

## RESEARCH COMMUNICATION

**Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta**Mark GRIFFITHS<sup>\*1</sup>, Sylvia Y. M. YAO<sup>†1</sup>, Fatima ABIDI<sup>\*</sup>, Simon E. V. PHILLIPS<sup>\*</sup>, Carol E. CASS<sup>‡</sup>, James D. YOUNG<sup>†</sup> and Stephen A. BALDWIN<sup>\*2</sup><sup>\*</sup>School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K., <sup>†</sup>Membrane Transport Research Group, Department of Physiology, University of Alberta, Edmonton T6G 2H7, Canada and <sup>‡</sup>Membrane Transport Research Group, Department of Oncology, University of Alberta, Edmonton T6G 2H7, Canada

Mammalian equilibrative nucleoside transporters are typically divided into two classes, *es* and *ei*, based on their sensitivity or resistance respectively to inhibition by nitrobenzylthioinosine (NBMPR). Previously, we have reported the isolation of a cDNA clone encoding a prototypic *es*-type transporter, hENT1 (human equilibrative nucleoside transporter 1), from human placenta. We now report the molecular cloning and functional expression in *Xenopus* oocytes of a cDNA from the same tissue encoding a homologous *ei*-type transporter, which we designate hENT2. This 456-residue protein is 46% identical in amino acid sequence with hENT1 and corresponds to a full-length form of the delayed-early proliferative response gene product HNP36, a

protein of unknown function previously cloned in a form bearing a sequence deletion. In addition to placenta, hENT2 is found in brain, heart and ovarian tissue. Like hENT1, hENT2 mediates saturable transport of the pyrimidine nucleoside uridine ( $K_m$   $0.2 \pm 0.03$  mM) and also transports the purine nucleoside adenosine. However, in contrast with hENT1, which is potently inhibited by NBMPR ( $K_i$  2 nM), hENT2 is NBMPR-insensitive ( $IC_{50} < 1$   $\mu$ M). It is also much less sensitive to inhibition by the coronary vasoactive drugs dipyridamole and dilazep and to the lidoflazine analogue draflazine, properties that closely resemble those reported for classical *ei*-type transport in studies with intact cells.

## INTRODUCTION

The uptake of nucleosides is essential for nucleotide synthesis by salvage pathways in mammalian cells, such as enterocytes and haemopoietic cells, that lack *de novo* biosynthetic pathways [1]. Protein-mediated transport is also the route of cellular uptake for many cytotoxic nucleosides used in the treatment of human neoplastic and viral diseases, including leukaemias and AIDS [2–4]. Nucleoside transport also plays a key role in the regulation of many physiological processes, including lipolysis, neurotransmission, platelet aggregation and coronary vasodilatation, through its effect on adenosine concentration at the cell surface [1,5].

The transport of nucleosides across the plasma membranes of mammalian cells occurs both by equilibrative (facilitated diffusion) processes and by concentrative (sodium-dependent) processes [1,2]. The latter are thought to be restricted to specialized cells such as intestinal and renal epithelia and liver, whereas equilibrative, sodium-independent processes are more widely distributed. These can be divided into two types on the basis of their sensitivity to inhibition by the 6-thiopurine ribonucleoside nitrobenzylthioinosine (NBMPR) [1,2]. Transporters of the *es* (equilibrative sensitive) type are inhibited by NBMPR, which binds with high affinities ( $K_d$  0.1–10 nM). By contrast, transporters of the *ei* (equilibrative insensitive) type are little affected by concentrations of NBMPR below 1  $\mu$ M. Similarly, *ei*-type

transporters are generally less susceptible to the coronary vasodilators dipyridamole and dilazep and to lidoflazine analogues than are the *es*-type transporters from the same species [1]. Both classes of transporter have a similar broad substrate specificity for purine and pyrimidine nucleosides, although some *ei*-type transporters have been reported to have lower affinities for nucleosides than *es*-type transporters from the same animal species [1] or even from the same cell type [6]. Although some cell types express only *es*- or *ei*-type transporters, many express both [1], and some workers have proposed that these activities may represent two forms of the same protein [7]. However, the isolation of an L1210 cell line that has selectively lost the *ei*-type transport activity suggests that the two activities are the products of distinct genes [8].

In a previous study we reported the cloning from human placenta of a cDNA encoding a prototypic *es*-type nucleoside transporter, which we designated hENT1 (human equilibrative nucleoside transporter 1) [9]. When expressed in *Xenopus laevis* oocytes, hENT1 mediated NBMPR-sensitive, sodium-independent uridine and adenosine uptake and was potently inhibited by the coronary vasodilator drugs dipyridamole and dilazep. This 456-residue glycoprotein is predicted to contain 11-membrane-spanning  $\alpha$ -helices and is unrelated to other known transport proteins, including the active sodium- and proton-linked nucleoside transporters of the mammalian CNT (concentrative nucleoside transporter) [10–12] and bacterial *nupC* [13] gene

Abbreviations used: *ei*, equilibrative-insensitive; *es*, equilibrative-sensitive; hENT1 and hENT2, human equilibrative nucleoside transporters 1 and 2; NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9- $\beta$ -D-ribofuranosylpurine}; TM, transmembrane.

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed.

The nucleotide sequence of hENT2 has been submitted to the GenBank™, EMBL and DDBJ Nucleotide Sequence Databases under the accession number AF029358.

family. The C-terminal two thirds of the hENT1 sequence shows strong similarity (approx. 44% identity) to the 36 kDa mouse and human HNP36 proteins, which are delayed-early proliferative response gene products [14]. These HNP36 proteins were reported to have a nucleolar location and were predicted to have only eight membrane-spanning segments [14]. However, we noted that the nucleotide sequence upstream of the assigned start codon in the human HNP36 cDNA included an open reading frame 49% identical in predicted amino acid sequence with the N-terminal region [transmembrane (TM) helices 1–2] of hENT1. We therefore suggested that the cDNA might have been 'artificially' truncated during its preparation, and that a full-length cDNA might encode a second mammalian passive-nucleoside-transporter isoform [9].

We report here the testing of this hypothesis by the PCR amplification of a cDNA containing the full-length coding region of human HNP36. Production of the encoded protein, which we designate hENT2, in *Xenopus* oocytes has revealed that it is indeed a functional transporter but, in contrast with hENT1, exhibits NBMPR-insensitive, *ei*-type nucleoside-transport activity. Although hENT2 shares with hENT1 the ability to transport adenosine and uridine, it is much less sensitive to inhibition both by the coronary vasodilator drugs dipyridamole and dilazep and by the lidoflazine analogue draflazine. It should now be possible to exploit the marked differences in activity between hENT1 and hENT2 to explore the structural features responsible for solute recognition in these physiologically important transport proteins.

## MATERIALS AND METHODS

### cDNA cloning and analysis

The cDNA encoding hENT2 was amplified from an oligo(dT)-primed human placental cDNA library [9] using a PCR approach. Initial amplification of the placental library by touchdown PCR using *Pwo* polymerase (Boehringer-Mannheim) employed primers corresponding to human HNP36 nucleotide positions 61–80 (sense, 5'-GCCATGGCCCCGAGGAGACGC-3') and 1551–1570 (antisense, 5'-TCCCAATCTTACTGGCCACC-3') [14] (GenBank™/EMBL/DDBJ accession no. X86681). A product from this amplification of size  $\approx 1.6$  kb was gel-purified and re-amplified using the same primers. The resultant  $\approx 1.6$  kb product, which contained the complete coding region of hENT2, was ligated into the *EcoRV* site of pBluescript II KS(+) to yield construct pHNPc7. This was sequenced on both strands by Taq DyeDeoxy terminator cycle sequencing using an Applied Biosystems model 373A DNA sequencer.

### *Xenopus*-expression and nucleoside-uptake assays

Plasmid DNA (pHNPc7 encoding hENT2 and clone 17.1 encoding hENT1 [9]) was linearized with *Not1* and transcribed with T3 RNA polymerase in the presence of  $m^7$ GpppG ( $P^1$ -5'-(7-methyl)guanosine- $P^3$ -5'-guanosine triphosphosphate) cap using the MEGAscript™ (Ambion) transcription system. *Xenopus* oocytes were injected with 10 ng of cRNA in 10 nl of water or 10 nl of water alone and then incubated for 3 days at 18 °C. Transport experiments were subsequently performed using radio-labelled uridine and adenosine and a transport buffer containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM Hepes, pH 7.5, exactly as previously described for hENT1 [9]. In adenosine-uptake experiments, the transport buffer also contained 1  $\mu$ M deoxycoformycin to inhibit adenosine deaminase activity. An uptake period of 1 min and a permeant concentration of 10  $\mu$ M were used unless otherwise indicated. For experiments

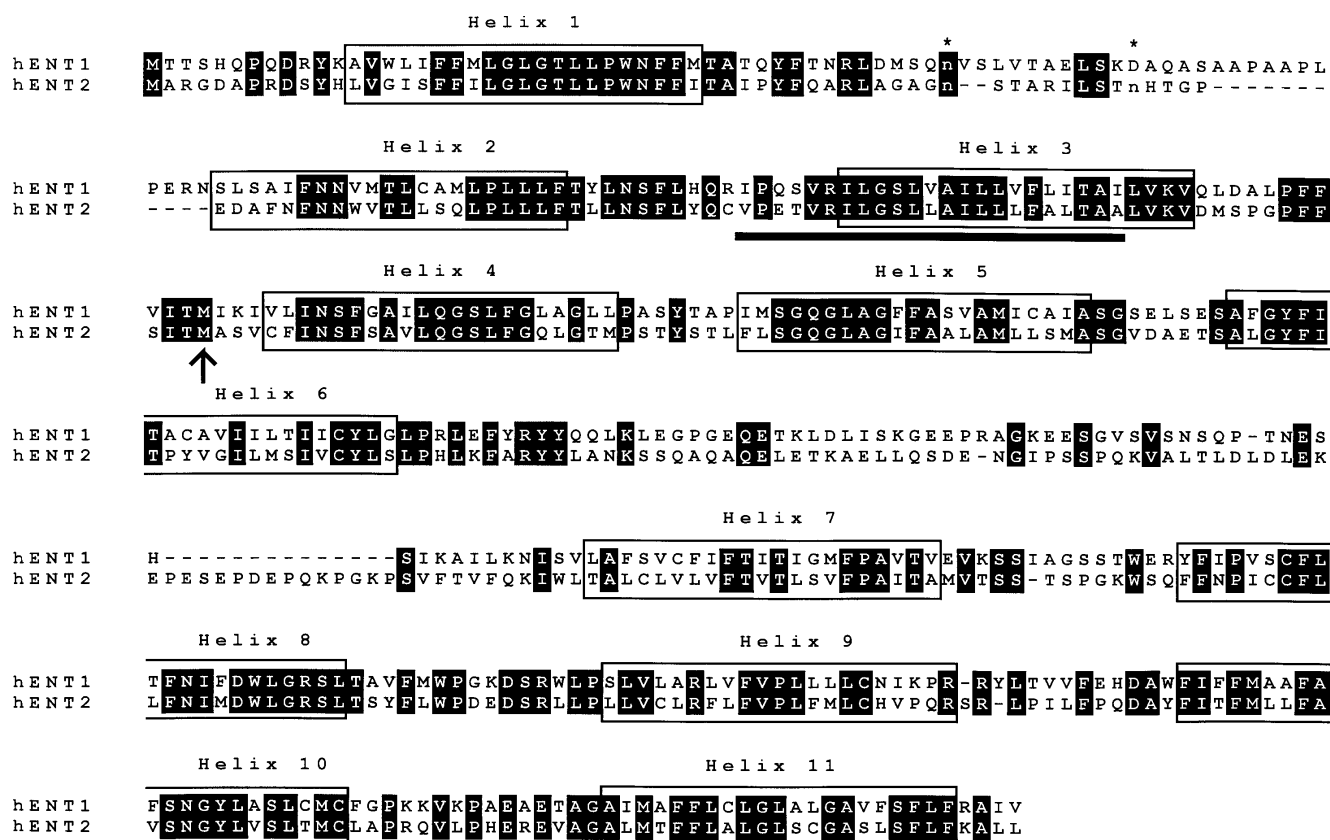
involving NBMPR, dilazep, dipyridamole and draflazine, oocytes were treated for 1 h with inhibitor before the addition of permeant. Each of the values shown for uptake experiments represents the mean  $\pm$  S.E.M. for 10–12 oocytes. Apparent  $K_m$  and  $V_{max}$  values for uridine influx were determined by non-linear regression analysis using the ENZFITTER software package (Elsevier-Biosoft, Cambridge, U.K.). Each experiment was performed at least twice on different batches of oocytes, yielding closely similar results.

## RESULTS AND DISCUSSION

The cDNA amplified from a human placental cDNA library with primers derived from the HNP36 nucleotide sequence contained a 1368 bp open reading frame that encodes a 456-residue protein of  $M_r$  50 173 (Figure 1). We have designated this protein hENT2. The nucleotide sequence was identical with the human HNP36 sequence, except that it contained an additional segment of 68 bp immediately following nucleotide position 337 in the HNP36 sequence. hENT2 is 46% identical in amino acid sequence with hENT1, and hydropathic analysis (results not shown) of the protein sequence predicts a similar TM topology of 11 hydrophobic TM  $\alpha$ -helices connected by hydrophilic loops (Figure 1). Most of the latter are predicted to be short, except for the loops connecting TMs 1 and 2 (28 residues) and TMs 6 and 7 (80 residues). The former (loop 1) is 13 residues shorter and the latter (loop 6) is 14 residues longer than the corresponding regions of hENT1. hENT2 is predicted to be a glycoprotein and contains two potential sites of N-linked glycosylation in the extramembranous loop connecting TMs 1 and 2, one of which, Asn<sup>48</sup>, is conserved in hENT1 (Figure 1).

To investigate the functional characteristics of hENT2 in comparison with those of hENT1, the recombinant proteins were expressed in *Xenopus* oocytes. At a substrate concentration of 10  $\mu$ M and at 20 °C, uptake of both the pyrimidine nucleoside [<sup>14</sup>C]uridine ( $0.231 \pm 0.012$  pmol/min per oocyte) and the purine nucleoside [<sup>14</sup>C]adenosine ( $0.612 \pm 0.021$  pmol/min per oocyte) was substantially greater in oocytes injected with the hENT2 transcript than in control oocytes injected with water alone ( $0.018 \pm 0.003$  and  $0.064 \pm 0.014$  pmol/min per oocyte for uridine and adenosine respectively). Uptake of both nucleosides was approximately half that seen in oocytes injected with the hENT1 transcript ( $0.422 \pm 0.008$  and  $1.023 \pm 0.089$  pmol/min per oocyte for uridine and adenosine respectively), although the significance of this observation with respect to the intrinsic kinetic properties of the two transporters remains unclear in the absence of any knowledge about their respective cell-surface concentrations. Both hENT1- and hENT2-RNA-injected oocytes exhibited fluxes of adenosine that were about 2.5-fold greater than the corresponding flux for uridine. Confirmation that hENT2 is a functional nucleoside transporter was provided by the demonstration that mediated influx of uridine, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable and conformed to simple Michaelis–Menten kinetics (Figure 2). The calculated apparent  $K_m$  value of  $0.20 \pm 0.03$  mM for hENT2 was essentially identical with the value of 0.24 mM that we have previously reported for hENT1 [9]. The  $V_{max}$  estimate for hENT2 was  $6.43 \pm 0.21$  pmol/min per oocyte.

As previously discussed, mammalian equilibrative- nucleoside-transport processes can be classified into *es* and *ei* types by virtue of their sensitivity or resistance to inhibition by NBMPR. hENT1 is a classic *es*-type transporter, its mediated influx of uridine (10  $\mu$ M) being inhibited by NBMPR with a calculated  $K_i$  value



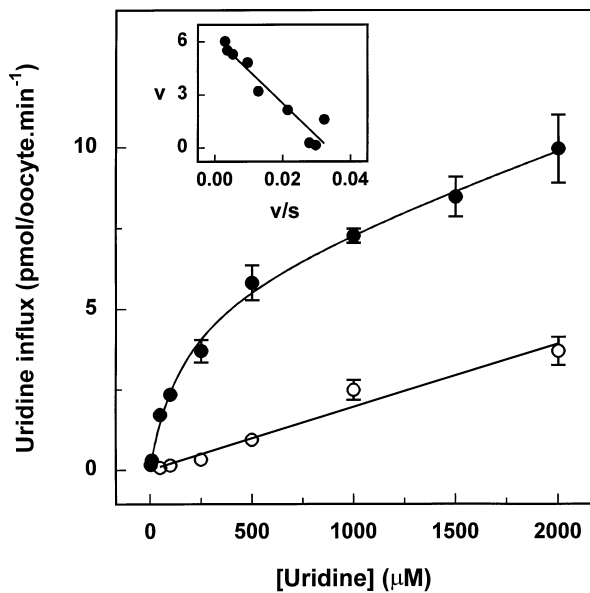
**Figure 1** Alignment of the predicted amino acid sequence of hENT2 with that of the human NBMPR-sensitive nucleoside transporter hENT1

The amino acid sequence of hENT2 was deduced from the nucleotide sequence of the open reading frame of the insert in clone pHNPc7. The sequence of hENT1 is from reference [9]. The underlined region of the hENT2 sequence is missing from the published sequence of human HNP36, and an arrow shows the position of the residue previously assigned as the N-terminus in that protein [14]. The positions of putative TM regions are shown as open rectangles and residues identical in both sequences are indicated by black boxes. Potential N-linked glycosylation sites in the hydrophilic segment linking putative TMs 1 and 2 of the proteins are shown as lower-case letters n, and their positions highlighted by an asterisk (\*) above the aligned sequences.

of 2 nM (Figure 3, Table 1 and [9]). By contrast, in the experiments shown in Figure 3 and Table 1, hENT2-mediated influx of uridine into *Xenopus* oocytes was inhibited by less than 15% in the presence of 1  $\mu$ M NBMPR and by only 70% in the presence of 10  $\mu$ M NBMPR. In five separate experiments, the mean ( $\pm$ S.E.M.) value for uridine influx in the presence of 1  $\mu$ M NBMPR was  $96 \pm 4\%$  of the control uninhibited uptake. hENT2-mediated adenosine transport exhibited a similar resistance to inhibition by NBMPR (results not shown). hENT2 is therefore an example of an NBMPR-insensitive, *ei* type of nucleoside transporter. Although in some cell types *ei* transporters are reported to have a lower affinity for substrates, including uridine, than the corresponding *es* transporters [1,6], no such differences were observed between hENT1 and hENT2. However, another reported characteristic of *ei*-type transport processes that distinguishes them from *es*-type transport processes in the same animal species is their relative insensitivity to inhibition by dipyridamole, dilazep and lidoflazine analogues such as draflazine [1]. Table 1 shows that while uridine (10  $\mu$ M) fluxes mediated by the *es*-type transporter hENT1 are potentially inhibited by all three compounds ( $\geq 94\%$  inhibition by 1  $\mu$ M inhibitor), hENT2-mediated uridine fluxes were poorly inhibited ( $\leq 71\%$  inhibition by 10 mM inhibitor).

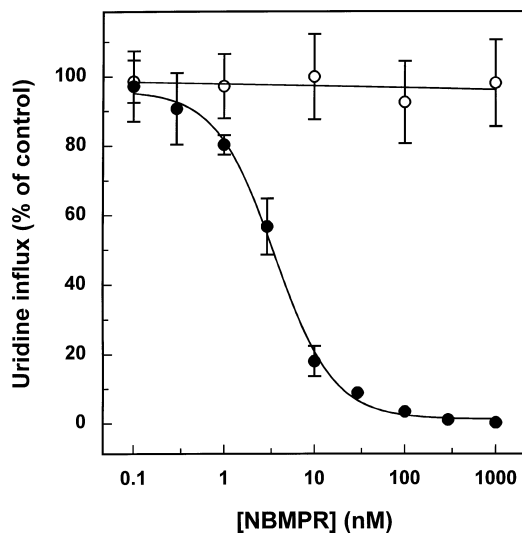
A search of the GenBank expressed-sequence-tag database showed that, in addition to placenta, hENT2 mRNA is expressed in adult ovary and ovarian tumours and in fetal brain and heart.

The truncated cDNA of hENT2 (human HNP36) previously reported [14] was isolated from an adult heart cDNA library. The physiological role(s) of hENT2 in these tissues is presently unclear. In the heart, adenosine is thought to play an important part in the regulation of myocardial  $O_2$  supply/demand balance [15], and there is considerable evidence that adenosine also exerts beneficial cardioprotective effects in the ischaemic/reperfused myocardium [16]. Over the last few years there has been increasing interest in the potential use of nucleoside-transport inhibitors as therapeutic agents in the heart [17]. For example, infusion of the lidoflazine analogue R-75231 before coronary-artery occlusion enhances local adenosine concentrations and post-ischaemic recovery of function while decreasing infarct size in the pig [18]. Preliminary trials of draflazine, an enantiomer of R75321, as a potential cardioprotective agent in humans have already been performed [19]. Although these agents appear to increase and prolong the effects of adenosine by inhibiting its uptake into metabolizing cells during ischaemia and delaying its wash-out during reperfusion [1,20], the precise roles of the different cell types in adenosine fluxes in the heart remain unclear. Our discovery that the heart expresses mRNA encoding not only the *es*-type transporter hENT1 [9], but also an *ei*-type transporter, hENT2, which is relatively insensitive to draflazine, is therefore of considerable interest. In particular, it will now be possible, using specific antibody and DNA probes for these two isoforms, to assess their distributions and individual contributions to the



**Figure 2** Concentration dependence of hENT2-mediated uridine influx

Oocytes were injected with water alone (○) or with water containing RNA transcript for hENT2 (●). After incubation for 3 days, the uptake of uridine was measured over the concentration range indicated, as described in the Materials and methods section. The inset shows a  $v$ -versus- $v/s$  plot of the transporter-mediated flux, calculated as influx in RNA transcript-injected oocytes minus influx in water-injected cells. Units of  $v$  and  $s$  are pmol of transporter-mediated uridine influx/min per oocyte and uridine concentration ( $\mu\text{M}$ ) respectively. Apparent  $K_m$  and  $V_{max}$  values, determined by non-linear regression analysis, are given in the text.



**Figure 3** Inhibition of hENT1 and hENT2 by NBMPR

Dose-response curves for the inhibition of uridine influx by NBMPR were produced in oocytes injected with RNA transcripts encoding hENT1 (○) or hENT2 (●). Fluxes were corrected for the small contribution of endogenous transport activity seen in water-injected oocytes, and are expressed as a percentage of the uninhibited hENT1 and hENT2 values, which were  $0.318 \pm 0.023$  and  $0.224 \pm 0.023$  pmol/min per oocyte respectively. The results for hENT1 have previously been reported [9].

regulation of extracellular adenosine concentration in cardiac and other tissues.

In conclusion, we have cloned and expressed a cDNA encoding

**Table 1** Inhibition of hENT1- and hENT2-mediated uridine fluxes by NBMPR, dipyridamole, dilazep and draflazine

Uridine influx ( $10 \mu\text{M}$ ,  $20^\circ\text{C}$ , 1 min flux) was measured 3 days after injection of oocytes with hENT1 or hENT2 RNA transcripts, as described in the Materials and methods section. Oocytes were pre-incubated with inhibitor for 1 h before addition of permeant. Fluxes were corrected for the small contribution of endogenous transport activity by subtraction of fluxes measured, under identical conditions, in oocytes injected with water alone instead of with RNA transcripts, and are expressed as a percentage of the uninhibited value. Abbreviation used: N.D., not determined.

Inhibitor	Concn. ( $\mu\text{M}$ )	Uridine flux (% of control)	
		hENT1-mediated	hENT2-mediated
None	—	$100 \pm 2.0$	$100 \pm 2.0$
NBMPR	1	$0.2 \pm 0.3$	$86.0 \pm 4.0$
	10	N.D.	$29.5 \pm 4.6$
Dipyridamole	1	$5.6 \pm 0.7$	$48.0 \pm 3.0$
	10	N.D.	$28.7 \pm 6.9$
Dilazep	1	$4.9 \pm 0.9$	$76.0 \pm 7.0$
	10	N.D.	$68.0 \pm 9.5$
Draflazine	1	$2.0 \pm 1.3$	$62.0 \pm 4.0$
	10	N.D.	$35.1 \pm 5.7$

an *ei*-type transporter, hENT2, from human tissues. Its initial identification as a delayed-early proliferative response gene product [14] may reflect the enhanced need for nucleotide precursors during cell proliferation. Although some workers have previously suggested that *es* and *ei* transporters may represent two forms of the same protein [7], our results establish that these two classes of transporters are the products of separate genes. Comparison of the sequences of hENT1 and hENT2 (Figure 1) shows the presence of conserved residues both within the putative transmembrane helices and in the hydrophilic loops connecting them, although the two large loops connecting TMs 1 and 2 and TMs 6 and 7 are poorly conserved. The conserved residues are likely to have structural and functional significance. The way is now open for determination of the residues that are responsible for the profound differences in the affinities of hENT1 and hENT2 for substrate analogues and inhibitors, through the production of chimaeras and other sequence modifications. Such knowledge should be of value in the rational design of novel nucleoside drugs with better selectivity for target tissues.

This work was supported by the Wellcome Trust (U.K.), by the Medical Research Council of Canada and by the Alberta Cancer Board. S.E.V.P. is an International Research Scholar of the Howard Hughes Medical Institute, C.E.C. is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada and J.D.Y. is a Heritage Medical Scientist of the Alberta Heritage Foundation for Medical Research. We thank M. Sundaram for the gift of the human placental cDNA library. Draflazine was generously given by the Janssen Research Foundation, Beerse, Belgium.

## REFERENCES

- Griffith, D. A. and Jarvis, S. M. (1996) *Biochim. Biophys. Acta* **1286**, 153–181
- Cass, C. E. (1995) in *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (Georgopapadakou, N. H., ed.), pp. 403–451. Marcel Dekker, New York
- Clumeck, N. (1993) *J. Antimicrob. Chemother.* **32**, 133–138
- Perigaud, C., Gosselin, G. and Imbach, J. L. (1992) *Nucleosides Nucleotides* **11**, 903–945
- Shryock, J. C. and Belardinelli, L. (1997) *Am. J. Cardiol.* **79**, 2–10
- Boleti, H., Coe, I., Baldwin, S. A., Young, J. D. and Cass, C. E. (1997) *Neuropharmacol.* **36**, 1167–1179
- Aronow, B., Allen, K., Patrick, J. and Ullman, B. (1985) *J. Biol. Chem.* **260**, 6226–6233

- 8 Belt, J. A. and Noel, L. D. (1988) *J. Biol. Chem.* **263**, 13819–13822
- 9 Griffiths, M., Beaumont, N., Yao, S. Y. M., Sundaram, M., Boumah, C. E., Davies, A., Kwong, F. Y. P., Coe, I., Cass, C. E., Young, J. D. and Baldwin, S. A. (1997) *Nat. Med.* **3**, 89–93
- 10 Ritzel, M. W. L., Yao, S. Y. M., Huang, M. Y., Elliott, J. F., Cass, C. E. and Young, J. D. (1997) *Am. J. Physiol.* **41**, C707–C714
- 11 Huang, Q.-Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E. and Young, J. D. (1994) *J. Biol. Chem.* **269**, 17757–17760
- 12 Che, M., Ortiz, D. F. and Arias, I. M. (1995) *J. Biol. Chem.* **270**, 13596–13599
- 13 Craig, J. E., Zhang, Y. and Gallagher, M. P. (1994) *Mol. Microbiol.* **11**, 1159–1168
- 14 Williams, J. B. and Lanahan, A. A. (1995) *Biochem. Biophys. Res. Commun.* **213**, 325–333
- 15 Mubagwa, K., Mullane, K. and Flameng, W. (1996) *Cardiovasc. Res.* **32**, 797–813
- 16 Lasley, R. D. and Mentzer, R. M. (1996) *Drug Develop. Res.* **39**, 314–318
- 17 Dennis, D. M., Raatikainen, P., Martens, J. R. and Belardinelli, L. (1996) *Circulation* **94**, 2551–2559
- 18 Martin, B. J., Lasley, R. D. and Mentzer, R. M. (1997) *Am. J. Physiol.* **41**, H1857–H1865
- 19 Rongen, G. A., Smits, P., Donck, K. V., Willemsen, J. J., Deabreu, R. A., Van Belle, H. and Thien, T. (1995) *J. Clin. Invest.* **95**, 658–668
- 20 Van Belle, H. (1993) *Drug Develop. Res.* **28**, 344–348

---

Received 16 October 1997/30 October 1997; accepted 3 November 1997