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Biochemistry

Cloning of *Leishmania* nucleoside transporter genes by rescue of a transport-deficient mutant

(purine salvage/drug resistance/leishmaniasis)

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ABSTRACT **All parasitic protozoa studied to date are incapable of purine biosynthesis and must therefore salvage purine nucleobases or nucleosides from their hosts. This salvage process is initiated by purine transporters on the parasite cell surface. We have used a mutant line (TUBA5) of *Leishmania donovani* that is deficient in adenosine/pyrimidine nucleoside transport activity (LdNT1) to clone genes encoding these nucleoside transporters by functional rescue. Two such genes, *LdNT1.1* and *LdNT1.2*, have been sequenced and shown to encode deduced polypeptides with significant sequence identity to the human facilitative nucleoside transporter hENT1. Hydrophobicity analysis of the LdNT1.1 and LdNT1.2 proteins predicted 11 transmembrane domains. Transfection of the adenosine/pyrimidine nucleoside transport-deficient TUBA5 parasites with vectors containing the *LdNT1.1* and *LdNT1.2* genes confers sensitivity to the cytotoxic adenosine analog tubercidin and concurrently restores the ability of this mutant line to take up [^{3}H]adenosine and [^{3}H]uridine. Moreover, expression of the *LdNT1.2* ORF in *Xenopus* oocytes significantly increases their ability to take up [^{3}H]adenosine, confirming that this single protein is sufficient to mediate nucleoside transport. These results establish genetically and biochemically that both *LdNT1* genes encode functional adenosine/pyrimidine nucleoside transporters.**

Parasitic protozoa of the genus *Leishmania* are the etiological agents of leishmaniasis, a disease that affects an estimated 12 million people worldwide (1) and ranges from the disfiguring cutaneous form to fatal visceral leishmaniasis (2). Because current empirically identified drugs suffer from many deficiencies, including toxicity and resistance, it is important to identify unique biochemical targets that could be exploited for rational development of improved therapies. Perhaps the most striking metabolic discrepancy between parasites and their hosts is the purine pathway. Whereas most mammalian cells synthesize purines *de novo*, all parasitic protozoa studied to date are unable to synthesize purines (3) and consequently must rely on purine acquisition from their hosts for survival and growth. The first step in this salvage pathway involves the transport of these substrates across the parasite plasma membrane. Moreover, these purine transporters initiate the uptake of certain pyrazolopyrimidine analogs of hypoxanthine and inosine that are toxic to both *Leishmania* and *Trypanosoma* (4). These pyrazolopyrimidines, such as allopurinol, allopurinol riboside, and formycin B, are subsequently metabolized to the nucleotide level by the parasite metabolic machinery and incorporated into RNA, metabolic transformations that do not occur in mammalian cells (4). Both the essential nutritional function of these transporters and their roles in mediating the toxicities of well-characterized antiparasitic agents provide compelling rationale to study these membrane permeases at the molecular level.

Biochemical and genetic studies have established that *Leishmania donovani* parasites express two distinct nucleoside transporters with nonoverlapping substrate specificities (5). One transporter mediates the uptake of adenosine and pyrimidine nucleosides and also transports tubercidin, a cytotoxic analog of adenosine, whereas the other transporter allows membrane permeation of guanosine, inosine, and formycin B (5). Parasites deficient in either or both transport activities have been isolated by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine followed by selection in tubercidin or formycin B (6). The availability of these null mutants provided a functional strategy for cloning genes encoding each of these nucleoside permeases.

In the present study, we have transfected the adenosine/pyrimidine nucleoside transport-deficient TUBA5 cell line with a cosmid library containing inserts of *L. donovani* genomic DNA (7) and screened individual transfectants for restoration of tubercidin sensitivity. Several tubercidin-sensitive transfectants were isolated and shown to contain distinct cosmids with overlapping inserts. Analysis of one of these cosmids has led to the identification of two ORFs encoding 491 amino acids, designated *LdNT1.1* and *LdNT1.2*, that mediate restoration of tubercidin sensitivity and [^{3}H]adenosine and [^{3}H]uridine transport capabilities to TUBA5 cells. Furthermore, expression of this *LdNT1.2* ORF in *Xenopus* oocytes stimulates the uptake of [^{3}H]adenosine in this heterologous system. These results establish that the LdNT1.1 and LdNT1.2 proteins are functional nucleoside transporters.

MATERIALS AND METHODS

Growth of Parasites and Nucleic Acid Preparation. The DI700 (8) and TUBA5 (5) strains of *L. donovani* were cultured at 26°C in DMEM-L (8) containing 10% fetal calf serum. Isolation of genomic DNA and RNA and preparation of Southern and Northern blots were performed as described (9).

Transfection and Screening for Tubercidin Sensitivity. To screen for cosmids containing the adenosine/pyrimidine nucleoside transporter genes, 30 separate transfections were performed as described (10) on ≈4 × 10^7 TUBA5 promas-

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tigotes using 10 μg of DNA from a cosmid library of genomic DNA from the Ld4 strain of *L. donovani* in the shuttle vector cLHYG (7). One day after transfection, parasites were plated onto 1% agar plates containing DMEM-L plus 10% fetal calf serum and 50 μg/ml hygromycin. After ≈2 weeks incubation at 26°C, colonies were picked and transferred into 96-well microtiter plates containing DMEM-L and 50 μg/ml hygromycin. After several days of growth, an aliquot from each well was inoculated into two replica microtiter wells, one of which contained 10 μM tubercidin. After 10–14 days of growth, replica microtiter plates were examined for cells that grew in medium lacking tubercidin but not in medium containing tubercidin. Cultures that tested positive by this screen were expanded, and the cosmids were isolated from the parasites by alkaline lysis (11). Approximately 2,000 transfectants were screened, yielding 5 positive clones, 1 of which contained the cosmid designated T1E1.

To localize the *LdNT1* genes within the T1E1 cosmid, restriction fragments were subcloned into the plasmid shuttle vector pSNAR (12). For characterization of the *LdNT1.1* gene, the 7.5-kb *Hin*dIII/*Xba*I fragment 1 (Fig. 1) was subcloned into pSNAR. For construction of the vector containing the *LdNT1.2* ORF, the 3.5-kb *Xba*I/*Eco*RI fragment 2 (Fig. 1) was digested with *Not*I, which has a site 29 bp upstream from the initiation codon, and with *Sph*I, which has a site 68 bp downstream from the termination codon. This *Not*I/*Sph*I fragment was blunted (13) with T4 DNA polymerase and subcloned into the blunted *Eco*RI site of pSNAR. Each recombinant plasmid was transfected into TUBA5 cells, and transfectants were selected in 100 μg/ml G418 and then tested for tubercidin sensitivity as described above.

DNA and Deduced Amino Acid Sequence Analysis. Manual sequencing of both strands of the *LdNT1.2* ORF in the *Xba*I/*Eco*RI fragment *2* (Fig. 1) was performed on single stranded DNA using the SequiTherm EXCEL DNA Sequencing Kit (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions. The *LdNT1.1* gene in the *Hin*dIII/*Xba*I fragment 1 (Fig. 1) was sequenced in both directions by the Oregon Health Sciences University Microbiology Core Facility using a model 377 Applied Biosystems automated fluorescence sequencer (Perkin–Elmer). Cycle sequencing was performed with AmpliTaq FS DNA polymerase using dichlororhodamine dye-labeled terminators (Perkin–Elmer). The GAP program from the University of Wisconsin Computer Genetics Group (14) was used for pairwise alignments of LdNT1.1 and LdNT1.2 with related mammalian nucleoside transporter sequences. Transmembrane segments were predicted using the TMPRED software (15).

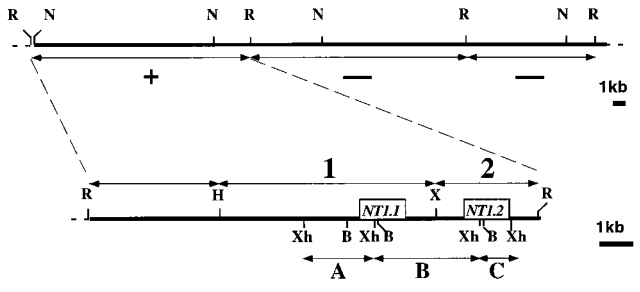

FIG. 1. Restriction map of the T1E1 cosmid. The map at the top includes the entire ≈40-kb T1E1 insert, whereas the expanded map below contains only the ≈15-kb *Eco*RI restriction fragment. Restriction fragments marked by arrows and designated 1, 2, A, B, and C are described in the text. □ designated *NT1.1* and *NT1.2* indicate the two *LdNT1* ORFs. The direction of translation of these ORFs is from left to right. The symbol "+" indicates that the relevant restriction fragment conferred sensitivity to 10 μM tubercidin when subcloned into the pSNAR shuttle vector and transfected into TUBA5 cells. The symbol "−" indicates that this restriction fragment did not confer sensitivity to tubercidin when transfected into TUBA5 cells. Symbols for restriction sites are as follows: R, *Eco*RI; H, *Hin*dIII, X, *Xba*I; N, *Not*I; B, *Bgl*II; Xh, *Xho*I. The *Hin*dIII, *Xba*I, *Bgl*II, and *Xho*I sites were mapped only within restriction fragments 1 and 2.

Uptake Assays. Uptake of [^{3}H]adenosine and [^{3}H]uridine was assayed by incubation of parasites with radiolabel followed by centrifugation through a cushion of dibutyl phthalate as described (16). Uptake was measured for a range of substrate concentrations over a time course ranging from 0 to 12 sec for cells expressing *LdNT1.1* and from 0 to 60 sec for cells expressing *LdNT1.2*. Initial uptake rates at each concentration were determined by linear regression analysis over the linear portion of the time course. These data were fitted to the Michaelis–Menten equation by least-squares analysis using the KALEIDAGRAPH program (Synergy Software, Reading, PA). For expression of *LdNT1.2* in *Xenopus* oocytes, cRNA was transcribed with T7 RNA polymerase from a linearized pL2.5 *Xenopus* expression vector (17) containing the *LdNT1.2* ORF, injected into oocytes, expressed for 6 days at 15°C, and assayed for uptake of 50 μM [^{3}H]adenosine as described (16).

RESULTS

Cloning of the *LdNT1* Adenosine/Pyrimidine Nucleoside Transporter Genes by Rescue of the Tubercidin Sensitivity Phenotype. Because the TUBA5 cell line is deficient in adenosine/pyrimidine nucleoside transport and is consequently resistant to micromolar levels of the adenosine analog tubercidin (5), the *LdNT1* nucleoside transporter genes were cloned by transfecting TUBA5 cells with a cosmid genomic library from *L. donovani* (7) and screening transformants for restoration of tubercidin sensitivity. One of five independent cosmids that rescued the wild-type tubercidin sensitivity phenotype with concomitant restoration of [^{3}H]adenosine transport capability (data not shown), T1E1, was chosen for detailed analysis. A restriction map of the T1E1 cosmid is shown in Fig. 1. To determine the location of the *LdNT1* transporter genes within the ≈40-kb cosmid insert, restriction fragments were subcloned into the pSNAR vector (12) and transfected into TUBA5 cells. The resulting transfectants were then tested for sensitivity to 10 μM tubercidin. Two adjacent restriction fragments, a 7.5-kb *Hin*dIII/*Xba*I fragment (fragment 1 in Fig. 1) and a 3.5-kb *Xba*I/*Eco*RI fragment (fragment 2 in Fig. 1), conferred both tubercidin sensitivity (Fig. 1) and [^{3}H]adenosine transport function (not shown) to transfected TUBA5 cells, suggesting that each restriction fragment contained at least one copy of *LdNT1*.

Deduced Amino Acid Sequence of the LdNT1.1 and LdNT1.2 Nucleoside Transporters. Partial sequence of the 7.5-kb *Hin*dIII/*Xba*I restriction fragment 1 (Fig. 1) revealed an ORF of 491 amino acids (Fig. 2) that contained 11 predicted hydrophobic transmembrane domains (15) and exhibited 33% amino acid identity with the human equilibrative nucleoside transporter hENT1 (18). Furthermore, the predicted topologies of LdNT1.1 and hENT1 are similar, including the conservation of a large hydrophilic loop between predicted transmembrane segments 6 and 7. Partial sequencing of the 3.5-kb *Xba*I/*Eco*RI restriction fragment 2 (Fig. 1) revealed a single copy of a closely related ORF, *LdNT1.2*, that differed from the *LdNT1.1* ORF at six amino acid positions, including the last three amino acids in the sequence (Fig. 2, asterisks and legend). Genomic Southern blots probed with the *LdNT1.2* ORF (Fig. 3*A*) revealed hybridizing fragments that are consistent with the map in Fig. 1. Thus the *Eco*RI genomic digest produced a single band of >15 kb, and the *Hin*dIII digest generated a single band of ≈17 kb, confirming that all the *LdNT1* genes were contained within the ≈11 kb *Hin*dIII/*Eco*RI fragment (fragments 1 and 2 together, Fig. 1). The *Xho*I digest produced three hybridizing bands, one of ≈3 kb corresponding to the *Xho*I fragment marked A in Fig. 1, one of ≈5 kb corresponding to the *Xho*I fragment marked B in Fig.

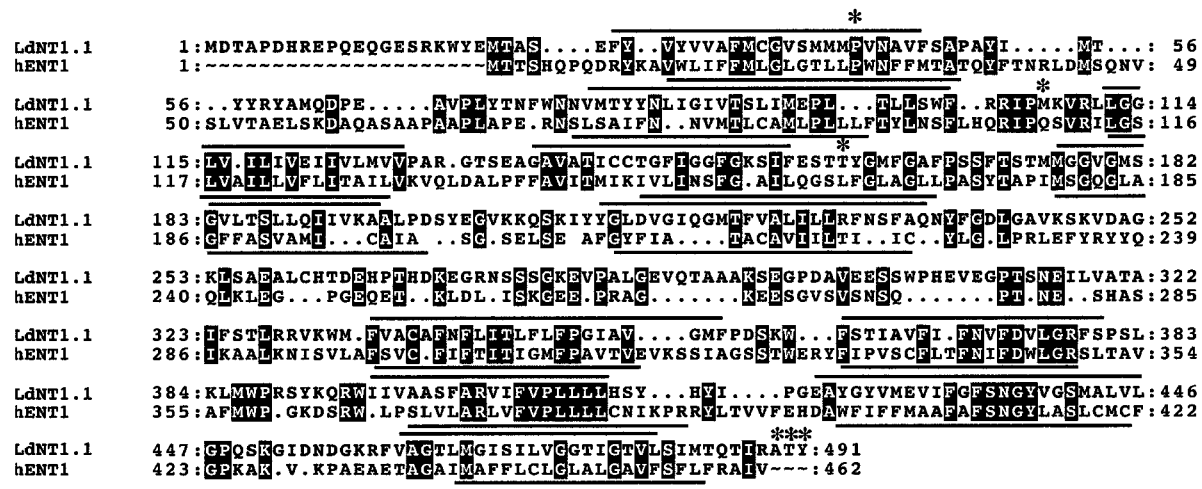

FIG. 2. Deduced amino acid sequence of LdNT1.1 (*Upper*) compared with the human equilibrative nucleoside transporter (18) hENT1 (*Bottom*) using the PILEUP program (14) with a gap weight of 4 and a gap length weight of 3. Amino acids that are identical between the two sequences are shown in white over a black background. The solid lines over the LdNT1 sequence and under the hENT1 sequence designate the predicted (15) transmembrane domains of each protein. The asterisks designate amino acids that are different in LdNT1.2. The amino acids P43, M107, T160, A489, T490, Y491 in LdNT1.1 are S43, I107, A160, E489, R490, H491 in LdNT1.2.

1, and one of ≈1 kb corresponding to the *Xho*I fragment marked C in Fig. 1. Finally, the DNA sequences of the *LdNT1* ORFs generated from fragments 1 and 2 both contain an *Xho*I site followed by a *Bgl*II site 122 bp downstream. These two closely linked *Xho*I and *Bgl*II sites occur at only one position within both fragments 1 and 2, confirming the existence of only two tandemly linked *LdNT1* genes.

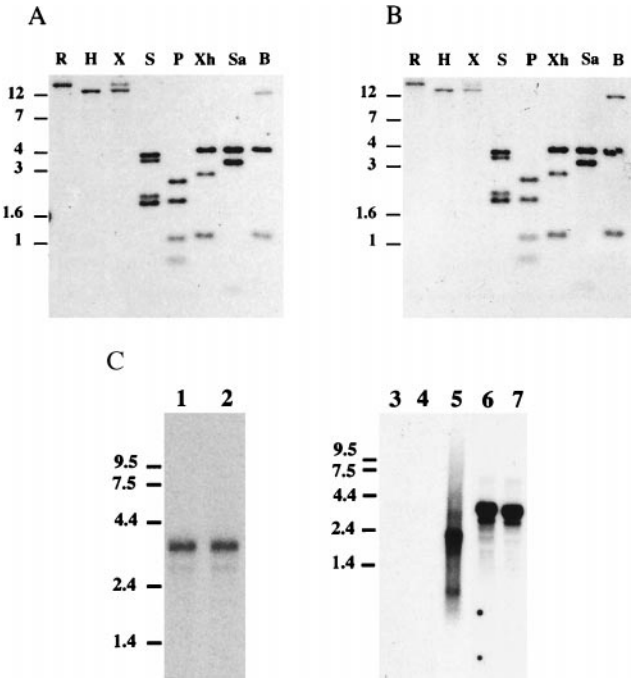

FIG. 3. Southern blots of genomic DNA and Northern blots of RNA from DI700 and TUBA5 cell lines. Genomic DNA (5 μg) from the DI700 (*A*) and TUBA5 (*B*) lines was digested with the indicated restriction enzymes: R, *Eco*RI; H, *Hin*dIII; X, *Xba*I; S, *Sac*I; P, *Pst*I; Xh, *Xho*I; Sa, *Sac*II; B, *Bgl*II. (*C*) Polyadenylylated RNA (4 μg) from the TUBA5 (lanes 1, 3, and 6) and DI700 (lane 2, 4, and 7) cell lines and 50 ng of *LdNT1.2* cRNA (lane 5) were separated on agarose-formaldehyde gels and transferred to a nylon membrane for hybridization. For each figure, the numbers at the left indicate the position of molecular mass markers with sizes given in kb pairs for *A* and *B* and kb for *C*. Blots were probed with the *LdNT1.2* ORF (lanes 1 and 2), a 750-bp *Sph*I/*Xba*I fragment from the 3′-untranslated region of *LdNT1.2* (lanes 3–5), and a 1-kb *Nde*I/*Xba*I fragment from the 3′-untranslated region of *LdNT1.1* (lanes 6 and 7).

Recently, the genes for another putative human equilibrative nucleoside transporter hENT2 (19), a homologous protein from mouse HNP36 (20), and two equilibrative nucleoside transporters from rat, rENT1 and rENT2 (21), have been cloned and sequenced. LdNT1.2 shows 28.9% identity to mHNP36, 29.3% identity to rENT1, and 30.9% identity to rENT2. Hence, the LdNT1.1 and LdNT1.2 transporters are members of an equilibrative nucleoside transporter family that spans the evolutionary range from primitive eukaryotes such as *Leishmania* to humans.

Functional Expression of the *LdNT1* Genes in *L. donovani* and in *Xenopus* Oocytes. To confirm that the LdNT1 polypeptides are functional nucleoside transporters, the *LdNT1.2* ORF alone was subcloned into the pSNAR vector and transfected into TUBA5 cells. Uptake assays using [^{3}H]adenosine and [^{3}H]uridine (Fig. 4 *A* and *B,* solid symbols) confirmed that the *LdNT1.2* ORF confers nucleoside transport activity when transfected into the adenosine/pyrimidine nucleoside transport-deficient TUBA5 cells. In contrast, TUBA5 cells transfected with the pSNAR vector alone took up only residual amounts of each nucleoside (Fig. 4 *A* and *B*, open symbols). Similarly, expression of the *LdNT1.1* gene also conferred the capacity to transport adenosine and uridine upon TUBA5 cells (Fig. 5 *A* and *B*).

To confirm that the LdNT1.2 polypeptide alone is a functional nucleoside transporter, *LdNT1.2* cRNA was expressed in the heterologous *Xenopus* oocyte system. Oocytes injected with *LdNT1.2* cRNA transported significantly more [^{3}H]adenosine than control oocytes injected with water (Fig. 4*C*), establishing that LdNT1.2 functions by itself as a nucleoside transporter.

Kinetic Characterization of LdNT1.1 and LdNT1.2. Substrate saturation curves for adenosine (Fig. 5 *A* and *C*) and uridine (Fig. 5 *B* and *D*) were determined for TUBA5 cells transfected with the *LdNT1.1* and *LdNT1.2* constructs. Least-squares fits to the Michaelis–Menten equation (17) of the data from at least three independent experiments yielded apparent K_m values (mean ± SD) for LdNT1.1 of 0.17 ± 0.09 μM (n = 4) adenosine and 5.6 ± 1.8 μM (n = 3) uridine and apparent K_m values for LdNT1.2 of 0.66 ± 0.15 μM (n = 3) adenosine and 40 ± 11 μM (n = 4) uridine. These results reveal that the LdNT1.1 transporter has significantly lower K_m values for both adenosine and uridine, compared with the LdNT1.2 trans-

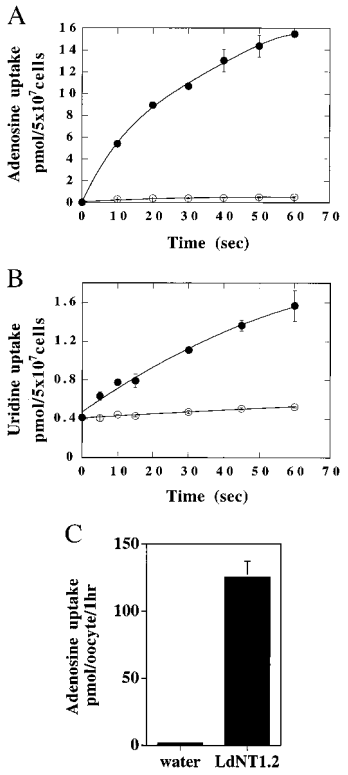

FIG. 4. Functional expression of *LdNT1* genes in TUBA5 cells and in *Xenopus* oocytes. TUBA5 cells transfected with the *LdNT1.2* ORF in the pSNAR vector (solid symbols) or with the pSNAR vector alone (open symbols) were assayed for uptake of 200 nM [^{3}H]adenosine (*A*) or 1 μM [^{3}H]uridine (*B*) (mean ± SD, $n = 2$). For each time point, samples were assayed in duplicate, and the results were plotted as the mean and SD (error bars). (*C*) *Xenopus* oocytes were microinjected with either water or ≈25 ng of *LdNT1* cRNA, incubated at 15°C for 6 days, and assayed (16) for uptake of 50 μM [^{3}H]adenosine over 1 h (mean ± SD, $n = 4$).

porter. Hence the six amino acid differences between the two permeases are likely to confer distinct kinetic properties upon each transporter.

Analysis of the *LdNT1* Locus and of *LdNT1* Transcripts in the DI700 Wild-Type and the TUBA5 Mutant Cell Lines. To determine whether the TUBA5 cell line had undergone a deletion or significant rearrangement at the *LdNT1* locus that resulted in loss of adenosine/uridine transport activity, we probed Southern blots of restriction digested genomic DNA from wild-type DI700 (Fig. 3*A*) and TUBA5 cells (Fig. 3*B*) with the *LdNT1.2* ORF. For all eight restriction enzymes examined, the hybridization patterns were identical for DI700 and TUBA5 cells, indicating the absence of large deletions or visible rearrangements at this locus. Similarly, Northern blots of polyadenylylated RNA from DI700 or TUBA5 promastigotes (Fig. 3*C*, lanes 1 and 2) probed with the *LdNT1.2* ORF revealed a single transcript of ≈3.5 kb that is expressed to equivalent levels in both strains, demonstrating that the mutant phenotype of the TUBA5 cells is not due to the absence of *LdNT1* transcripts. Northern blots hybridized with probes for the 3′-untranslated regions of the *LdNT1.2* gene (Fig. 3*C*, lanes 3 and 4) or the *LdNT1.1* gene (Fig. 3*C*, lanes 6 and 7) revealed a ≈3.5-kb *LdNT1.1* transcript in both wild-type and TUBA5 cells but did not detect any transcript for the *LdNT1.2* gene in either cell line. A positive control containing *LdNT1.2* cRNA (Fig. 3*C*, lane 5) demonstrated that the *LdNT1.2* 3′-untranslated region probe was able to detect homologous transcripts, confirming that the absence of a signal in lanes 3 and 4 was due to the absence of appreciable levels of *LdNT1.2* transcript in these parasites. The organisms used in this study were cultured promastigotes, similar to the life cycle stage that lives within the gut of the sand fly vector. It is possible that the *LdNT1.2* transcript is expressed exclusively in the amastigotes, the life cycle stage that resides inside the phagolysosomes of the vertebrate host macrophages. However, in the promastigote stage of the parasite life cycle, the *LdNT1.1* RNA is the major stable transcript from the *LdNT1* locus. Additional studies will be required to determine the relative levels of each mRNA in amastigotes.

DISCUSSION

In the present study, we have cloned the genes for the LdNT1.1 and LdNT1.2 adenosine/pyrimidine nucleoside transporters of *L. donovani* by rescuing the mutant phenotype of the adenosine/pyrimidine transport-deficient TUBA5 cell line. This is a powerful technique that may be applied to the cloning of genes for other transporters and proteins for which a strong negative genetic selection is available. Using this approach, we have also cloned genes encoding the LdNT2 guanosine/inosine transporter using the guanosine/inosine transport-deficient FBD5 cell line (manuscript in preparation). In addition, a similar approach has been used to clone several genes involved in biosynthesis of a major surface glycoconjugate (22, 23) and a gene required for biogenesis of the glycosome (24), an organelle involved in glycolysis and other metabolic interconversions in kinetoplastid protozoa.

The LdNT1.1 and LdNT1.2 transporters are members of a family of permeases currently represented by several mammalian equilibrative nucleoside transporters and possibly other proteins from *Caenorhabditis elegans* and yeast (18). Our results demonstrate that, similar to families for other classes of transporters (25), this family is represented across a large phylogenetic range from primitive eukaryotes like *Leishmania* to humans. One advantage of obtaining sequences from a diverse array of family members is the potential to identify a limited number of residues that are conserved over a large phylogenetic distance and that may represent functionally critical amino acids. It should be possible to test the potential roles of such highly conserved amino acids by site-directed mutagenesis.

One notable difference between LdNT1.1 and LdNT1.2 compared with the mammalian equilibrative nucleoside transporters is the limited substrate specificity of the parasite transporters. Whereas LdNT1.1 and LdNT1.2 transport adenosine and pyrimidine nucleosides (5), most mammalian equilibrative transporters have broad substrate specificities that accept all of the nucleosides (21). Furthermore, although the LdNT1 transporters are related to other equilibrative nucleoside transporters, it is not yet clear whether they are facilitative transporters or whether they are active transporters that might utilize the strong proton electrochemical gradient across the parasite plasma membrane (26, 27) to concentrate nucleosides inside these purine requiring organisms. It is notable that the MIT *myo*-inositol transporter from *L. donovani* is a proton symporter (28), even though it is a member of a superfamily containing the mammalian facilitative glucose transporters (29). In addition, previous experiments (5) have revealed partial inhibition of nucleoside transport in *L. donovani* by proton ionophores such as carbonylcyanide *m*-chlorophenylhydrazone. Whether the LdNT1 transporters are proton symporters will be investigated in future studies.

The genomic arrangement of the *LdNT1* genes (Fig. 1) reveals the presence of two closely related tightly linked genes. Tandemly repeated genes containing from two to dozens of identical or closely related members are common among the kinetoplastid protozoa such as *Leishmania* (30). However, the presence of an *LdNT1.1* and an *LdNT1.2* gene on each of two homologous chromosomes of this diploid organism suggests that all four genes may have been inactivated in the adeno-

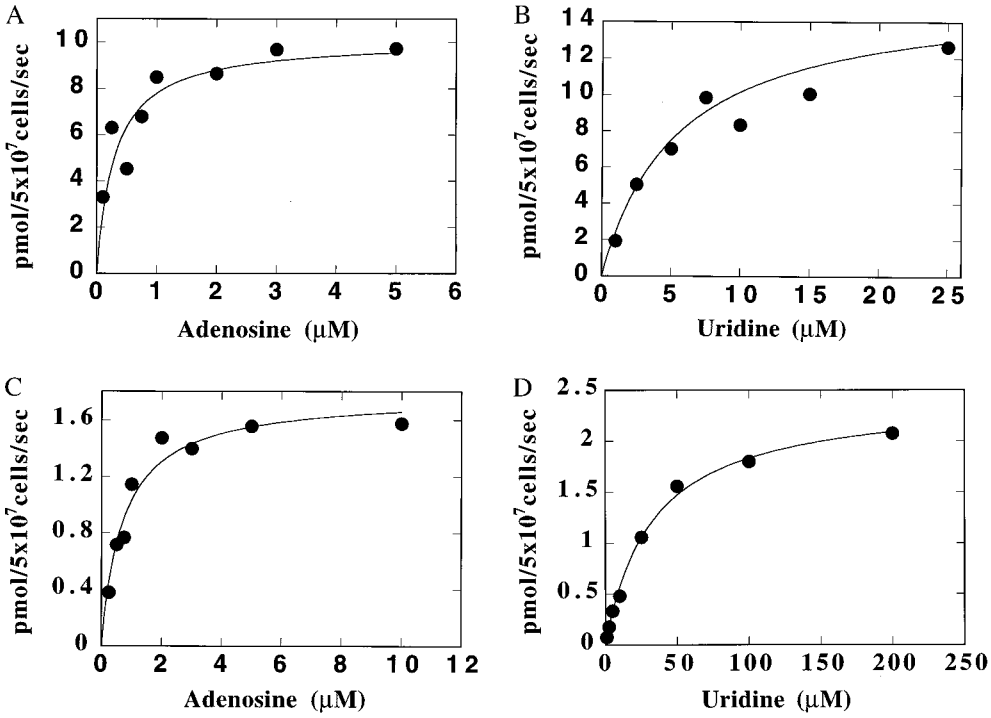

FIG. 5. [^{3}H]adenosine (*A* and *C*) and [^{3}H]uridine (*B* and *D*) substrate saturation curves for TUBA5 cells transfected with constructs containing *LdNT1.1* (fragment 1 in Fig. 1) (*A* and *B*) and *LdNT1.2* (either fragment 2 in Fig. 1 or the LdNT1.2 ORF) (*C* and *D*) in the pSNAR vector.

sine/pyrimidine nucleoside transport-deficient TUBA5 cell line. One theoretical possibility is that the original mutation resulted in a deletion or rearrangement of a region containing both the *LdNT1.1* and the *LdNT1.2* genes that led to either a loss of both ORFs or to unstable *LdNT1* RNAs. However, the absence of detectable deletions or rearrangements at this locus in TUBA5 cells (Fig. 3*B*) and the presence of stable *LdNT1.1* transcripts in both mutant and wild-type lines (Fig. 3*C*) render either of these explanations unlikely. Furthermore, the presence of the *LdNT1.1* RNA in the TUBA5 cells rules out mutations affecting transcription, RNA processing, or RNA stability.

There are several alternative explanations for the null transport phenotype of the TUBA5 cells. (*i*) Both the *LdNT1.1* and *LdNT1.2* genes could have obtained different point mutations generating either stop codons or functionally compromising missense mutations. The wild-type alleles on the homologous chromosome might then have been converted to the mutant genotype by "loss of heterozygosity", a phenomenon that has been experimentally demonstrated in both *L. major* (31) and *L. donovani* (32). (*ii*) A mutation may have been introduced into one of the *LdNT1* genes initially and then rapidly disseminated throughout other members of the family. Such "gene correction" events have been documented in mammalian cells (33). (*iii*) A missense or nonsense mutation in a single *LdNT1* gene could generate a dominant-negative phenotype if the LdNT1 permeases function as oligomers. (*iv*) The mutation in the TUBA5 cells that inactivates LdNT1 transport activity may not be in the *LdNT1* genes themselves but in another gene that affects transport activity. In this case, expression of exogenous copies of the *LdNT1* genes would rescue the transport-deficient phenotype by suppression rather than by complementation. Although distinguishing between these or other hypotheses is beyond the scope of the present work, it should be possible ultimately to elucidate the TUBA5 genotype by cloning and sequencing the *LdNT1.1* and *LdNT1.2* genes from the TUBA5 cell line. Furthermore, if debilitating missense mutations are found within the *LdNT1* ORFs of TUBA5 cells, these results would validate a forward genetic approach to identify residues in the LdNT1 transporters that are critical for function. It should then be possible to isolate and characterize numerous independent adenosine/pyrimidine nucleoside transport-deficient cell lines containing different missense mutations within the *LdNT1* locus.

The functional rescue strategy employed here for restoration of tubercidin sensitivity/nucleoside transport activity and another rescue protocol previously accomplished to isolate genes required for assembly of the glycosome in *L. donovani* (24) both involved screening >1,000 transfectants for restoration of a wild-type phenotype in a mutant background. In the present study, ≈1,250 cosmids would constitute one genome equivalent (22). The success of these two large scale screens underscores the utility of this genetic strategy and suggests that similar screens could be designed to identify genes involved in a variety of biological processes among the kinetoplastid protozoa.

Along with a gene for a related transporter from *Toxoplasma gondii* (34), *LdNT1.1* and *LdNT1.2* represent the first genes for nucleoside transporters to be cloned from any parasite. The central role of these permeases in the uptake and salvage of purines by these purine requiring parasites, and their potential involvement in the development of drug resistance (5, 35), underscores the importance of studying these intriguing membrane proteins at the molecular level. The cloned *LdNT1.1* and *LdNT1.2* genes can now serve as cornerstones for investigating the structure, function and pharmacological importance of these transporters.

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