2 were unaffected by dipyridamole or dilazep at concentrations up to 1 μM (19).

Since hENT1 and rENT1 share extensive protein sequence similarity, but display distinct functional differences with respect to dipyridamole and dilazep inhibition, generation of chimeric molecules between the two proteins should help identify structural domains of hENT1 involved in the binding of vasoactive compounds. Furthermore, since vasodilators and the substrate analog NBMPR interact at common or overlapping sites (1, 2, 16, 17), such chimeras should also shed light on the site of NBMPR (and permeant) binding. Therefore, in the present investigation, a series of chimeric transporters were generated between hENT1 and rENT1 using recombinant DNA techniques. The chimeric proteins were expressed in Xe-

EXPERIMENTAL PROCEDURES

Nomenclature and Construction of Chimeric Transporters—The different chimeric transporters created between hENT1 and rENT1, which have 456 and 457 amino acid residues, respectively (Fig. 1), are illustrated in Fig. 2. The additional amino acid residue present in rENT1 is a cysteine, located just before TM 8. A four-letter nomenclature was chosen to represent each chimera. The numbers of Rs and Hs in the name indicate the approximate percentage of each wild-type cDNA in a particular construct, where the letter R represents the DNA and encoded amino acid sequence of hENT1 and the letter H denotes that of rENT1. For instance, RHHH is a 50:50 chimeric transporter whose amino-terminal half is rENT1 and whose carboxyl-terminal half is hENT1; RRHH is a 25:75 chimeric transporter whose amino-terminal one-quarter is rENT1 and whose carboxyl-terminal three-quarters is hENT1. The two junction points are represented by arrows A and B in Fig. 1.

The hENT1 and rENT1 cDNAs (GenBank™ accession numbers U81375 and AF015304) used to construct the chimeras were cloned in our laboratories as described previously (18, 19) into the vectors pBlue-Script II KS(+)(Stratagene) and pGEM-T (Promega), respectively. Both cDNAs have a common EcoRI restriction site in the middle of their coding sequence which, in our topology model, corresponds to the start of the central cytoplasmic loop between TM 6 and TM 7 (arrow B in Fig. 1). To make use of this EcoRI site for the construction of chimeras, EcoRI sites present in the non-coding regions of the hENT1 cDNA were

FIG. 1. Topographical model of hENT1 and rENT1. Hydropathy profiles for the predicted amino acid sequences of hENT1 and rENT1 were determined by the method of Eisenberg et al. (14). Potential membrane-spanning α-helices in the topographical model are numbered, and putative glycosylation sites in hENT1 and rENT1 are indicated by solid and open stars, respectively. Residues identical in the two proteins are shown as solid circles. Splice sites used for the construction of chimeras are represented by arrows A and B.

FIG. 2. Schematic representation of hENT1/rENT1 chimeric molecules. A, diagrammatic representation of the rENT1 series of chimeras, constructed by sequential transplantation of hENT1 fragments into corresponding regions of rENT1. B, illustration of the hENT1 series of chimeras, generated by successively replacing regions of hENT1 with the rat counterparts. The nomenclature used in this study is indicated, and the precise amino acids that constitute each chimera are shown by numbers. Hatched regions indicate the hENT1 sequence, and unfilled spaces represent the rENT1 sequence. The EcoRI site located in the middle of the coding regions of the hENT1 and rENT1 cDNAs, which was used for subcloning fragments, is also shown.
removed by deletion PCR (22). Thus, 219 base pairs of the 5'-untranslated region of the hENT1 cDNA and most of the 3'-untranslated region (up to three nucleotides downstream of the stop codon) were eliminated. This deleted version of the hENT1 cDNA, which was functionally identical to the original clone when expressed in *Xenopus* oocytes, was used in subsequent studies. Plasmids pKS(+)hENT1 and pGEMT-rENT1 also share a SalI site upstream of the cDNAs they carry. This SalI site and the common internal EcoRI site were used to interchange the 5'-halves of the hENT1 and rENT1 cDNAs to create the 50:50 chimeras RRHH and HHRR. All other chimeric transporters were produced in two steps by the overlap extension PCR method (23) by using *Pyrococcus furiosus* DNA polymerase. The hybrid molecules generated by PCR were subcloned into the SalI and EcoRI sites of either pKS(+)hENT1 or pGEMT-rENT1 depending on the type of chimera under construction. All chimeras were sequenced in both directions to ensure that no mutations had been introduced.

**In Vitro Transcription and Expression in Xenopus Oocytes**—pKS(+)hENT1 and pKS(+ ) containing chimeras RRHH, RRHH, and RRHH were linearized with SaeI and transcribed with T3 polymerase using the mMESSAGE mMACHINE™ (Ambion) transcription system. pGEMT-rENT1 and pGEMT containing chimeras HHRR, HRHH, and HHRR were linearized with SphI and transcribed with SP6 polymerase using the MEGAscript™ kit (Ambion). Defolliculated stage VI Xenopus oocytes (19) were injected with 20 nl of water or 20 nl of water containing capped RNA transcript (20 ng) and incubated in modified Barth’s medium (changed daily) at 18 °C for 72 h prior to the assay of transport activity.

**Transport Assays**—Transport experiments were performed as described previously (18, 19) on groups of 10 oocytes at 20 °C using [14C]uridine (Amersham Pharmacia Biotech) (1 μCl/ml) in 200 μl of transport buffer containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, pH 7.5. Except where otherwise indicated, initial rates of uridine uptake (10 μM) were determined using an incubation period of 1 min (19). To ensure maximum effect, oocytes were pretreated with NBMPR, dipyridamole, or dilazep (1 μM unless otherwise stated) for 1 h before the addition of permeant (18, 19). The influx values shown represent the means ± S.E. of 8–10 oocytes, and each experiment was performed at least twice on different batches of oocytes.

**RESULTS AND DISCUSSION**

Comparison of the predicted amino acid sequences and hydropathy profiles of hENT1 (456 residues) (18) and rENT1 (457 residues) (19) show that the two proteins are close structural homologs with 78% sequence identity and 88% similarity (Fig. 1). Functionally, hENT1 and rENT1 display es-type nucleoside transport activity with similar apparent K_m values for uridine influx (0.2 mM) and similar IC_50 values for NBMPR inhibition (4 nM at 10 μM uridine) (18, 19). In contrast, the two transporters differ markedly in their sensitivity to inhibition by vasoactive drugs (18, 19). We have exploited this difference to identify regions of hENT1 responsible for vasoactive drug binding. Since vasoactive drugs compete with NBMPR and nucleosides for binding to the transporter (1, 2, 16, 17), such regions are likely to be localized within, or adjacent to, the permeant-binding site. Ligand binding studies have established that radiolabeled NBMPR and dipyridamole bind to es-type transporters from guinea pig lung (which are dipyridamole-sensitive) with the same stoichiometry (1:1), whereas the corresponding dipyridamole-insensitive es-type transporters from rat lung bind only NBMPR (24).

By using the topology model shown in Fig. 1 as a guide, six chimeric transporters (Fig. 2) composed of reciprocal domain swaps were generated between hENT1 and rENT1. To increase the probability of obtaining functional chimeras, the two graft-
ing sites (Fig. 1) were engineered in regions at the beginning of putative cytoplasmic domains, thereby minimizing disruption to native TMs and loops. Graft site A was close to the start of the first putative cytoplasmic loop between residues 99 and 100 of h/rENT1. Graft site B was close to the start of the large central putative cytoplasmic loop between residues 231 and 232.

RNA transcript from each chimeric cDNA was synthesized by *in vitro* transcription and microinjected into *Xenopus* oocytes, which were then assayed for nucleoside transport activity (10 μM uridine influx) in the presence and in the absence of inhibitors as described under “Experimental Procedures” (18, 19). Endogenous nucleoside transport activity was measured in water-injected oocytes (typically <3% that in RNA transcript-injected oocytes). As shown in Figs. 3 and 4, all of the chimeras were functional and inhibited by NBMPR (1 μM) to a similar extent as the parent proteins, suggesting that the native conformation was generally retained in all constructs. However, chimera HRHH consistently displayed a substantially lower level of functional expression than the other chimeras, and an incubation period of 30 min was required to obtain transport activity comparable to the wild-type proteins. One reason for such a low level of expression could be improper plasma membrane targeting. Uridine influx (10 μM) in oocytes expressing recombinant HRHH was in the range 0.6–1.2 pmol/oocyte·min, compared with 0.43–0.84 pmol/oocyte·min for hENT1, rENT1, and the other chimeric constructs.

The ability of the chimeras to interact with vasoactive compounds was investigated, in the first instance, using dipyridamole (a model vasodilator) at a single high concentration of 1 μM (Figs. 3 and 4). This concentration of dipyridamole caused almost complete inhibition of hENT1 but had no effect on rENT1 (Figs. 3A and 4A) (18, 19). For clarity, the results obtained for different chimeras are discussed in two groups, the rENT1 series (panel A in Fig. 2) and the hENT1 series (panel B in Fig. 2).

With the rENT1 series (where pGEMT-rENT1 was the par-
ent plasmid), regions of hENT1 were sequentially transplanted into equivalent positions in rENT1 to determine whether the dipyridamole inhibition function of hENT1 could be transferred to rENT1. The results are presented in Fig. 3 and summarized Table I. The first of the series, HHRR, was a 50:50 chimera in which the amino-terminal half of rENT1 (incorporating TMs 1–6) was replaced with that of hENT1 (the splice site is shown as arrow B in Fig. 1). Dipyridamole (1 μM) was able to inhibit most of the uridine transport mediated by this chimera (Fig. 3B), and the extent of residual HHRR-mediated uridine influx was very similar to that observed with hENT1 (Fig. 4A), suggesting that the site of drug interaction is within the amino-terminal half of hENT1.

Therefore, the next splice site was designed about the middle of TMs 1–6 (arrow A in Fig. 1) with a view to localizing dipyridamole recognition domains within the amino-terminal half of the protein. This resulted in two chimeric transporters, namely HRRR and RHRR. The construct HRRR, composed of hENT1 from the amino terminus to the end of TM 2 and rENT1 for the rest of the protein, displayed only modest inhibition by dipyridamole (Fig. 3C), narrowing the region of interest to TMs 3–6 (including 12 residues of the preceding intracellular loop). For simplicity, we will hereafter refer to this region as TMs 3–6. The abbreviation TMs 1–2 will be used to describe the domain from the amino terminus to the end of TM 2.

The next chimera in the series, RHRR, was created by transplanting TMs 3–6 of hENT1 into the corresponding region of rENT1. The resulting chimera was strongly inhibited by dipyridamole (Fig. 3D) but to a lesser extent than chimera HHRR. Thus, the rENT1 series of chimeras suggested that dipyridamole inhibition, and hence binding, may involve two domains present in the amino-terminal half of hENT1, with TMs 3–6 being the major site of interaction.

With this information, three mirror image chimeras, desig-
nated as the hENT1 series, were generated in pKS(+)-hENT1. In this series, regions of rENT1 were successively transplanted into hENT1 to determine whether dipyridamole insensitivity could be transferred from rENT1 to hENT1 and, if so, by which regions of sequence. Results are illustrated in Fig. 4 and summarized in Table I. Dipyridamole (1 μM) was unable to inhibit RRHH-mediated uridine transport (Fig. 4B), confirming that dipyridamole interacts with the amino-terminal half of hENT1, as noticed with the reciprocal 50:50 chimera HHRR. Replacing TMs 1–2 of hENT1 by those of rENT1 resulted in construct RHHH, which was dipyridamole-sensitive, but exhibited significantly higher residual uridine influx in the presence of dipyridamole than did wild-type hENT1 (Fig. 4C). In other words, there was a small decrease in dipyridamole inhibition when hENT1 lacked its amino-terminal region containing TMs1–2, indicating that this region may play a minor role in determining dipyridamole sensitivity as suggested by chimera HRRR. The last of the reciprocal chimeras, HRHH, was generated by substituting TMs 3–6 of hENT1 with the corresponding region of rENT1. Functionally, chimera HRHH showed a large decrease in dipyridamole inhibition compared with native hENT1 (Fig. 4D), implicating TMs 3–6 as the major site of dipyridamole interaction and confirming the results obtained with chimera RHRR. Similar patterns of inhibition were obtained when transport assays were performed on the two series of chimeras in the presence of 1 μM dilazep (Figs. 3 and 4).

Table II

| Transporter | IC50 (nM) | Hill coefficient |
|-------------|-----------|------------------|
| HHHH (hENT1) | 198 ± 24 | 1.25 ± 0.12 |
| RRRR (rENT1) | ≥10,000 | |
| HHRR | 211 ± 23 | 1.04 ± 0.10 |
| HRRR | 1940 ± 19 | 0.90 ± 0.10 |
| RRHR | 339 ± 52 | 1.10 ± 0.14 |
| RRHH | ≥10,000 | |
| RHHH | 298 ± 15 | 1.20 ± 0.05 |
| HRHH | ND | |

*ND, not determined.

Chimera HHRR displayed a dipyridamole IC50 value (211 nM) similar to that of hENT1 (198 nM), demonstrating that structural domains responsible for dipyridamole sensitivity are indeed confined to the amino-terminal half of hENT1. This was further confirmed by the reciprocal chimera RRHH, which showed an IC50 value comparable to rENT1 (≥10,000 nM).
Chimera RHRR exhibited an IC$_{50}$ value (298 nM) slightly higher than hENT1, consistent with TMs 1–2 having a small effect on dipyridamole binding affinity. A similar result was noticed with the reciprocal chimera HRRR, where an IC$_{50}$ value of 1940 nM was obtained, compared with $\approx$10,000 nM for rENT1. Chimera RHRR, generated by grafting TMs 3–6 of hENT1 into rENT1, displayed an IC$_{50}$ value (339 nM) approaching that of hENT1, highlighting the major importance of this region of the transporter.

As shown in Fig. 1, the major protein sequence differences between hENT1 and rENT1 lie in the large putative extracellular loop between TMs 1 and 2, in the putative central cytoplasmic loop between TMs 6 and 7, and in TM 9. It might be anticipated, therefore, that these regions could be responsible for the different dipyridamole sensitivities displayed by the two transporters. The present chimeric study has eliminated involvement of the central cytoplasmic loop and, as well, the carboxy-terminal half of the protein (including TM 9). It has also been demonstrated that the amino terminus up to the end of TM 2, and including large extracellular loop, contributes only minimally to dipyridamole inhibition. Instead, the key structural requirements for dipyridamole sensitivity reside in TMs 3–6 (residues 100–231). Since dipyridamole is a competitive inhibitor of es transport and NBMPR binding, TMs 3–6 are also likely to form part of the NBMPR and nucleoside binding pocket. Consistent with this interpretation, photoaffinity labeling studies with purified erythrocyte hENT1 have shown that the site of covalent attachment of NBMPR is within 16 kDa of the site of N-linked glycosylation (predicted to be Asn-48 in the extracellular loop between TMs 1 and 2) (25).

Since hENT1 and rENT1 exhibit very similar activity profiles with respect to uridine transport and NBMPR binding, the overall characteristics of the binding pocket should be the same in both proteins. That of rENT1 may, however, lack specific amino acid side chains involved in dipyridamole binding and/or contain residues that sterically hinder binding of this and other vasodilator drugs. The amino acid sequences of hENT1 and rENT1 in TMs 3–6 (between splice sites A and B in Fig. 1) are 83% identical and 95% similar, with differences between the two proteins occurring mainly as single residue substitutions across the region. Thus, introduction of point mutations to this region of hENT1 (rather than microdomain swaps) will provide more specific information on vasoactive drug binding. Another outcome of the present study is that it opens the way to identification of hENT1 residues involved in NBMPR (and per- meant) binding.

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