Cloning of the Human Equilibrative, Nitrobenzylmercaptopterine Riboside (NBMPR)-insensitive Nucleoside Transporter \(ei\) by Functional Expression in a Transport-deficient Cell Line*  

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Charles R. Crawford‡, Divyen H. Patel‡, Clayton Naeva§, and Judith A. Belt‡¶  
From the ‡Department of Molecular Pharmacology and §Center for Biotechnology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105  

Mammalian cells obtain nucleic acid precursors through the de novo synthesis of nucleotides and the salvage of exogenous nucleobases and nucleosides. The first step in the salvage pathway is transport across the plasma membrane. Several transport activities, including equilibrative and concentrative mechanisms, have been identified by their functional properties. We report here the functional cloning of a 2.6-kilobase pair human cDNA encoding the nitrobenzylmercaptopterine riboside (NBMPR)-insensitive, equilibrative nucleoside transporter \(ei\) by functional complementation of the transport deficiency in a subline of CEM human leukemia cells. Expression of this cDNA conferred an NBMPR-insensitive, sodium-independent nucleoside transport activity to the cells that exhibited substrate specificity and inhibitor sensitivity characteristic of the \(ei\) transporter. The cDNA contained a single open reading frame that encoded a 456-residue protein with 11 potential membrane-spanning regions and two consensus sites for N-glycosylation in the first predicted extracellular loop. The predicted protein was 50% identical to the recently cloned human NBMPR-sensitive, equilibrative nucleoside transporter ENT1 and thus was designated ENT2. Surprisingly, the carboxyl-terminal portion of the ENT2 protein was nearly identical to a smaller protein in the GenBank™ data base (human HNP36, 326 residues) that has been identified as a growth factor-induced delayed early response gene of unknown function. Comparison of the ENT2 and HNP36 nucleotide sequences suggested that HNP36 was translated from a second start codon within the ENT2 open reading frame. Transient expression studies with the full-length ENT2 and a 5’-truncated construct that lacks the first start codon (predicted protein 99% identical to HNP36) demonstrated that only the full-length construct conferred uridine transport activity to the cells. These data suggest that the delayed early response gene HNP36 is a truncated form of ENT2 and that the full-length open reading frame of ENT2 is required for production of a functional plasma membrane \(ei\) transporter.

Nucleoside transporters play an important role in the salvage of exogenous physiological nucleosides such as thymidine and uridine and in the uptake of antitumor and antiviral nucleoside analogs. At least five distinct nucleoside transport activities have been identified that differ in their permeant selectivity, sensitivity to inhibitors, and distribution in normal tissues and tumors (reviewed in Refs. 1 and 2). Two of these are equilibrative mechanisms that mediate both the influx and efflux of nucleosides across the plasma membrane, whereas the other three are concentrative, sodium-dependent mechanisms that under physiological conditions mediate only the influx of nucleosides. The major equilibrative carrier in most cells, \(es\) (equilibrative, sensitive), is highly sensitive to the inhibitor nitrobenzylmercaptopterine riboside (NBMPR) with \(IC_{50}\) values of 0.1 to 1 nM. Many cells, however, have a second equilibrative transporter \(ei\) (equilibrative, insensitive) that is insensitive to nanomolar concentrations of NBMPR but can be inhibited by higher (\(\mu M\)) concentrations. Both of the equilibrative transporters accept a broad range of physiological nucleosides and their cytotoxic and antiviral analogs as permeants, although there appear to be differences in the affinity of the two transporters for some nucleosides. In addition to the equilibrative transporters, there are at least three sodium nucleoside co-transporters that can mediate the concentrative uptake of nucleosides. These transporters are insensitive to NBMPR and differ from each other in their permeant specificity. Two of the concentrative transporters have been cloned from rat and human tissues (3–5) and found to belong to the \(nupC\) family of bacterial nucleoside transporters. Another potential nucleoside transporter belonging to the sodium/glucose co-transporter family has also been cloned (6). More recently the human NBMPR-sensitive equilibrative nucleoside transporter \(es\) has been cloned (7). The predicted protein has 10–11 potential membrane-spanning regions and appears to represent a new family of membrane transport proteins designated ENT for equilibrative nucleoside transporter.

We report here the cloning of a human cDNA that encodes the NBMPR-insensitive equilibrative nucleoside transport ac-
tivity \( \text{ei} \). This cDNA is highly homologous to ENT1, with 69% similarity of the deduced amino acid sequences, and has therefore been designated ENT2. A preliminary report of these studies has been presented (8).

**EXPERIMENTAL PROCEDURES**

**Cells and Growth Conditions—**HeLa S3 and COS-1 cells from the American Type Culture Collection were grown at 37 °C in a humified air and 5% CO\(_2\) atmosphere in DMEM supplemented with 10% heat-inactivated fetal calf serum (HeLa S3) or with 5% heat-inactivated fetal calf serum plus 5% NuSerum IV (COS-1). A nucleoside transport-deficient subline (CEM/ARCAC-8C) (9) of CEM human leukemia cells was provided by Dr. Buddy Ullman (Oregon Health Sciences University). This cell line and its derivatives were grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and the following additions: CEM/ARCAC-8C: 0.25 \( \mu \)M tubercidin, 0.5 \( \mu \)M Ara-C; CEM/C19: 0.25 \( \mu \)M tubercidin, 0.5 \( \mu \)M Ara-C, 50 \( \mu \)g/ml G418; CEM/N1–7: 20 \( \mu \)M DUP-785, 100 \( \mu \)M uridine.

**Isolation of the EBNA-1-positive, Nucleoside Transport-deficient Cell Line CEM/C19—**To attain a high frequency of stable transfection for the functional selection of cells expressing rare nucleoside transporter cDNAs, the Epstein-Barr virus-based vector pDR2 was utilized (10). This vector requires the use of a human cell line expressing the EBNA-1 gene product in trans. Thus an EBNA-1-positive, nucleoside transport-deficient human cell line was constructed by co-transfection of the EBNA-1 expression cassette pCMV-EBNA with pRSVneo (20:1 molar ratio) into CEM/ARCAC-8C cells. After selection in G418 (200 \( \mu \)g/ml Geneticin; Life Technologies), surviving cells were cloned in soft agarose (0.35%) as described previously (11). Colonies were transferred to charged nylon membranes (Hybond-N, Amersham Corp.). A clone designated CEM/C19 had a stable transfection frequency of approximately 10\(^{-2}\), which was 4 orders of magnitude greater than that of the parental EBNA-negative cell line (data not shown).

**Expression Cloning—**A CLONTECH HeLa S3 cDNA library in the pDR2 vector was transfected into CEM/C19 cells (2 \( \times \) 10\(^{5}\)) by electroporation. The library was constructed from 960 micrograms of total RNA from HeLa S3 cells. Twenty-four hours after delivery of the electrocompetent cells, surviving colonies were selected in bulk culture in medium containing 200 \( \mu \)g/ml hygromycin (14 days), 20 \( \mu \)M DUP-785, 100 \( \mu \)M uridine (27 days), and finally 20 \( \mu \)M DUP-785, 100 \( \mu \)M uridine, 1 \( \mu \)M NBMPR (14 days). Plasmids were extracted (QIA-prep spin plasmid miniprep kit; Qiagen) from the surviving cell population and rescued back into electrocompetent WM 1100 (Bio-Rad). Plasmids from individual \( E. \ coli \) colonies were analyzed for inserts and individually introduced into CEM/C19 cells, which were then selected as before.

**Sequencing of the pDR2/N1–71 Insert—**Both strands of the insert were sequenced to a level of 3-7-fold redundancy by Taq DyeDeoxy terminator cycle sequencing on an automated Model 373A DNA Sequencer (Applied Biosystems).

**Northern Analysis—**Poly(A\(^{+}\)) RNA was isolated from the indicated cell lines using the FastTrack 2.0 kit from Invitrogen. RNA (5 \( \mu \)g/lane) was separated on formaldehyde-reducing 1% agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp.). Hybridization was for 16 h at 42 °C in 50% formamide containing 10% dextran sulfate. The blot was washed at high stringency (0.2 \( \times \) saline/sodium phosphate/EDTA at 65 °C) and analyzed using a PhosphorImager and ImageQuant software. Two human multiple tissue blots (CLONTECH) with 2 \( \mu \)g of poly(A\(^{+}\)) RNA/lane were also probed under identical conditions.

**RESULTS**

**Cloning Strategy—**Since the NBMPR-insensitive equilibrative nucleoside transport protein had not been identified, and there were no antibodies or probes available, a cloning strategy based on the functional expression of \( \text{ei} \) transport activity in a nucleoside transport-deficient cell line was devised. The primary selecting agent was DUP-785, an inhibitor of \( \text{de novo} \) uridylate synthesis (13), in combination with uridine. The toxicity of DUP-785 can be circumvented by the salvage of exogenous uridine in transport-competent cells (13) but not in the transport-deficient CEM/C19 cell line (data not shown). Thus, it was expected that the combination of DUP-785 with uridine would provide a strong positive selection for transfected cells expressing a functional nucleoside transporter. The choice of a cDNA library from HeLa cells was based on previous observations that these cells have high levels of both \( \text{ei} \) and \( \text{es} \) transport activity (14) but do not have any detectable sodium-dependent nucleoside transport activity.\(^2\) After transfection, batch cultures of cells were subjected to sequential selection as follows: 1) hygromycin to select for transfected cells, 2) DUP-785 plus uridine to select for cells expressing any nucleoside transporter, and 3) DUP-785 plus uridine and NBMPR to select for cells expressing NBMPR-insensitive uridine transport activity. Surviving cell cultures were found to be positive for NBMPR-insensitive uridine uptake (data not shown). By rescue of plasmids from positive cultures back into \( E. \ coli \) followed by reintroduction into the transport deficient CEM/C19 cells, a plasmid (pDR2/N1–71) containing a 2.6-kbp insert was identified that permitted survival of CEM/C19 cells in DUP-785/uridine/NBMPR selection medium. A stable cell line expressing the 2.6-kbp N1–71 insert was established and designated CEM/N1–7. This cell line was used for further functional characterization of the plasmid.

**Uridine Transport in CEM/N1–7 Cells—**To confirm the presence of transport activity in the transfected cells, uridine influx was compared in CEM/N1–7 and CEM/C19 cells (Fig. 1A). CEM/N1–7 cells displayed a large component of uridine influx that was at least 10-fold greater than that of the recipient CEM/C19 cells. The addition of a large excess of unlabeled uridine blocked uridine transport in the transfected cells, indicating the presence of a saturable, carrier-mediated process. In contrast, uridine had no effect on nucleoside uptake in CEM/C19 cells, suggesting that the slow rate of uptake in these cells was due to simple diffusion. Neither removal of sodium (Fig. 1A) nor the addition of low concentrations of NBMPR (0.1 \( \mu \)M) (Fig. 1B) had a significant effect on transport in CEM/N1–7. These data strongly suggested that the cDNA insert encodes the NBMPR-insensitive equilibrative nucleoside transporter \( \text{ei} \). Partial inhibition of transport at higher concentrations of NBMPR (Fig. 1B) was also consistent with the properties of the human \( \text{ei} \) transporter in HeLa cells where the IC\(_{50}\) values for inhibition of uridine transport via \( \text{es} \) and \( \text{ei} \) are 1 \( \mu \)M and 6 \( \mu \)M, respectively (14). Also consistent with the \( \text{ei} \) transporter of HeLa cells (14), transport in CEM/N1–7 cells was completely blocked by 10 \( \mu \)M dipyridamole (Fig. 1B). As expected from the permeant selectivity of \( \text{ei} \) (1), uridine influx in CEM/N1–7 cells was inhibited by both purine and pyrimidine nucleosides (Fig. 1C) but not by the corresponding nucleotides (data not shown). Interestingly, uridine transport in CEM/N1–7 cells was inhibited by the nucleobase hypoxanthine (Fig. 1C) but not by adenine or uracil. Although \( \text{ei} \) is generally considered a nucleoside transporter, previous studies have suggested that it may also transport hypoxanthine (1). This was recently confirmed by Jarvis and co-workers (15) using direct measurements of hypoxanthine transport in human vascular endothelial cells. Thus, the uridine transport activity in CEM/N1–7 cells was \( \text{Na}^{+}\)-independent, inhibited by physiological nucleosides, hypoxanthine, and dipyridamole but relatively insensitive to inhibition by NBMPR. All these features are consistent with those of the \( \text{ei} \) transporter.

\(^2\)  J. A. Belt and C. R. Crawford, unpublished data.
and C, assays and have been corrected for extracellular water space. The values shown are means of duplicate experiments.

The open reading frame was 61% identical to the recently cloned human NBMPR-sensitive equilibrative nucleoside transporter ENT1 (7), and the deduced proteins exhibited 69% similarity (Fig. 2).

There was no significant homology to the sodium-dependent nucleoside transporters (3, 4, 6). Based on its similarity to ENT1, ENT2 has 11 potential membrane-spanning regions (Fig. 2). As seen with other membrane transporter families (16), the most highly conserved regions of the ENT proteins fall in the transmembrane domains. ENT2 has three potential N-glycosylation sites, two of which lie in a predicted (17) extracellular loop between transmembrane domains 1 and 2.

Surprisingly, the carboxy two-thirds of the ENT2 protein was nearly identical to a 326-residue putative protein (human HNP36) in the GenBank™ data base (X86681). The HNP36 cDNA (18) was isolated as the human homolog of a mouse delayed early response gene (DER12 (18, 19)) that is induced when quiescent cells are stimulated into cycle by serum or growth factors. The function of this protein has been unknown. Inspection of the human HNP36 nucleotide sequence revealed the presence of two potential open reading frames, with the HNP36 protein translated from the second Kozak consensus site (20) (Fig. 2). As shown in Fig. 2, the 5’-end of the first open reading frame, human HNP36orf1, is also identical to ENT2. Comparison of the nucleotide sequences revealed that the HNP36 cDNA differed from the coding region of ENT2 by only a 68-bp deletion beginning at position 359 of HNP36 with 2 base changes at positions 69 and 595. The deletion resulted in a frameshift relative to ENT2 with an in-frame stop codon. Thus HNP36 appears to encode an amino-truncated form of the ENT2 protein. To determine whether the ei transport activity encoded by ENT2 could be due to the corresponding 36-kDa protein translated from the second Kozak site of ENT2, a 5’-truncated cDNA fragment (bp 393–2610) lacking the first Kozak site and a fragment (bp 233–2610) encompassing the full coding region were cloned into pcDNA3 and compared in transient expression experiments in COS-1 cells. As shown in Fig. 3, only the full-length pcDNA3/ENT2 construct conferred uridine transport activity to COS-1 cells. Uptake in cells transfected with the truncated construct was not different from that of control cells transfected with the empty plasmid. These data indicate that the plasma membrane ei transport protein is encoded by the full open reading frame of ENT2.

Expression of ENT2 in Human Cell Lines and Tissues—Northern blots of poly(A)+ RNA were probed at high stringency with a BamH1/Nhel fragment of pDR2/N1–7. In the HeLa cell line from which the cDNA library was derived, a single transcript of approximately 2.6 kb was observed (Fig. 4A). A slightly larger transcript (approximately 3.0 kb) was found in the stable transfectant CEM/N1–7. The larger size in CEM/N1–7 cells could be accounted for by the fact that the message is derived from transcriptional start and termination sites of the pDR2 vector, which add ~460 nucleotides to the message. These data suggest that N1–7 is a full-length, or nearly full-length, cDNA for the ei transporter. As shown in Fig. 4A, no new message was detected in the transport-deficient recipient cell
The tissue distribution of the \( \text{ei} \) transporter was examined using human multiple tissue blots from CLONTECH (Fig. 4B). As expected from previous functional studies of \( \text{ei} \) transport activity in cultured cell lines, \( \text{ENT2} \) was found to be expressed in a number of tissues, and the level of expression was variable among tissues. A message of about 2.6 kbp was detected in most tissues, but a larger transcript (\( \sim 4 \) kbp) was also observed in thymus, prostate, heart, brain, lung, skeletal muscle, and pancreas. The highest level of \( \text{ENT2} \) expression was in skeletal muscle, with the 2.6-kbp message predominating. The high level of expression in skeletal muscle was unexpected, as this tissue is composed of nondividing, terminally differentiated cells. It is possible, however, that the \( \text{ENT2} \) transporter plays a role in the efflux of inosine and hypoxanthine from muscle cells during the net degradation of purine nucleotides that occurs during strenuous exercise and/or in the re-uptake of these purines during the recovery process (21, 22).

**DISCUSSION**

Cloning of a human cDNA encoding the \( \text{NBMPR} \)-insensitive equilibrative nucleoside transporter \( \text{ei} \) was accomplished by...
and tissues (2 μCi/mg) of leukemia (9). The conclusion that the ENT2 cDNA encodes the functional complementation of the nucleoside transporter in a mutant cell line derived from the human CEM T-cell line (7) but lacks homology to the concentrative nucleoside transport protein ENT1 (7) but lacks homology to the concentrative nucleoside transporters (3, 4, 6) or other known membrane transport proteins. Although these studies demonstrate that ENT1 and ENT2 belong to the same gene family, they also confirm our previous hypothesis that the es and ei nucleoside transport activities are mediated by separate and distinct proteins rather than isoforms of a single gene product produced by alternate splicing or post-translational modification (1, 2, 23, 24).

The CEM/N1–7 cell line established in these studies, which stably expresses ENT2 in a nucleoside transport-deficient background, will provide a valuable tool for further characterization of this transporter. This is the first cell line available that has ei as its sole nucleoside transport activity. Whereas several cell lines have been identified that have endogenous ei activity, they all have additional nucleoside transport activities that have complicated the characterization of the ei transporter. Thus, the functional characterization of ei has lagged behind that of es, where several good model systems are available. It is also anticipated that the CEM/N1–7 cell line and additional derivatives of CEM/C19 expressing single recombinant human nucleoside transporters will provide valuable tools to screen for transportability in the development of antitumor and antiviral nucleoside analogs.

Although our studies have demonstrated that the delayed early response gene HNP36 encodes a truncated form of the nucleoside transporter ENT2, the functional relationship between these two proteins is still unclear. The full-length human ENT2 cDNA conferred uridine transport activity to COS-1 cells, whereas a 5′-truncated construct encoding the 36-kDa protein did not (Fig. 3). The lack of activity of the truncated construct could reflect an integral role for the amino terminus of the protein in the transport process or in the proper insertion of the protein into the membrane. On the other hand, the 36-kDa protein might play another role in the cell that is not detected in the uridine uptake assays used here, perhaps the transport of nucleosides or related compounds between intracellular compartments. Further studies are required to determine if a 36-kDa protein is produced and whether it has a role in nucleoside transport. It is also unclear whether ENT2 is a delayed early response gene as reported for mouse HNP36 (18, 19). In preliminary studies using serum stimulation of the human fibroblast cell line IMR-90, we have been unable to detect regulation of ENT2, although regulation of cyclin D1 was clearly evident.2 We have also been unable to detect regulation of ENT2 in phytohemagglutinin/interleukin-2-stimulated peripheral blood lymphocytes. Furthermore, previous studies of nucleoside transport in a bone marrow-derived murine macrophage cell line have demonstrated colony stimulating factor 1 stimulation of es transport activity (ENT1) with no change in ei transport activity (ENT2) (25). Thus, a role for the ENT2 nucleoside transporter in the proliferative response is unclear and will require further study. During the revision of this manuscript, Young and co-workers (26) cloned a rat ENT2 cDNA. They concluded that ENT2 is the full-length form of HNP36 and that the previously described delayed early response gene (18, 19) is therefore the ei nucleoside transporter. As discussed above, this may not be the case, and further experimental evidence is needed to determine whether ei is a delayed early response gene and to clarify the functional relationship between ENT2 and HNP36.

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