Cloning of a Novel Inosine-Guanosine Transporter Gene from *Leishmania donovani* by Functional Rescue of a Transport-deficient Mutant*

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Nicola S. Carter‡, Mark E. Drew§, Marco Sanchez§, Gayatri Vasudevan$$, Scott M. Landfear§, and Buddy Ullman¶

From the ‡Department of Biochemistry and Molecular Biology and the §Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201

Purine transport is an indispensable nutritional function for protozoan parasites, since they are incapable of purine biosynthesis and must, therefore, acquire purines from the host milieu. Exploiting a mutant cell line (FBD5) of *Leishmania donovani* deficient in inosine and guanosine transport activity, the gene encoding this transporter (LdNT2) has been cloned by functional rescue of the mutant phenotype. LdNT2 encodes a polypeptide of 499 amino acids that shows substantial homology to other members of the equilibrative nucleoside transporter family. Molecular analysis revealed that LdNT2 is present as a single gene copy within the leishmanial genome and encodes a single transcript of 3 kilobase pairs. Transfection of FBD5 parasites with LdNT2 re-established their ability to take up inosine and guanosine with a concurrent restoration of sensitivity to the inosine analog formycin B. Kinetic analyses reveal that LdNT2 is highly specific for inosine ($K_m = 0.3 \text{ M}$) and guanosine ($K_m = 1.7 \text{ M}$) and does not recognize other naturally occurring nucleosides. Expression of LdNT2 cRNA in *Xenopus* oocytes significantly augmented their ability to take up inosine and guanosine, establishing that LdNT2 by itself suffices to mediate nucleoside transport. These results authenticate genetically and biochemically that LdNT2 is a novel nucleoside transporter with an unusual and strict specificity for inosine and guanosine.

*Leishmania donovani* is a protozoan parasite and the etiologic agent of visceral leishmaniasis, a devastating and invariably fatal disease if untreated. The parasite exhibits an intricate life cycle in which the extracellular, flagellated promastigote exists in the phlebotomine sandfly vector, and the intracellular amastigote resides in the phagolysosome of macrophages and other reticuloendothelial cells of the mammalian host. Drugs are the only defense against visceral leishmaniasis, but the efficacy of these empirically derived agents is compromised both by drug toxicity and resistance (1). Thus, it is increasingly imperative to identify new and unique biochemical targets in the parasite for potential therapeutic exploitation.

Among the most conspicuous metabolic differences between parasites and their mammalian hosts is the purine pathway. Whereas animal cells synthesize purine nucleotides *de novo*, all protozoan parasites are incapable of synthesizing purines and depend upon purine acquisition from their hosts to survive and proliferate (2). Hence, each genus of parasite has evolved a unique complement of purine salvage enzymes in order to scavenge purines from the host milieu (2). The first step in this salvage process involves the translocation of purines across the parasite plasma membrane, a process mediated by membrane permeases. These permeases also initiate the uptake of pyrazolopyrimidine nucleobase and nucleoside analogs of hypoxanthine and inosine that are selectively toxic to *Leishmania* spp. (3, 4). Thus, purine transporters play vital roles in both purine nutrition and antiparasitic drug targeting intimating that these membrane proteins could be targets for either inhibitor or cytotoxic ligand development.

Genetic and biochemical investigations have demonstrated that *L. donovani* promastigotes express two nucleoside transport activities of nonoverlapping ligand specificity (5). The first, LdNT1, transports adenosine, pyrimidine nucleosides, and the cytotoxic adenosine analog tubercidin, and the second, LdNT2, recognizes inosine, guanosine, and the cytotoxic inosine isomer formycin B (FoB)1, (5, 6). Mutant *L. donovani* clones deficient in LdNT1 or LdNT2 activity have been isolated by virtue of their resistance to either tubercidin or FoB, respectively (5). The availability of these transport-deficient mutants and the ability to transfect *Leishmania* with cosmid expression libraries (7) provided an avenue for cloning the genes encoding nucleoside transporter proteins by selecting for functional recovery of the wild type drug sensitivity phenotype and, thus, nucleoside transport capability. This functional rescue scheme was previously employed to clone the *LdNT1* locus (8).

Functional rescue has now been exploited to isolate LdNT2. LdNT2 is present as a single copy gene within the leishmanial genome and encodes a transcript of ∼3 kb. Functional expression of LdNT2 in nucleoside transport-deficient *L. donovani* and in *Xenopus laevis* oocytes revealed LdNT2 to be a novel high affinity inosine-guanosine transporter with a singular predicted membrane topology.

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† Scholar in Molecular Parasitology.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, OR 97201. Tel.: 503-494-8437; Fax: 503-494-8393; E-mail: ullmanb@ohsu.edu.

1 The abbreviations used are: FoB, formycin B; DME-L, Dulbecco’s modified Eagle’s-Leishmania; FBS, fetal bovine serum; HYG, hygromycin B; ORF, open reading frame; ENT, equilibrative nucleoside transporter; TM, transmembrane domain; NBMPR, 4-nitrobenzyl-6-thioinosine.
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EXPERIMENTAL PROCEDURES

Chemicals, Materials, and Reagents—Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs, Inc. (Beverly, MA), Roche Molecular Biochemicals, or Life Technologies, Inc. Radiolabeled d-[2,8-3H]inosine (31.3 Ci mmol⁻¹) and d-[8-3H]guanosine (5.0 Ci mmol⁻¹) were purchased from Moravek (Brea, CA). a-[32P]CTP (3000 Ci mmol⁻¹) was procured from ICN Biomedicals (Costa Mesa, CA). Hygromycin B was purchased from Roche Molecular Biochemicals and G418 from BioWhittaker (Walkersville, MD). All other chemicals, materials, and reagents were of the highest grade commercially available and bought from either Aldrich or Sigma.

Parasite Cell Culture—L. donovani wild type (D700) and LdNT2 deficient (FB5D5) parasites (5) were propagated at 26 °C in Dulbecco’s modified Eagle’s-Leishmania (DME-L) medium (9) containing 10% calf serum. The FB5D5 cells were maintained continuously in DME-L supplemented with 1 μM FoB to ensure that the population did not harbor any wild type revertants. Transfectants were propagated in DME-L supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 50 μg ml⁻¹ of hygromycin B (DME-L/FBS/HYG).

Transfection and Isolation of Cosmids—To isolate cosmids containing LdNT2, 30 independent transfections were performed, using previously reported parameters (10), on exponentially growing FB5D5 cells resuspended at 1 × 10⁶ cells ml⁻¹ in electroporation buffer (10). FB5D5 promastigotes were transfected with 10 μg of DNA prepared from a cosmid library of L. donovani L44 strain DNA in the shuttle vector cLHYG, which encompasses the hygromycin phosphotransferase gene (7). Transfectants were maintained in the absence of hygromycin B for 24 h post-transfection, after which time they were plated on semi-solid (1%) DME-L/FBS/HYG supplemented with 25 μg ml⁻¹ G418. Uptake of [3H]inosine (0.31 Ci mmol⁻¹) and [3H]guanosine (0.05 Ci mmol⁻¹) was measured in FB5D5 cells harboring either LdNT2-ALTN0 or pALT-NEO as described previously (6). Briefly, FB5D5 promastigotes (4 × 10⁶ cells ml⁻¹), resuspended in phosphate-buffered saline, pH 7.4, and supplemented with 10 mM Na-glucose, were mixed with radiolabel for various times. Uptake was terminated by a modified oil-stop technique using a dibutyl phthalate cushion (6). All transport measurements on the LdNT2 transfectants were performed on stationary phase parasites, since inosine transport into exponentially growing parasites was nonlinear at concentrations proximal to the Km value even after a few seconds. Technical limitations of the transport assay precluded the use of fewer cells. Initial rates for each nucleoside concentration were determined by linear regression analysis over the linear portions of the assay, and Km values were determined by Hanes analysis. Competition experiments were performed in the same buffer containing 1 μM [3H]inosine (0.31 Ci mmol⁻¹) and 100 μM unlabeled inhibitor.

Nucleoside Uptake into X. laevis Oocytes—Oocytes were dissected and defolliculated as described previously (26, 27) and maintained at 16 °C in frog Ringer’s solution supplemented with 2.5 mM pyruvate, 0.5 mM theophylline, and 50 μg/ml gentamycin (Life Technologies, Inc.). The pLdNT2-OG-1 plasmid was linearized with NotI and capped cRNA synthesized in the presence of the cap analog m’GppG by T7 polymerase (28). Stage V–VI oocytes were microinjected with 5–20 ng of cRNA 1 day after defolliculation. Control oocytes were injected with equivalent volumes of water. [3H]inosine (31.3 Ci mmol⁻¹) and [3H]guanosine (5.0 Ci mmol⁻¹) uptake was assayed after 3 days of cRNA expression. Each oocyte was dissolved in 0.25 ml of 5% sodium dodecyl sulfate, and oocyte-associated radiolabel incorporation was quantitated by liquid scintillation spectrometry.

RESULTS

Cloning of LdNT2 by Functional Rescue of the FoB Sensitivity Phenotype—The cloning of LdNT2 was prejudicated on the functional restoration of a wild type phenotype (FoB-sensitive, nucleoside transport-competent) in a FoB-resistant, nucleoside transport-deficient (FB5D5) background (5). Six thousand independent hygromycin B-resistant colonies representing more than five genome equivalents of leishmanial DNA (7) were picked after transfection with a leishmanial cosmid library and selection in 20 μg ml⁻¹ of hygromycin B (DME-L/FBS/HYG). Of these, only two colonies exhibited a wild type FoB-sensitive phenotype, and both were subsequently determined to be inosine transport-proficient (data not shown). The cosmids, designated F30F12 and F44H9, were recovered from the two FoB-sensitive transfectants and subjected to restriction endonuclease analysis with the enzymes EcoRI, NotI, HindIII, XbaI, SacII, EcoRV, BglII, and BamHI. Restriction mapping revealed that both cosmids were inserts that both contained a common 11-kb EcoRI fragment. The cosmids F30F12 (Fig. 1A) was selected for further analysis. The location of the LdNT2 gene was determined by subcloning fragments of F30F12 DNA into the leishmanial transfection vector pSNAR (11), transfecting them back into FB5D5 cells, and testing for concomitant restoration of FoB sensitivity and inosine transport capability (Fig. 1). A 5-kb EcoRI fragment conforming to the appropriate phenotype was sequenced in its entirety, and an ORF (LdNT2) was identified that upon conceptual translation encoded a hydrophobic polypeptide with

Vectors for Transport Assays—LdNT2-mediated transport was measured both in FB5D5 L. donovani and X. laevis oocytes. For expression in oocytes, a 2.2-kb HindIII fragment encompassing the entire LdNT2 ORF, ~100 bp of 5'-untranslated region, and 600 bp of 3'-untranslated region was excised from the 5-kb EcoRV fragment and subcloned into the pOOG-1 oocyte expression vector (24), creating pLdNT2-OOG-1. For expression in L. donovani, an ~2.5-kb BamHI-NotI fragment from pLdNT2-OOG-1 encompassing the 2.2-kb HindIII LdNT2 fragment and ~300 bp of pOOG-1 oocyte-derived sequence encoding the 3'-untranslated region of the X. laevis β-globin gene was subcloned into the appropriate sites within the leishmanial expression vector pALTNEO (25) generating pLdNT2-ALTNEO.

Nucleoside Uptake into FB5D5 Cells Expressing LdNT2—FB5D5 promastigotes were transfected with either pLdNT2-ALTNEO or pALT-NEO, and the resulting transfectants were maintained continuously in DME-L/FBS supplemented with 25 μg ml⁻¹ G418. Uptake of [3H]inosine (0.31 Ci mmol⁻¹) and [3H]guanosine (0.05 Ci mmol⁻¹) was measured in FB5D5 cells harboring either LdNT2-ALTNEO or pALT-NEO as described previously (6). Briefly, FB5D5 promastigotes (4 × 10⁶ cells ml⁻¹), resuspended in phosphate-buffered saline, pH 7.4, and supplemented with 10 mM Na-glucose, were mixed with radiolabel for various times. Uptake was terminated by a modified oil-stop technique using a dibutyl phthalate cushion (6). All transport measurements on the LdNT2 transfectants were performed on stationary phase parasites, since inosine transport into exponentially growing parasites was nonlinear at concentrations proximal to the Km value even after a few seconds. Technical limitations of the transport assay precluded the use of fewer cells. Initial rates for each nucleoside concentration were determined by linear regression analysis over the linear portions of the assay, and Km values were determined by Hanes analysis. Competition experiments were performed in the same buffer containing 1 μM [3H]inosine (0.31 Ci mmol⁻¹) and 100 μM unlabeled inhibitor.

Isolation of LdNT2—Cosmids F30F12 and F44H9 were subjected to restriction digestion with BamHI, BglII, EcoRI, EcoRV, HindIII, NotI, SacII, and XhoI. To localize the LdNT2 gene within the F30F12 cosmid, various restriction fragments were subcloned into the leishmanial shuttle vector pSNAR, encompassing the neomycin phosphotransferase gene (11), and tested for restoration of FoB sensitivity after transfection into FB5D5 cells and selection in 20 μg ml⁻¹ G418. An ~5-kb EcoRV fragment that conferred the appropriate phenotype was sequenced in its entirety. The se-Quent_analysis_Based on the results obtained from sequencing, the LdNT2 gene was determined by subcloning from standard procedures (22). Restriction enzyme-digested DNA was blotted onto GeneScreen Plus® hybridization transfer membrane (NEN Life Science Products) and hybridized to a 2.2-kb HindIII fragment derived from the 5-kb EcoRV fragment in pSNAR that contains the LdNT2 ORF.

RNA Extraction and Northern Blotting—Total cellular RNA was isolated from ~5 × 10⁸ exponentially growing L. donovani promasti- goites using the RNeasy Midi kit (Qiagen Inc., Valencia, CA). Poly(A) RNA, prepared from total RNA using the Oligotex mRNA mini kit (Qiagen Inc., Valencia, CA), was subjected to denaturing agarose electrophoresis, transferred to a GeneScreen Plus® hybridization transfer membrane (NEN Life Science Products), and probed with the 2.2-kb HindIII fragment described above. Signals were normalized by hybridization to probes corresponding to Leishmania enrietti α-tubulin gene (23).
significant homology to other parasite and mammalian equi-
librative nucleoside transporters (8, 13–16, 26, 29).

**Sequence Analysis of LdNT2**—The LdNT2 ORF comprises
1,497 bp and predicts a polypeptide of 499 amino acids (Fig. 2).
A multisequence alignment of LdNT2 with the human equili-
brative nucleoside transporters, hENT1(13) and hENT2 (14,
15), the L. donovani LdNT1.1 transporter (8), and the Trypa-
nosoma brucei TbNT2 transporter (16) is depicted in Fig. 2.
Pairwise alignments between LdNT2 and each of the other
nucleoside transporters in Fig. 2 showed amino acid identities
between 25 and 44%. Hydropathy predictions by three inde-
pendent algorithms (19–21) suggest that LdNT2 accommod-
ates nine transmembrane domains (TMs) with a large hydro-
philic loop between TMs 5 and 6 (Figs. 2 and 3). LdNT2 also has
two potential Asn-linked glycosylation sites, Asn226 and Asn430
(Fig. 3), although only Asn226 is predicted to be within an
exposed loop.

**Molecular Characterization of LdNT2 in Wild Type and
FBD5 Cells**—Southern blot analysis of L. donovani genomic
DNA digested with a battery of restriction enzymes that cut
either within (PstI, PvuI, and SalI) or outside (EcoRI and
HindIII) the ORF indicated that LdNT2 was a single copy gene
(Fig. 4A). This result is compatible with the nucleotide se-
quence of the 5-kb EcoRV fragment that encompasses LdNT2.
The Southern blot of FBD5 genomic DNA hybridized with the
LdNT2 ORF was identical to that of wild type DNA (Fig. 4A),
demonstrating that neither a gross deletion nor rearrange-
ment of the LdNT2 locus confers the nucleoside transport-deficient
phenotype on FBD5 cells.

Northern analysis of wild type poly(A)+ RNA revealed a
major LdNT2 transcript of ~3 kb (Fig. 4B). A fainter hybrid-
izing band at ~5 kb was also observed, which could conceivably
be an unprocessed mRNA. FBD5 cells, as well as FBD5 cells
transfected with either pALTNEO or pLdNT2-ALTNEO, also
expressed the major and minor transcripts, indicating that
loss of LdNT2 function in FBD5 cells cannot be attributed to
a lack of LdNT2 transcription (Fig. 4B). As expected, LdNT2
is overexpressed in the FBD5 strain transfected with pLdNT2-
ALTNEO. The size of this transcript, which arises from splice
acceptors within the vector and insert, is also ~3 kb. Levels of
poly(A)+ RNA were normalized for each cell line using the L.
enriettii α-tubulin gene (23). There is an additional band that
hybridizes to the α-tubulin probe in the pLdNT2-ALTNEO
lane, suggestive of an alternatively spliced transcript, the
reason for which is unclear.

**Functional Characterization of LdNT2 in L. donovani**—To
establish that LdNT2 is a functional nucleoside transporter,
LdNT2 was subcloned into the pALTNEO leishmanial expres-
sion vector and transfected into FBD5 cells. Uptake assays
using 5 μM [3H]inosine confirmed that transfection with
LdNT2 bestows a robust inosine transport phenotype on FBD5
cells (71 pmol/s/10^8 cells) (Fig. 5). FBD5 cells transfected with
pALTNEO alone displayed only minimal inosine transport ca-
Bility (1 pmol/s/10^8 cells) (Fig. 5). Substrate saturation
curves with FBD5 pLdNT2-ALTNEO cells revealed that [3H]
inosine and [3H]guanosine transport displayed Michaelis-Men-
ten kinetics with an apparent K_m value of 0.3 ± 0.1 (n = 4) and
1.7 ± 0.5 μM (n = 3), respectively. Representative experiments
for both inosine and guanosine are displayed in Fig. 6. These
values are comparable to those determined for wild type para-
sites (data not shown and Ref. 6).

An inhibition profile determined for LdNT2-mediated trans-
port into pLdNT2-ALTNEO FBD5 transfecants revealed that
1 μM [3H]inosine transport was inhibited by a 100-fold excess of
nonradiolabeled inosine (96%), guanosine (96%), and several
analogues, including 8-aminoguanosine (95%), 6-thioguanosine
(94%), 6-thiopurinol riboside (54%), and allopurinol riboside (44%). No inhibitory effects were observed with 9-dea-
zainesine (5%) and 2-aminopurine riboside (~1%) (Fig. 7). By
contrast a 100-fold excess of the naturally occurring nucleo-
sides, adenosine (~1%), xanthosine (35%), uridine (33%), cyti-
dine (~1%), and thymidine (14%), as well as the nucleobases,
adename (6%), hypoxanthine (24%), guanine (26%), xanthine
(12%), thymine (~1%), and uracil (1%), only inhibited 1 μM
[3H]inosine transport marginally (Fig. 7). LdNT2-mediated inosine
transport was also not impacted by 100 μM 4-nitrobenzyl-6-
thioinosine (NBMPR) (~1%) (Fig. 7), a potent inhibitor of mam-
malian nucleoside transport at nanomolar concentrations (30).

The phenotypic consequence of restored nucleoside transport
proficiency in FBD5 cells was also assessed by growth phen-
type in FoB. The effective concentration of FoB that inhibited
growth of the LdNT2 transfectants by 50% (EC_{50}) value was
4.5 ± 3.9 nM (n = 4), a value similar to that determined for wild
type promastigotes (5.1 ± 3.5 nM) (n = 4) (5). In contrast, FBD5
cells transfected with the empty pALTNEO vector exhibited an
EC_{50} value of 5.7 ± 0.9 μM (n = 4).
Functional Expression of LdNT2 in X. laevis Oocytes—To confirm that LdNT2 by itself encodes a functional inosine-guanosine transporter, LdNT2 cRNA was expressed in Xenopus oocytes. Oocytes injected with the LdNT2 cRNA transported [3H]inosine and [3H]guanosine 10–20-fold more efficiently than water-injected control oocytes (Fig. 8). Rates of uptake were 0.36 and 0.32 pmol min^{-1} (oocyte)^{-1} for inosine and guanosine, respectively.

**DISCUSSION**

LdNT2 encoding the L. donovani inosine-guanosine transporter was identified and cloned after screening for restoration of a wild type phenotype in inosine-guanosine transport-deficient FBD5 cells following transfection with an LdNT2-containing cosmid. A similar functional rescue strategy has also been exploited to pinpoint and isolate the LdNT1 locus encoding the L. donovani adenosine-pyrimidine nucleoside transporters (8) and offers a powerful genetic approach toward the isolation of any gene for which a selection or screen can be devised. Other leishmanial genes that have been isolated by complementation of mutant phenotypes include several involved in the biosynthesis of lipophosphoglycan (31, 32), an important cell surface glycoconjugate and one crucial for biosynthesis of the glycosome (33), a unique kinetoplastid peroxisomal-like microbody that accommodates glycolytic and other nutritional enzymes (34).

The predicted amino acid sequence indicates that LdNT2 is a member of the ENT family. This family includes transporters...
from mammalian cells (13–15), a variety of protozoan parasites (8, 16, 26, 29, 35), and as yet many functionally uncharacterized ORFs from eukaryotic cells uncovered among a variety of genome sequencing projects (13). ENTs are distinct from bacterial nucleoside transporters (36, 37), as well as from mammalian concentrative nucleoside transporters (38, 39) both in their primary sequences and topological profiles. Multiphase alignment of LdNT2 with other members of this family revealed that LdNT2 shares a number of common residues, most of which reside in predicted membrane-spanning domains, including the charged residues aspartate 389 and arginine 393 within predicted TM 7 (Fig. 2). Hydrophathy predictions suggest that LdNT2 exhibits an unusual nine membrane-spanning topology with a large hydrophilic loop between TMs 5 and 6, whereas other members of the ENT family are conjectured to possess 11 membrane-spanning domains. The inferred model for LdNT2 lacks a membrane-spanning domain equivalent to TM 2 of other members of the ENT family and predicts that TM 8 encompasses two of the carboxyl-terminal membrane-spanning domains of the other transporters. If this model is correct, it suggests that the large hydrophilic loop would be on the opposite side of the membrane from the amino terminus, unlike the other ENTs. Whether this loop is exo- or endofacial in any of these transporters, however, is unknown, since these topological predictions have yet to be confirmed experimentally for any ENT member. The topological predictions for LdNT2 were made by three independent algorithms (19–21) in which individual parameters for length of membrane-spanning regions, hydropobicity, and surface probability were considered. These same algorithms predict 11 membrane-spanning regions for other ENT members.

Functional characterization of LdNT2 indicates that it is a novel high affinity transport system for both inosine and guanosine (Fig. 6) that excludes other purine and pyrimidine nucleosides and bases (Fig. 7). This unusual ligand specificity and affinity are distinct from all previously characterized ENTs from both mammalian cells and parasites. For instance, hENT1 and hENT2 both exhibit a broad ligand specificity for all naturally occurring purines and pyrimidine d-nucleosides (30), but the affinities of the human transporters for these nucleoside ligands are much lower than for either LdNT1 (8) or LdNT2. It is worth noting, however, a sodium-dependent nucleoside transport activity that appears to be specific for guanosine has been described in human acute promyelocytic leukemia cells (40).

LdNT2 also recognizes a variety of cytotoxic inosine and guanosine analogs, although large excesses of 2-aminopurine riboside and 9-deazaguanosine failed to impede inosine entry (Fig. 7). The inability of LdNT2 to recognize efficiently either 2-aminopurine riboside or adenosine implies that the exocyclic oxygen on C-6 of the purine ring is a critical determinant for ligand recognition by LdNT2. The inosine analog NBMPR, in which the C-6 hydroxyl is replaced by a nitrobenzyl moiety, does not inhibit LdNT2 at 100 μM, a concentration 3 orders of magnitude greater than that required to inhibit hENT1 (30).

Southern analysis revealed that LdNT2 is present as a single copy within the leishmanial genome and that there are no gross anomalies in the LdNT2 locus of the mutant FBD5 line. Moreover, LdNT2-specific transcripts of a comparable size and intensity were observed in both wild type and mutant parasites. These results indicate that loss of LdNT2 function in FBD5 cells is most likely due to minor deletions or point mutations
whether this loss of function is due to mutations within both \textit{LdNT2} alleles or to mutations within one allele with a concomitant loss of heterozygosity, as has been previously observed for \textit{Leishmania} (41, 42), is unknown. Isolation and sequence analysis of the mutant \textit{LdNT2} locus from FBD5 cells should differentiate between these two models.

The availability of a molecular clone encoding \textit{LdNT2}, a mutant strain deficient in \textit{LdNT2} activity, and both homologous and heterologous expression systems in which to assess \textit{LdNT2} function provide a foundation for a thorough analysis of nucleoside transport activity in \textit{Leishmania}. \textit{LdNT2}-deficient mutants, the ability to create further mutants, and the identification of conserved residues among nucleoside transporters provide an avenue for the genetic dissection of nucleoside transport both by forward and reverse genetic techniques and suggest \textit{LdNT2} as a paradigm for the study of nucleoside transport in higher eukaryotes. Particularly noteworthy is the ability to generate nonlethal loss-of-function mutants with facility.

Finally, it is worth noting that functional differences between parasite and mammalian transporters might be exploited therapeutically. Indeed, the selective toxicity of the drugs melarsoprol and pentamidine, both currently employed in the treatment of African trypanosomiasis, is mediated by their uptake on a novel adenine-adenosine transporter (P2) (43, 44). Since \textit{LdNT2} differs from its human counterparts in terms of ligand specificity, ligand affinities, and inhibitor profiles, these discrepancies might ultimately be availed of pharmacologically.

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