We have previously shown that the human equilibrative nucleoside transporter 1 (hENT1) is expressed and functional in the mitochondrial membrane and that this expression enhances the mitochondrial toxicity of the nucleoside drug, fialuridine (FIAU) (Lai, Y., Tse, C. M., and Unadkat, J. D. (2004) J. Biol. Chem. 279, 4490–4497). Here we report on identification of the mitochondrial targeting sequence of hENT1. Using confocal microscopy and different truncated and point mutants of hENT1-YFP (yellow fluorescent protein) expressed in Madin-Darby canine kidney cells, we identified amino acid residues Pro71, Glu72, and Asn74 (the PE motif) of hENT1 as important in mitochondrial targeting of hENT1. Identification of this mitochondrial targeting sequence provides a possible explanation for the dramatic difference in mitochondrial toxicity of FIAU between humans and rodents. Although the mouse ENT1 (mENT1), expressed in Madin-Darby canine kidney cells, can transport FIAU, confocal microscopy showed that mENT1-GFP (green fluorescent protein) was not localized to the mitochondria. Consistent with this observation, mitochondria isolated from mouse livers did not transport FIAU. Sequence alignment of hENT1, mENT1, and rat ENT1 (rENT1) showed that the PEXN motif of hENT1 was substituted with a PAXS motif in both mENT1 and rENT1. Substitution of PAXS in mENT1 with PEXN (to create mENT1-PEAXN-GFP) and of PEXN in hENT1 with PAXS (to create hENT1-PAXS-YFP) resulted in partial mitochondrial localization of mENT1-PEAXN-GFP and loss of mitochondrial localization of hENT1-PAXS-YFP. This is the first time that the mitochondrial targeting signal of hENT1 has been identified. Our data suggest that the lack of mitochondrial toxicity of FIAU in mice is due to the lack of mENT1 targeting to and expression in the mitochondria.

The human nucleoside transporter, hENT1, plays an important role in modulating the physiological activity of nucleosides and in the transport of many therapeutic nucleoside drugs used in the treatment of cancer (e.g., 5-fluorouridine) and viral diseases (e.g., ribavirin) (2). hENT1 is an equilibrative nucleoside transporter that is ubiquitously expressed (3). Two additional members of the equilibrative nucleoside transporter family have been identified, hENT2 and hENT3 (4, 5). hENT2 is expressed in a selective number of tissues, such as the muscle (3), whereas hENT3 appears to be an intracellular transporter, expressed primarily in the lysosomes (5). We have recently reported that hENT1 is also functionally present in intracellular compartments, specifically in the mitochondrial membrane (1). In addition, we have shown that the presence of hENT1 on the mitochondrial membrane facilitates the entry of fialuridine (FIAU) into the mitochondria, where it is phosphorylated to produce its mitochondrial toxicity.

FIAU is a uridine analog that was developed for treatment of hepatitis B. Administration of this antiviral nucleoside to 15 patients with hepatitis B resulted in severe multisystem toxicity, due to mitochondrial damage, including fatal hepatotoxicity, pancreatitis, neuropathy, or myopathy (6). Among seven patients showing severe FIAU-mediated hepatotoxicity, five died, and two survived only after emergency liver transplant (6). hENT1, expressed in the mitochondrial membrane, facilitates the entry of the hydrophilic FIAU (1). Once in the mitochondria, FIAU is metabolized by thymidine kinase 2, a mitochondrial specific enzyme, to the monophosphate that is the rate-limiting step in the formation of the triphosphate (TP), a potent inhibitor of mitochondrial DNA polymerase-γ (7). The mitochondrial hepatotoxicity of FIAU is widely accepted to be due to inhibition by FIAU-TP of the mitochondrial DNA polymerase γ, resulting in depletion of mitochondrial DNA and cell death (8–10).

Interestingly, the lethal mitochondrial toxicity of FIAU observed in the clinic was not predicted from preclinical toxicity studies in rodents (rats or mice), even at doses that were 1,000-fold of those used in the human study (8, 11–13). This lack of predictive accuracy is troubling and important as the rodents are extensively used in drug development to predict human toxicity. These preclinical data on FIAU toxicity raise an important question. What is the mechanistic basis for this dramatic interspecies difference in FIAU toxicity? Understanding the mechanistic basis of the interspecies differences in FIAU toxicity will guide the use of appropriate species for future development of nucleoside drugs, including for preclinical toxicity studies. The lack of hepatotoxicity of FIAU in the rodents cannot be explained by a difference in the metabolism of FIAU. Rodent thymidine kinase 2 is capable of metabolizing FIAU (14), and FIAU-TP can inhibit rodent mitochondrial DNA polymerase-γ (15).

Although hENT1 is expressed on the mitochondrial as well as the plasma membrane, the signal sequences that target this protein to the mitochondria have not been identified. Here, we report the identification of a mitochondrial targeting signal sequence for hENT1, PEXN, which is present in the N terminus of the protein. In addition, we have found that mENT1 is not expressed in the mitochondria in either mouse livers or MDCK cells expressing mENT1-GFP. This lack of expression of mENT1 is due to the absence of the mitochondria.
drial targeting signal in mENT1 but present in hENT1. These findings suggest that the lack of toxicity of FIAU in mice is due to lack of targeting, and therefore, lack of expression and activity of mENT1 in the mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Construction of Truncated and Full-length hENT1-YFP Mutants—Although a consensus sequence for mitochondrial membrane targeting of proteins has not emerged, several studies have suggested that mitochondrial targeting signals often have a proline-rich region surrounded by positively charged residues (16–19). Therefore, we searched and found that the extracellular N-terminal region between the first and second transmembrane domain of hENT1 has proline residues at 66th, 69th, and 71st positions flanked by positively charged residues at positions 58 (Lys) and 73 (Arg). To test whether this region contained the mitochondrial targeting sequence of hENT1, we constructed a series of four progressively truncated versions of the human intestinal hENT1 gene (20). The sequence of all constructs was confirmed by automated sequence analysis using BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA).

Stable Expression of Nucleoside Transporters in MDCK Cells—MDCK cell lines were transfected with each constructed plasmid, either alone (for transport studies) or together with pDsRed2-Mito vector (Clontech), a vector that expresses a red fluorescent protein (henceforth referred to as MitoRed) that localizes only to the mitochondria (21). The sequences of all constructs were confirmed by automated sequence analysis using BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA).

FIGURE 1. Construction of hENT1-YFP truncated mutants and their mitochondrial localization. A, the following truncated versions of hENT1-YFP were constructed and expressed in MDCK cells. The numbers refer to amino acid positions starting from the N-terminal, the position of the 74th amino acid residue within the predicted secondary structure of the first three of 11 membrane-spanning domains of hENT1 (34) is shown. C, mitochondrial localization of truncated versions of hENT1-YFP (green) when co-expressed with MitoRed (red) in MDCK cells. hENT180-YFP or hENT174-YFP and MitoRed co-localized to the mitochondria (yellow), but hENT170-YFP and hENT134-YFP showed diffuse localization within the cells. Bar = 10 μm.

(A) hENT1-YFP (wt)
- YFP
- hENT1

(B) hENT180-YFP
- YFP
- hENT1

(C) hENT174-YFP
- YFP
- MitoRed
- Merged

hENT1180-YFP
hENT174-YFP
hENT170-YFP
hENT134-YFP

fragment was amplified using PfuTurbo DNA polymerase ( Stratagene, La Jolla, CA) and digested with BglII and KpnI restriction enzymes followed by ligation into pEYFP-C1 vector (Clontech) cut with the same enzyme. The constructs were introduced into the pEYFP-C1 vector and fused to YFP. A previously cloned 1.4-kb human intestinal hENT1 gene, contained in the pEYFP-C1 vector, was used as the wild type hENT1 (20). Mouse ENT1 (mENT1) gene (GenBank TM accession number AF131212) was amplified from pGEM-mENT1 plasmid (a gift from Prof. James Hammond, University of Western Ontario, London, Ontario, Canada) (21), and introduced into the pEGFP-C1 vector (Clontech). Mutants of hENT180-YFP (Fig. 1A), hENT1-YFP, and mENT1-GFP (green fluorescent protein) were constructed using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by automated sequence analysis using BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA).

Stable Expression of Nucleoside Transporters in MDCK Cells—MDCK cell lines were transfected with each constructed plasmid, either alone (for transport studies) or together with pDsRed2-Mito vector (for confocal microscopy, Clontech), a vector that expresses a red fluorescent protein (henceforth referred to as MitoRed) that localizes only to
the mitochondria (22). Stable expression was achieved using G418 selection as described previously (1). To enrich cells expressing YFP, GFP, or MitoRed, cells were selected by a cell sorter (BD Biosciences FACSVantage SE). The selected cells were routinely maintained in minimum Eagle’s medium with L-glutamine containing 10% fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in 95% air, 5% CO2 with 95% humidity as described previously (1).

Visualization of Expression of hENT1-YFP, mENT1-GFP, or Their Mutants in MDCK Cells — The cells were grown on Lab-Tek borosilicate cover glass chambers (Nalge Nunc International Corp., Naperville, IL) for 2–3 days. The living cells were observed and photographed using a Leica TCS NT laser-scanning confocal fluorescent microscope equipped with a krypton/argon laser as the light source. The YFP or GFP images were captured by excitation at 488 nm and emission at 510–540 nm. The MitoRed images were captured by excitation at 568 nm and emission at 600–640 nm.

Nucleoside Transport Assay — Transport of radiolabeled uridine or FIAU into MDCK cells was determined as described before (20). Briefly, when the cells were confluent in a 24-well plate, the cells were incubated with 1 μM [3H]uridine or 8 μM [14C]FIAU (Moravek Biochemicals, Brea, CA) in sodium-free transport buffer (20 mM Tris-HCl, 3 mM K2HPO4, 1 mM MgCl2·6H2O, 2 mM CaCl2, 5 mM glucose, 130 mM N-methyl-D-glucamine, pH 7.4) with or without 20 mM uridine. After incubating for 10 min at room temperature, nucleoside transport by the cells was rapidly terminated by washing the cells three times with ice-cold transport buffer. The cells were lysed by adding 1 N NaOH, neutralized by adding an equal volume of 1 N HCl, and then counted on a scintillation counter. All transport experiments were carried out in triplicate.

Mitochondria Isolation — Mitochondria were isolated from human or mouse liver tissue or MDCK cells using a mitochondria isolation kit (Sigma). Human livers were procured, prepared, and stored as described previously (23, 24). Liver tissue (100 mg) or 1–5 × 10⁶ cells (pooled from 8 to 10 culture dishes) were homogenized on ice with 2 ml of the manufacturer’s extraction buffer using a Teflon-glass homogenizer. As recommended by the vendor, to obtain a more purified “heavy” mitochondrial fraction, the low and high speed centrifugation steps listed in the manufacturer’s guide were modified to three rounds of 1,000 and 3,500 g, respectively. This modification reduced the contamination from lysosomes and peroxisomes.

Nucleoside Transport into Mitochondria — To measure nucleoside transport into mitochondria, the mitochondria (0.1 mg of protein) were incubated with 0.2 μM [3H]uridine or 8 μM [14C]FIAU in transport buffer for 5 min at room temperature in the presence or absence of 20 mM uridine. Nucleoside transport by the mitochondria was rapidly terminated by filtration followed by washing the filtered mitochondria three times with ice-cold transport buffer. The mitochondria retained on the filter were solubilized with 0.5% Triton X-100 and then counted on a scintillation counter. All transport experiments were carried out in triplicate.

Data Analysis — Data were expressed as mean ± S.D. of triplicate experiments. Data shown were representative of a minimum of two experiments carried out on different days using different batches of cells.
RESULTS

Mitochondrial Localization of Truncated and Mutated hENT1-YFP Proteins—When MDCK cells co-expressing the truncated hENT1-YFP proteins and MitoRed were visualized by confocal fluorescent microscopy, only hENT170-YFP and hENT134-YFP were found to be localized to the mitochondria (Fig. 1C). hENT170-YFP and hENT134-YFP, showed no mitochondrial localization and instead showed diffuse localization in the cytoplasm similar to that observed for MDCK cells expressing the YFP vector alone (mock cells; data not shown). These data suggested that amino acid residues 71–74 of hENT1 contained the mitochondrial targeting signal of hENT1. When each of these residues, proline, glutamic acid, arginine, or asparagine, was mutated to a conservative residue (e.g. proline to leucine or asparagine to glutamine) or a residue having a different characteristic (e.g. proline to aspartic acid) and co-expressed with MitoRed in MDCK cells, only R73G was localized to the mitochondria. The other mutants, hENT170-P71D-YFP or P71L-YFP, hENT134-E72G-YFP or E72R-YFP, hENT170-R73K-YFP, and hENT134-N74L-YFP or N74Q-YFP, were not localized to the mitochondria, partially localized to the mitochondria, or diffusely expressed (Fig. 2). These results suggested that residues Pro71, Glu72, and Asn74 of this N-terminal PERN motif were likely important for mitochondrial targeting of hENT1.

Localization of Point Mutants of the N-terminal PERN Motif of the Wild Type hENT1-YFP—To confirm the importance of Pro71, Glu72, or Asn74 amino acid residues in mitochondrial targeting of hENT1, we created and expressed, in MDCK cells, point mutants at each of these residues in the full-length hENT1-YFP (WT) (Fig. 3A). In addition, we also created mutants that have substitutions at either 2 (PGGN) or all 4 residues (LGGQ) of the PERN motif (Fig. 3A). As observed with the truncated mutants, unlike hENT1-YFP (WT), the single point mutants, hENT1-P71L-YFP, hENT1-E72G-YFP, or hENT1-N74Q-YFP, showed no or poor localization to the mitochondria, indicating that the substitution of the 71st, 72nd, or 74th amino acid residue disrupted the mitochondrial targeting ability of hENT1-YFP. In addition, as observed previously with the truncated mutants, R73G was localized to the mitochondria. Moreover, the double mutant, PGGN, or the quadruple mutant, LGGQ, resulted in a complete lack of mitochondrial targeting of the transporter. These results suggested that the PEXN motif, spanning the 71st to 74th amino acid residues of hENT1, contained the mitochondrial targeting signal of hENT1. Interestingly, all the mutants of full-length hENT1-YFP appeared to be localized to the plasma membrane. To confirm that these mutants were functional there, we conducted transport experiments with the double and quadruple mutants. The transport of [3H]uridine by these mutants was comparable with that of hENT1-YFP (Fig. 3B). Similar results were obtained when transport of [3H]adenosine was measured (data not shown).

Mouse ENT1 (mENT1-GFP) Does Not Localize to the Mitochondria in MDCK Cells—When mENT1-GFP was co-expressed with MitoRed in MDCK cells, mENT1-GFP was expressed in the plasma membrane but not in the mitochondria (Fig. 4A). mENT1-GFP in the plasma membrane was functional as demonstrated by its ability to transport [14C]FIAU with activity comparable with that of hENT1-YFP (Fig. 4B). To determine whether mENT1 was not localized to the mitochondria due to the absence of the mitochondrial targeting PEXN motif found in hENT1, we compared the first 80 amino acid residues of hENT1, mENT1, and rat ENT1. Indeed the PERN motif of hENT1 was substituted in both species with PARS (Fig. 5A). To confirm that this was the basis of the lack of mitochondrial expression of mENT1-GFP, we created microchimeras of mENT1 and hENT1. When the PERN sequence of hENT1 was replaced with PARS of mENT1 (to create the microchima hENT1-PARS-YFP), hENT1-PARS-YFP was not localized to the mitochondria (Fig. 5B). In addition, when the amino acid residues, PARS, in mENT1 were replaced with the mitochondrial targeting signal PERN (to create mENT1-PERN-GFP), mENT1-PERN-GFP was significantly expressed in the mitochondria (Fig. 5B) as shown by the yellow color in the merged image. Mitochondria isolated from mENT1-PERN-GFP-expressing MDCK cells showed no [3H]uridine transport activity (Fig. 5C). However, both chimeras retained plasma membrane expression (Fig. 5B) and functional activity (Fig. 5D).

FIAU Transport by Mitochondria Isolated from Mouse or Human Livers—To confirm that the untagged mENT1 was also not localized to the mitochondria in vivo, we determined the transport of [14C]FIAU into mitochondria isolated from mouse or human livers. Although mitochondria isolated from human liver robustly transported [14C]FIAU, those from the mouse liver showed no transport activity (Fig. 6).
Mitochondrial Targeting Signal of hENT1

(A) GFP MitoRed Merged

(B) without inhibitor with inhibitor

**FIGURE 4. Expression and transport activity of mENT1-GFP.** A, co-expression of mENT1-GFP (green) and MitoRed (red) in MDCK cells showed that mENT1-GFP was not localized to the mitochondria. Bar = 10 μm. B, mENT1-GFP or hENT1-YFP, when expressed in MDCK cells, show similar capacity to transport [14C]FIAU in the absence (filled bars) or presence of the inhibitor, 20 mM uridine (open bars). Data shown are mean ± S.D. of triplicate determinations.

**DISCUSSION**

In a previous report, we showed that hENT1 is expressed in the mitochondria as well as in the plasma membrane (1). This mitochondrial localization of hENT1 mediates the entry of FIAU into the mitochondria, where its metabolite, the triphosphate, produces significant mitochondrial toxicity resulting in hepatotoxicity, pancreatitis, and myopathy (1). This observation led us to conduct experiments to elucidate the mitochondrial targeting sequence of hENT1.

Several studies have suggested that mitochondrial membrane targeting signals of proteins often have a proline-rich region surrounded by positively charged residues (16–19). Consistent with this observation, we found that the 18PERN74 motif of hENT1 contained the mitochondrial targeting signal when truncated hENT1-YFPs (Fig. 1A) were expressed in MDCK cells and imaged using a confocal microscope (Fig. 1C). Within this PERN motif, except for arginine, substitution of all other amino acid residues in the PERN motif resulted in either complete lack of, or poor targeting to, the mitochondria (Fig. 1C). This was the case irrespective of whether Pro, Glu, or Asn in the motif was substituted with an amino acid residue of similar or dissimilar characteristics (Fig. 3A). Henceforth, this motif will be referred to as the PEXN motif. However, all substitutions of the PEXN motif allowed plasma membrane localization of the proteins. These plasma membrane proteins were functional as shown by the ability of the double and the quadruple mutants of the PEXN motif, PGGN or LGGQ, to transport [3H]uridine comparable with the wild type hENT1-YFP (Fig. 3B). These data indicated that the PEXN motif of hENT1 is critical for mitochondrial targeting of hENT1-YFP but is not necessary for plasma membrane targeting.

Many proteins are encoded by the nucleus but expressed in the mitochondria or in the outer or inner membrane of the mitochondria. These proteins contain mitochondrial targeting signal(s) that direct them to the mitochondria. Two major classes of mitochondrial targeting signals have been elucidated, those contained in the N-terminal presequence and those contained within the protein (25). The presequence consists of about 80 amino acid residues that form α-helices, has a net positive charge, and is cleaved by the mitochondrial protein peptidase during or after import into the mitochondria. In contrast, the mitochondrial membrane proteins have internal targeting sequence(s). These mitochondrial sequences are less well characterized, are not cleaved, and may be distributed in different parts of the polypeptide chain such as in the ADP/ATP carrier (25–28). We hypothesize that hENT1 belongs to the second class of proteins and is localized in the inner mitochondrial membrane and that the PEAXN motif identified here appears to be the dominant mitochondrial targeting signal within the transporter. Furthermore, the first 80 amino acid residues of hENT1, containing the PEXN motif, carries a net positive charge and has an α-helical transmembrane domain. These features have been shown to be important for mitochondrial targeting of other presequence-independent proteins (29–31).

After the fatal hepatotoxicity of FIAU was observed in the clinic, national inquiries led by the FDA (11) and the Institute of Medicine (12) asked the following question. Why was this toxicity not observed in preclinical studies in rodents? These inquiries stimulated additional experiments by the company sponsoring the FIAU clinical trial (Eli Lilly and Co.). These experiments revealed that rodents do not display hepatotoxicity to FIAU when administered the drug for 10 weeks, even at 1,000-fold higher doses than those administered to the hepatitis B patients (12). Therefore, these experiments did not shed any light on the mechanisms by which significant interspecies difference in hepatotoxicity of FIAU was observed. Studies from other laboratories showed that this difference could not be explained by differences in activation of FIAU to the triphosphate as, in vitro, rodent thymidine kinase 2 is capable of phosphorylating FIAU, and rodent DNA polymerase γ is inhibited by FIAU-TP (14, 15). The identification of the mitochondrial targeting signal sequence of hENT1 presented here led us to propose the hypothesis that the lack of mitochondrial toxicity of FIAU in the mouse is due to the absence of mENT1 in the mouse mitochondrial membrane. The absence of mENT1 in the mouse mitochondrial membrane will limit the entry of the drug into the mouse mitochondria and therefore prevent the toxicity of this drug in this rodent.

To test this hypothesis, we compared the sequence of mENT1, rENT1, and hENT1 at positions 71–74, where the PEXN motif is found in hENT1. We found that the mitochondrial signal of hENT1 (PEAXN) was not conserved in the rodents. Both the mouse and the rat sequence at these positions were PARS. Clearly, as we have shown above, the Glu and the Asn in hENT1 are required for mitochondrial targeting of hENT1. As expected, mENT1 was not expressed in the mitochondria (Fig. 4A). Consistent with this observation, mitochondria isolated from the mouse livers did not transport [14C]FIAU, but mitochondria isolated from human livers did (Fig. 6). Since mENT1 on the plasma membrane transported [14C]FIAU efficiently, we conclude from these data that mENT1 is not functional in the mitochondrial membrane as it is not localized there. As mENT1 antibodies are not available to us, we cannot directly test this conclusion with mitochondria isolated from mouse livers. To determine whether substituting the PARS motif in mENT1 with the PERN motif of hENT1 (to create mENT1-PERN-GFP)
and vice versa (to create hENT1-PARS-YFP) would respectively redirect mENT1-GFP to the mitochondria and hENT1-YFP away from the mitochondria, we created and expressed these microchimeras together with MitoRed in MDCK cells. Interestingly, the PERN motif of hENT1 significantly localized mENT1-PERN-GFP to the mitochondria, whereas the PARS motif abolished the mitochondrial localization of hENT1. Mitochondria isolated from mENT1-PERN-GFP-expressing cells did not transport uridine, indicating either that the expression of mENT1-PERN-GFP was too low to determine the transport activity or that the protein localized there was not functional (perhaps due to incorrect folding in the mitochondrial membrane). However, mENT1-PERN-GFP in the plasma membrane was capable of transporting \([3H]\)uridine.

The physiological function of ENT1 in the mitochondria is not known. Clearly, ENT1 expression in the mitochondria is not necessary for mitochondrial function as mice do not have such expression. In fact, ENT1 expression on the plasma membrane also appears not to be essential for life as demonstrated by survival of ENT1(H11002/14/H11002/H11002) mice who do not demonstrate any overt pathology (32). Therefore, the needs of mitochondria for nucleotides (including ATP synthesis) must be satisfied by alternate transporters such as the ADP/ATP carrier or the deoxynucleotide carrier (33).

FIGURE 5. Expression and transport activity of hENT1 and mENT1 microchimeras. A, multiple sequence alignment of 80 amino acid residues of N terminus of human, rat, or mouse ENT1 (GenBank accession numbers AF079117, AF015304, or AF131212, respectively). The PERN motif of hENT1 is substituted with a PARS motif in the mouse or the rat ENT1. The alignment was created by Clustal W (version 1.83). * indicates sequence homology. B, the PERN motif of hENT1 was substituted with the PARS motif of mENT1 to create the microchimera hENT1-PARS-YFP. The PARS motif of mENT1 was substituted with the PERN motif of hENT1 to create the microchimera mENT1-PERN-GFP. Co-expression in MDCK cells of the microchimeras, mENT1-PERN-GFP or hENT1-PARS-YFP, and MitoRed showed significant localization of mENT1-PERN-GFP to the mitochondria (yellow) but lack of mitochondrial localization of hENT1-PARS-YFP. Each image is shown in the x-y plane (upper image) as well as the z plane (lower image). Both chimeras showed plasma membrane localization. Bar = 10 μm. C, mitochondria isolated from MDCK cells expressing mENT1-PERN-GFP (showing some localization to the mitochondria) did not show greater \([3H]\)uridine transport than those isolated from hENT1-LGGQ-YFP-expressing cells (showing no localization to the mitochondria) or mock cells (mock). In contrast, mitochondria isolated from hENT1-YFP (WT) showed robust \([3H]\)uridine transport activity. D, transport of \([3H]\)uridine into MDCK cells by mENT1-PERN-GFP, mENT1-GFP, or hENT1-YFP (WT) is similar but greater than that into mock cells. \([3H]\)Uridine transport activity was measured in the absence (filled bars) or presence (open bars) of the inhibitor, 20 μM uridine. Data shown are mean ± S.D. of triplicate determinations.

FIGURE 6. Mitochondria isolated from mouse liver showed no \([14C]\)FIAU transport activity, whereas those isolated from human liver showed robust \([14C]\)FIAU transport activity. Transport activity was measured in the absence (filled bars) or presence (open bars) of the inhibitor, 20 μM uridine. Data shown are mean ± S.D. of triplicate determinations.
Mitochondrial Targeting Signal of hENT1

In summary, this is the first time that the mitochondrial targeting signal of hENT1 has been identified as well as a dramatic interspecies difference in mitochondrial expression of a membrane transporter. Moreover, our data give rise to the intriguing hypothesis that the clinical mitochondrial toxicity of FIAU was not predicted from toxicity studies in the mice due to the lack of expression of mENT1 in the mitochondria. This hypothesis will need to be definitively tested by creating transgenic mice expressing hENT1 and then conducting FIAU toxicity studies in these mice. Such studies are currently underway in our laboratory. Our data also highlight a hitherto poorly recognized phenomenon, that of the role of intracellular transporters in the hepatotoxicity of drugs and the potential role of variability in expression of such transporters in understanding interspecies differences in the toxicity and efficacy of drugs.

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