Purine transport into the protozoan parasite *Toxoplasma gondii* plays an indispensable nutritional function for this pathogen. To facilitate genetic and biochemical characterization of the adenosine transporter of the parasite, *T. gondii* tachyzoites were transected with an insertional mutagenesis vector, and clonal mutants were selected for resistance to the cytotoxic adenosine analog adenine arabinoside (Ara-A). Whereas some Ara-A-resistant clones exhibited disruption of the adenosine kinase (AK) locus, others displayed normal AK activity, suggesting that a second locus had been tagged by the insertional mutagenesis plasmid. These Ara-A AK+ mutants displayed reduced adenosine uptake capability, implying a defect in adenosine transport. Sequences flanking the transgene integration point in one mutant were rescued from a genomic library of *Ara-A* AK+ DNA, and Southern blot analysis revealed that all *Ara-A* AK+ mutants were disrupted at the same locus. Probes derived from this locus, designated *TgAT*, were employed to isolate genomic and cDNA clones from wild-type libraries. Conceptual translation of the *TgAT* cDNA open reading frame predicts a 462 amino acid protein containing 11 transmembrane domains, a primary structure and membrane topology similar to members of the mammalian equilibrative nucleoside transporter family. Expression of *TgAT* cRNA in *Xenopus laevis* oocytes increased adenosine uptake capacity in a saturable manner, with an apparent *Km* value of 114 μM. Uptake was inhibited by various nucleosides, nucleoside analogs, hypoxanthine, guanine, and dipyridamole. The combination of genetic and biochemical studies demonstrates that *TgAT* is the sole functional adenosine transporter in *T. gondii* and a rational target for therapeutic intervention.

\* This work was supported by National Institutes of Health Grants P30-AI-27767 (to C. M. W.), AI-31808 (to D. S. and B. U.), and AI-29848, AI-39550, and AI-42975 (to M. H. K. and F. N. M. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM} and EBI Data Bank with accession number(s) AF013137 (cDNA) and AF061580 (genomic DNA).

§ Present address: The Vanderbilt Cancer Center, Nashville, TN 37211.

** Burroughs Wellcome Fund Scholars in Molecular Parasitology.

§§ To whom correspondence should be addressed: Division of Geographic Medicine, University of Alabama at Birmingham, 845 19th St. South BBRB 203, Birmingham, Alabama 35294-2170. Tel.: 205-975-7608; Fax: 205-933-5671; E-mail: cwilson@uab.edu.

\*\* The abbreviations used are: AK, adenosine kinase; HGXPR, hypoxanthine-guanine-xanthine phosphoribosyltransferase; Ara-A, arabinosyl adenine; Ara-As, Ara-A-resistant; CBSS, Carter basal salt solution; kb, kilobase; DPA, dipyridamole; NBMPR, 4-nitrobenezylthioinosine; h, human; r, rat.

**The Journal of Biological Chemistry**

Vol. 274, No. 49, Issue of December 3, pp. 35255–35261, 1999

Printed in U.S.A.
The adenosine analog abinoxalin adenine (Ara-A) is transported and metabolized by wild-type T. gondii, leading to death of the parasite. Resistance to Ara-A has been employed to isolate T. gondii mutants deficient in AK activity (13), and insertional mutagenesis was used to genetically tag and clone the AK gene (14). Several Ara-A-resistant (Ara-A') insertional mutants exhibited normal AK activity, however, and these mutants define a second locus encoding the T. gondii adenosine transporter, as described below. Flanking sequences from this locus were employed to isolate cDNA clones predicted to encode a polypeptide with significant similarity to eukaryotic equilibrative nucleoside transporters, and microinjection into Xenopus laevis oocytes confers adenosine transporter activity exhibiting appropriate kinetic properties and substrate specificity. This TgAT cDNA provides a molecular cornerstone for biochemical and pharmacological studies on nucleoside transporter function in T. gondii and other important pathogens.

EXPERIMENTAL PROCEDURES

Parasite Reagents and General Procedures—All experiments were performed using cultured tachyzoites of a clonal isolate of the virulent T. gondii RH strain. Parthenogenetic stages for routine cultivation of parasites and host cells in vitro have been described previously (8).

Molecular Techniques—Isolation of high molecular weight genomic DNA and procedures for library screening, Southern hybridization, and Northern hybridization (on 1% formaldehyde-agarose gels) were performed according to standard protocols (15). Genomic libraries for the RH wild-type and RD3 mutant (see below) strains were constructed in DASH-II (Stratagene, La Jolla CA) and EMBL3 (Promega, Madison WI), respectively, according to procedures recommended by the manufacturers. A T. gondii RH ZAP-II cDNA library was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Ogden Bioservices Corp, Rockville MD; catalog number 1896). Genomic DNA for Southern blot analysis was prepared using QiAamp Tissue kits (Qiagen, Valencia, CA), and plasmid DNA was isolated using the plasmid Maxi Kit (Qiagen). Poly(A)+ RNA was obtained from parasites using the Poly(A) Pure mRNA purification system (Ambion, Austin TX). 32P-Labeled DNA probes were radiolabeled using the Prime-a-Gene Labeling or DNA 5' End-Labeling system (Promega).

Insertional Mutagenesis—Two sets of insertional mutagenesis experiments were performed using plasmids containing a cDNA-derivative allele of T. gondii dihydropicolinate reductase-histidine synthase that confers resistance to pyrimethamine (16). Ara-A' mutants designated with the "RD" prefix were isolated following transfection with plasmid pTgDHFR-TSc3/2M32 (Ogden Bioservices catalog number 2854), and "BS" series mutants were generated using plasmid pTgDHFR-TSc3ABP, which contains additional restriction sites designed to facilitate plasmid rescue (10). As described previously (10), 2 × 10^7 parasites were electroporated with 50 μg of plasmid and 2 μg of HindIII-linearized plasmid, inoculated onto a monolayer of human foreskin fibroblasts, and selected with 1 μM pyrimethamine to enrich for transfection events. After 2–3 days, emerging parasites were inoculated onto a fresh host cell monolayer in medium containing both pyrimethamine and 110 μM Ara-A. Parasites were passaged 2–3 times in Ara-A-containing medium and cloned by limiting dilution (8–10).

Identification and Analysis of Genomic and cDNA Clones—Recombinant genomic clones containing the locus tagged by the insertional mutagenesis plasmid in parasite clone RD3 were isolated by screening the RD3EMBL3 library with pBluescript II KS (+) at high stringency. Two overlapping clones containing pBluescript DNA were identified and designated AT7-1 and AT8-1. The wild-type RH strain genomic library was screened using a ∼3-kb SalI fragment derived from AT7-1 (pSal3) and a ∼4-kb SalI fragment from AT8-1 (pSal4) as hybridization probes, yielding genomic clones AT7-1 and AT8-1 (see Fig. 2). Screening of the T. gondii ZAP-II cDNA library with a 4.3-kb BamHI genomic fragment from AT7-1 (pB4.3, Fig. 2) yielded five cDNA clones, which were sequenced using dye-primer reactions (Applied Biosystems model 373 sequencer).

Analysis of DNA and protein sequences was carried out using DNA-SIS 2.0 (Hitachi Software Engineering), and the membrane topographical model of TgAT was predicted according to published methods (17, 18). Similarity searches were conducted using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information, and pairwise (19) and multiple sequence alignments (20) were performed and analyzed by standard algorithms.

Adenosine Transporter from T. gondii—A modified oil-stop technique was used to measure adenosine uptake into extracellular T. gondii tachyzoites (7). Purified parasites were resuspended in RPMI-derived Carter basal salt solution (CBSS) (21) at a concentration of 2 × 10^7/ml. Uptake was initiated by addition of 100 μl of parasite suspension into a microcentrifuge tube containing 100 μl of CBSS and 20 μCi/ml [2,8-3H]adenosine (Amersham Pharmacia Biotech), overlaying an inert oil mixture (84% 550 silicon oil + 16% light paraffin oil). Exposure to exogenous radiolabel was terminated by centrifugation of parasites through the oil layer at 13,000 × g for 30 s in a microcentrifuge. The aqueous layer was then aspirated and the surface of the oil washed twice with CBSS. After aspiration of the final CBSS wash and most of the oil, the cell pellet was solubilized in 100 μl of Trition X-100, and radiolabel was quantitated by liquid scintillation spectrometry.

Expression of TgAT in X. laevis Oocytes—Oocytes were dissected from ovariian fragments of X. laevis, defolliculated for 1 h at ambient temperature using 1 mg/ml collagenase (Sigma), and were maintained at 18 °C in frog Ringer's solution supplemented with 2.5 mM pyruvate, 0.5 mM theophylline, and 50 μg/ml gentamycin (Life Technologies, Inc.). To optimize expression in oocytes, the TgAT cDNA was subcloned into pOG1 (22) as a BamHI-KpnI fragment, thereby providing 5' - and 3'-flanking regions derived from Xenopus globin mRNA. This vector was linearized with NotI and transcribed in the presence of the Cap analog m7GpppG using T7 polymerase as described (23). 10–50 ng of transcribed cRNA was injected into stage V–VI oocytes 1 day after harvest. Transfected oocytes and injected oocytes were incubated for 2 days in supplemented frog Ringer's buffer containing 3% horse serum, followed by incubation for 1 additional day in buffer without serum to allow expression of TgAT activity. Control oocytes were injected with equivalent volumes of water.

Adenosine uptake was measured in un-supplemented frog's Ringer buffer using various concentrations of [2,8-3H]adenosine (48 Ci/mmol, Moravek Biochemicals, Brea, CA) as indicated. Competition experiments with unlabeled purines or inhibitors of mammalian nucleoside transport were conducted in the same buffer containing 10 μM [2,8-3H]adenosine (2.4 Ci/mmol) and either 500 μM unlabeled purine or pyridoxamine or 1 μM dipyridamole (DPA) or 4-nitrobenzyloxynucleosine (NBMPR), both of which are potent inhibitors of mammalian nucleoside transport activity (24). To ascertain whether adenosine transport mediated by TgAT is Na+-dependent, oocytes injected with either TgAT mRNA or H2O were washed once in a Na+-free buffer consisting of 10 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES-Tris, pH 7.5, followed by 30 min incubation at room temperature in the same buffer containing 10 μM [2,8-3H]adenosine (2.4 Ci/mmol, Moravek Biochemicals). All uptake assays were terminated by removing the oocytes from radiolabeled buffer and washing three times with ice-cold buffer. Each oocyte was dissolved in 0.25 ml of 5% SDS, and oocyte-associated [3H]adenosine incorporation was quantitated by liquid scintillation spectrometry.

RESULTS

Isolation and Biochemical Characterization of Ara-A' Mutants—Fiveteen clonal isolates of Ara-A' T. gondii tachyzoites were isolated following insertional mutagenesis using plasmids that integrate at random into the parasite genome by non-homologous recombination (8, 10). The frequency of integration events employing these plasmids has proven sufficient to tag single copy loci in haploid T. gondii tachyzoites (11, 12, 14). Eleven of the 15 Ara-A' clones exhibit very low or undetectable AK activity, and at least five of these were shown to be tagged at the AK locus (14). The four remaining clones (RD3, BS6, BS9, and BS16) express wild-type levels of AK activity, and further analysis reveals that adenosine transport is impaired in at least three of these, as shown in Fig. 1 (clone BS16 has not been tested for transport capability).

Identification of the Genomic Locus Tagged by Insertional Mutagenesis—In order to isolate and identify the tagged locus in an Ara-A' AK+ mutant, genomic libraries were constructed from Ara-A' clone RD3, as transgene integration in clone RD3 involved significant reorganization of the vector DNA, precluding direct plasmid rescue (11) and complicating inverse-polymerase chain reaction strategies (10) for isolating the TgAT locus. Because RD3 was the only Ara-A' AK+ clone available at the time these studies were initiated, prior to the isolation of

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
Adenosine Transporter from T. gondii

Wild-type Parasites and Ara-A Mutants Deficient in AT Activity—To investigate the genomic organization of the TgAT locus in other Ara-A clones, DNA from several clonal Ara-A lines was digested with BamHI and EcoRI, blotted, and probed with AT7-1 subclone pB4.3 (Fig. 2). As shown in Fig. 5, all three of the Ara-A AK+-adenosine transport-deficient clones examined (RD3, BS6, and BS9) were tagged at the TgAT locus by the insertional mutagenesis plasmid, whereas TgAT was not disrupted in any of the four Ara-A AK–lines tested (BS4, BS12, BS15, and BS20).

Northern blot analysis reveals the presence of a single ~3.1-kb TgAT transcript in wild-type RH strain poly(A)+ RNA (Fig. 6), and this transcript was not observed in poly(A)+ RNA isolated from the Ara-A RD3 mutant. Faint bands of 2.5 and 1.6 kb were detected in mutant RD3 (Fig. 6, 2nd lane). These bands, which were not detected in wild-type parasites, also hybridize with pBluescript vector DNA (not shown) and probably represent truncated transcripts from the disrupted TgAT locus. Given the lack of transport activity in RD3 parasites (Fig. 1), it is unlikely that these aberrant transcripts are translated into functional protein.

Expression of TgAT in X. laevis Oocytes—Xenopus oocytes are highly efficient at translating native or synthetic mRNA after microinjection and normally express only modest levels of nucleoside transport activity. The oocyte expression system has therefore been useful for characterizing numerous mammalian nucleoside transporter genes (26, 27, 31, 32). As shown in Fig. 7, oocytes injected with 10 ng of TgAT transcript exhibited a significant increase in adenosine transport capability compared with oocytes injected with an equivalent volume of H2O. This stimulation does not require the presence of external Na+ ions, as equally strong expression of adenosine transport activity was observed in oocytes incubated in Na+-free medium. Kinetic analysis of transport activity in X. laevis oocytes indicated that TgAT-mediated adenosine transport followed Michaelis-Menten kinetics with an apparent Km of 114 ± 37 μM for adenosine, as determined by Hanes analysis (Fig. 8).

Competition experiments (Fig. 9) showed that transport of 10 μM [2,8-3H]adenosine was inhibited by a 50-fold excess of unlabeled adenosine, inosine, and guanosine (78, 64, and 48%, respectively) and also by the inosine analogs formycin B and allopurinol riboside (82 and 67%, respectively). The purine bases hypoxanthine and guanine suppressed incorporation of [2,8-3H]adenosine into oocytes by 37%, whereas adenine and pyrimidine nucleosides had little effect. Inhibitors of mammalian adenosine transport (24) were also evaluated, and DPA and NBMPR were found to inhibit adenosine uptake by 81 and 31%, respectively.

**DISCUSSION**

We have exploited insertional mutagenesis (analogous to transposon tagging in other genetic systems) to tag the locus encoding the TgAT adenosine transporter of T. gondii, and probes derived from sequences flanking one transgene insertion enabled the isolation of wild-type TgAT genomic and cDNA clones (Fig. 2). Insertional mutagenesis provides a generally applicable strategy for the isolation of any gene for which a strong negative selection is available, and this approach has previously been exploited to identify several purine and pyrimidine salvage enzymes in T. gondii (5, 33) as follows: uracil phosphoribosyltransferase, HXGPR, and AK (11, 12, 14). This report demonstrates the efficacy of such approaches for the isolation of transport mutants as well.

That the TgAT gene encodes a functional adenosine transporter is supported by several lines of evidence. First, disruption of the TgAT locus in Ara-A–insertional mutant RD3 (which contains only a single transgene) is associated with high level uptake impaired in AK+ insertional mutants. Wild-type (RH-strain) tachyzoites and Ara-A AK+ insertional mutants were incubated with [3H]adenosine as described under "Experimental Procedures." Incorporation was stopped at 1-min intervals, and initial rates of adenosine uptake were calculated by linear regression analysis over the first 5 min.

BS series clones), the tagged locus was sought by constructing an RD3 genomic library and probing for sequences unique to the insertional mutagenesis vector. Six genomic phage clones containing bacterial vector sequences (introduced as part of the insertional mutagenesis vector) were isolated and purified from the RD3 EMBL3 library. Southern blot analysis of these clones revealed two distinct groups with minimal overlap. A 3-kb SalI fragment (pSal3) and a 4-kb SalI fragment (pSal4) were subcloned from genomic clones AT7-1 and AT8-1 (see Fig. 2) and used to screen a RH strain DASH-II library in order to obtain wild-type genomic clones. Southern analysis of restriction digests indicates that two genomic clones, designated AT7-1 and AT8-1, encompass 4.3 kb of overlapping sequence (corresponding to pB4.3 in Fig. 2).

Isolation and Sequence Analysis of TgAT cDNA Clones—Screening of a TgAT cDNA library with genomic subclone pB4.3 (Fig. 2) yielded five cDNA clones up to 3.1 kb in length. Three of these clones span a 1386-base pair open reading frame predicted to encode a protein of 462 amino acids, designated TgAT (Fig. 3). This open reading frame is preceded by an in-frame termination codon 73–75 nucleotides 5' to the predicted initiation methionine codon. Comparison of cDNA and genomic sequences reveals a single 313-base pair intron, predicted to be flanked by sequences that match the genomic splice consensus (25).

The deduced amino acid sequence of TgAT exhibits significant similarity to several sequences in GenBank®, including human equilibrative nucleoside transporters hENT1 and hENT2 (26, 27), and the adenosine-pyrimidine nucleoside transporter family (31, 32) was detected, although significant sequence similarity to bacterial nucleoside transporters (29, 30) or members of the mammalian Na+-dependent nucleoside transporter family (31, 32) was not observed. Although several functionally uncharacterized open reading frames from Caenorhabditis elegans and Saccharomyces cerevisiae exhibit similarity to TgAT, hydropathy analysis predicts 11 transmembrane domains, separated into two clusters by a large hydrophilic loop connecting membrane spanning domains 6 and 7 (Figs. 3 and 4). This membrane topology is virtually identical to that proposed for hENT1 (Fig. 3), hENT2, and LdNT1 (26–28).

Analysis of TgAT Genomic Organization and Expression in ...
Ara-A resistance and a complete abrogation of adenosine transport (Fig. 1). The ability to isolate a transport-defective mutant after insertion of only a single transgene into the parasite genome provides genetic proof that all adenosine entry into the parasite is mediated by a single gene. Second, expression of TgAT cRNA in X. laevis oocytes increases adenosine transport activity by more than 2 orders of magnitude, and this induced transport is saturable, indicative of a facilitated carrier mechanism. Third, the deduced primary structure of TgAT sequence is similar to well characterized equilibrative nucleoside transporters from mammals (26, 27) and L. donovani (28), and the 11 membrane spanning domains predicted for TgAT are consistent with the predicted membrane topology of other equilibrative nucleoside transporters (26–28). Like other members of the equilibrative nucleoside transporter family, TgAT is not related to members of the mammalian Na\(^{+}\)-dependent concentrative nucleoside transporter family (21, 31, 32) or to prokaryotic nucleoside transporters (29, 30) in terms of either amino acid sequence or predicted membrane topology.

As TgAT and LdNT1 are evolutionarily distant from the mammalian homologs, the primary structures of these protozoan transporters should be particularly useful for the identification of key amino acids vital for transport function and/or ligand specificity. A multisequence alignment among TgAT and the human and leishmanial equilibrative nucleoside transporters reveals only 26 amino acids common to all four proteins (Fig. 3). Twenty two of the 26 universally conserved residues are found within putative membrane spanning domains, and 9 of these are located within transmembrane segments 7 and 8, suggesting their importance in ligand permeation or substrate recognition. Interestingly, chimeric constructs between the human (h) and rat (r) equilibrative nucleoside transporters hENT1 and rENT1 has implicated membrane spanning domains in the binding of DPA and other coronary vasodilator...
drugs, although this study implicated domains 3–6 (34). Of the 26 amino acids common to the 4 aligned nucleoside transporters, 8 were glycine, 6 were phenylalanine, and 4 were proline residues. Conserved aromatic residues have been shown to play important roles in mediating binding and recognition of ring substrates of the human glucose transporter GLUT1 (35–37), whereas a mutation in a conserved proline perturbed the glucose transport activity (38), presumably by reducing conformational flexibility. Only 4 of the conserved amino acids, an aspartate, arginine, and threonine, were charged or hydrophilic. It is worth noting that despite the enormous amino acid sequence divergence among eukaryotic equilibrative nucleoside transporters, the predicted membrane topologies are strikingly similar, including 11 membrane spanning domains and a long hydrophilic intracellular loop between putative domains 6 and 7 (Fig. 4). These comparisons suggest that higher order structure among nucleoside transporters is far more important for function than primary sequence.

Previous studies (7) identified a non-concentrative adenosine transporter activity in intact extracellular T. gondii tachyzoites. These workers reported that transport was inhibited by an 80-fold excess of either inosine, formycin B, or hypoxanthine but not by pyrimidine nucleosides, implying that the T. gondii adenosine transporter is purine-selective but recognizes a broad spectrum of purine nucleosides and nucleobases (7). Adenosine transport was also inhibited by DPA and (marginally) by NBMPR (7). Furthermore, the apparent $K_m$ of TgAT for adenosine determined in oocytes of 114 $\mu$M (see Fig. 8) was virtually identical to the apparent $K_m$ value of 120–150 $\mu$M obtained in intact parasites (7). Taken together, our genetic evidence that TgAT is the sole relevant transporter of adenosine in T. gondii and the similar substrate specificities, kinetic parameters, and inhibitor profiles of TgAT assayed in Xenopus oocytes (Fig. 9) argues that we have cloned the adenosine transporter previously defined by Schwab et al. (7). Adenosine is the preferred substrate for purine salvage by T. gondii (5), but this nucleoside is not particularly abundant in the host cell cytosol (<1 $\mu$M; Ref. 24). It is possible, however, that metabolic flux through adenosine in the host is sufficient to provide for parasite survival. Alternatively (or in addition), adenosine may be produced by parasite-mediated hydrolysis of host ATP pools (~5 mM). Intracellular T. gondii tachyzoites reside within a parasitophorous vacuole that contains high

FIG. 4. *Predicted membrane topology of TgAT*. TgAT membrane topology was predicted from standard algorithms (17, 18) and includes an intracellular NH$_2$ terminus, 11 transmembrane domains, and a large intracellular loop between domains 6 and 7. Open circles indicate hydrophobic residues; solid circles denote uncharged polar residues, and charged residues are indicated by + or −.

FIG. 5. Disruption of the TgAT locus in three independent Ara-A′ AK+ parasite mutants. Hybridization analysis was carried out on BamHI and EcoRI-digested genomic DNA from wild-type T. gondii parasites (RH), three Ara-A′ AK+ mutants (RD3, BS6, and BS9) and four Ara-A′ AK− mutants (BS4, BS12, BS15, and BS20). TgAT probe pB4.3 (see Fig. 2) reveals that all three of the Ara-A′ AK+ mutants exhibit disruptions at this locus, which is unaltered (identical to wild type) in other insertional mutants.

FIG. 6. Aberrant TgAT transcripts in mutant RD3. 2 $\mu$g of poly(A)$^+$ RNA from wild-type RH and Ara-A′ mutant RD3 parasites was separated on a 1% formaldehyde-agarose gel, and Northern blot analysis was performed according to standard procedures (15), using full-length TgAT cDNA as a probe. A 1-kb polymerase chain reaction product encompassing the T. gondii SAG1 coding sequence (42) was used as a control for mRNA loading and integrity. The 3.1-kb TgAT mRNA present in wild-type parasites is absent from mutant RD3, although two short, novel transcripts were detected after long exposure.
levels of nucleotide-degrading enzymes (39, 40). As this vacuole is freely permeable to small molecules (41), these nucleoside triphosphate hydrolases (combined with a plasma membrane 5'-nucleotidase) could produce adenosine for the parasite to transport. Of course, both of these hypotheses presume a plasma membrane location for the transporter, which remains to be demonstrated, and the absence of 9-nucleotidase activity casts some uncertainty about the role of host adenylate pools as a direct source of adenosine.2

Although inactivation of the TgAT locus eliminates virtually all adenosine transport, this genetic lesion is not fatal, indicating that the parasites remain able to transport other purine nucleosides and/or nucleobases. Although it is likely that failure to transport adenosine precludes access of exogenous purines to AK, HGXPRT provides an alternative route for purine salvage (4–6, 12). One other purine transport activity has been identified in T. gondii, an inosine translocator that is inhibited by excess formycin B and hypoxanthine but not by adenosine, adenine, uridine, or thymidine (7). The existence of multiple nucleoside transporter activities within a given parasite species is not unique to T. gondii, as a multiplicity of transporters has been distinguished both biochemically and genetically in L. donovani (43, 44), Trypanosoma cruzi (45), Trypanosoma brucei (46), Giardia (47–49), and Trichomonas vaginalis (50). Moreover, mammalian cells clearly express multiple different nucleoside transporters, both equilibrative (26, 27) and concentrative (21, 31, 32) in nature.

The substrate specificities and affinities for ligands among nucleoside transporters from T. gondii and animals are quite different. The equilibrative nucleoside transporters of mammals have broad specificity for purine and pyrimidine nucleosides and vary in both their ability to recognize purine bases and their sensitivity to inhibitors such as DPA, NBMPR, and dilazep (24). Recent studies in T. gondii indicated that NBMPR is phosphorylated by the AK of T. gondii and is selectively toxic to T. gondii-infected cells suggesting the uptake of NBMPR by T. gondii (42). Whether or not NBMPR is a substrate for TgAT remains to be determined. Members of the Na+-dependent concentrative nucleoside transporter family can be either purine- or pyrimidine-specific (32, 38, 51, 52) and are insensitive to the inhibitors of the equilibrative nucleoside transporter family. The molecular and evolutionary basis for this divergence is unknown, although a single serine residue has been implicated in substrate discrimination among members of the Na+-dependent nucleoside transporter family (53).

Whether adenosine transport will ultimately prove useful as a therapeutic target is presently unclear. Purine salvage is essential for parasite survival, but adenosine transport-deficient T. gondii are clearly viable, suggesting that inhibition of purine transport as a therapeutic option would require targeting of multiple purine permeases. The logical conjecture is that other purine permeases function to funnel exogenous purines through HGXPRT, and T. gondii certainly express the metabolic machinery to convert purine nucleosides into HGXPRT

2 E. O. Ngo, H. M. Ngo, and K. A. Joiner, submitted for publication.

---

**FIG. 7.** TgAT-mediated adenosine transport. Uptake of 10 μM [2,8-3H]adenosine (2.4 Ci/mmol) by either TgAT- (10 ng/oocyte) (●, ■) or water (○, □)-injected oocytes was assayed in buffer containing either 96 mM sodium (●, ○) or 96 mM choline (■, □) over a 120-min period. Each time point is the mean ± S.D. of 5 individual oocytes.

**FIG. 8.** Kinetics of TgAT-mediated adenosine transport. Uptake of [2,8-3H]adenosine (0.096 Ci/mmol) by TgAT-injected oocytes (10 ng/oocyte) was determined over a 120-min period for a range of substrate concentrations (0.05–1 mM). The rate of adenosine uptake was determined at each substrate concentration by linear regression analysis. The results represented as a Hanes analysis are expressed as a Hanes analysis versus adenosine (nM)/rate of adenosine transport (pmol/min/oocyte).

**FIG. 9.** Substrate specificity and inhibition profile of TgAT-mediated adenosine transport. Uptake of 10 μM [2,8-3H]adenosine (2.4 Ci/mmol) by TgAT cRNA-injected oocytes (10 ng/oocyte) was measured over a 60-min time course in the presence or absence of an excess of purge or pyrimidine (500 μM) or 1 μM DPA or NBMPR as described under “Experimental Procedures.” Results are expressed as percent uptake compared with non-competed 10 μM [2,8-3H]adenosine uptake, and each value is the mean ± S.D. of 5 individual oocytes.
Adenosine Transporter from *T. gondii*

substrates (4–6, 12). It should be possible to identify and characterize these transporters using insertional mutagenesis methods similar to those employed to clone the TgAT locus. Further studies on TgAT and other transporters are likely to provide insight into the mechanisms of purine transport and salvage by *T. gondii* and facilitate the assessment of purine transporters and salvage enzymes as valid chemotherapeutic targets for the treatment of toxoplasmosis.

Acknowledgments—We thank Drs. Dale Benos, Catherine Fuller, Honglong Ji, and Navid Madani for valuable discussions regarding *Xenopus* oocyte experiments and Christie Browne for excellent assistance with oocyte microinjection.

REFERENCES

1. Luft, B. J., and Remington, J. S. (1988) *J. Infect. Dis.* 157, 1–6
2. Roos, D. S., Sullivan, W. J., Striepen, B., Bohne, W., and Donald, R. G. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7770–7776
3. Berens, R. L., Krug, E. C., and Marr, J. J. (1995) *Biochemistry and Molecular Biology of Parasites* (Marr, J. J. and Muller, M., eds) pp. 89–117, Academic Press Ltd., London
4. Schwartman, J. D., and Pfefferkorn, E. R. (1982) *Exp. Parasitol.* 57, 76–86
5. Krug, E. C., Marr, J. J., and Berens, R. L. (1989) *J. Biol. Chem.* 264, 10601–10607
6. Pfefferkorn, E. R., and Pfefferkorn, L. C. (1977) *Exp. Parasitol.* 41, 95–104
7. Schwab, J. C., Alifi Alifi, M., Pizzorno, G., Handschumacher, R. E., and Joiner, K. A. (1995) *Mol. Biochem. Parasitol.* 70, 59–69
8. Ros, D. S., Donald, R. G. K., Morrisette, N. S., and Moulton, A. L. C. (1994) *Methods Cell Biol.* 45, 27–63
9. Boothyroyd, J. C., Kim, K., Pfefferkorn, E. R., Sibley, L. D., and Soldati, D. (1995) *Methods Mol. Genet.* 6, 1–29
10. Ross, D. S., Sullivan, W. J., Striepen, B., Bohne, W., and Donald, R. G. K. (1997) *Methods* 13, 112–122
11. Donald, R. G. K., and Roos, D. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 5749–5753
12. Donald, R. G. K., Carter, D., Ullman, B., and Roos, D. S. (1996) *J. Biol. Chem.* 271, 14010–14019
13. Pfefferkorn, E. R., and Pfefferkorn, L. C. (1976) *J. Biol. Chem.* 251, 993–999
14. Sullivan, W. J., Chiang, C. W., Wilson, C. M., Donald, R. G. K., Naguib, F. N. M., el Kouni, M. H., and Roos, D. S. (1999) *Mol. Biochem. Parasitol.* 103, 1–14
15. Ramakrshna, S., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Donald, R. G. K., and Roos, D. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 11703–11707
17. von Heijne, G. (1992) *J. Mol. Biol.* 225, 487–494
18. Hofmann, K., and Stoffel, W. (1993) *Bioch. Chem. Hoppe-Seyler* 374, 166
19. Needleman, S. B., and Wunsch, C. D. (1970) *J. Mol. Biol.* 48, 443–453
20. Feng, D. F., and Doolittle, R. F. (1987) *J. Mol. Evol.* 25, 351–360
21. Fairlamb, A. H., Carter, N. S., Cunningham, M., and Smith, K. (1992) *Mol. Biochem. Parasitol.* 53, 215–222
22. Krieg, P. A., and Melton, D. A. (1984) *Nucleic Acids Res.* 12, 7657–7670
23. Seyfang, A., Kavagna, M. P., and Landfear, S. M. (1997) *J. Biol. Chem.* 272, 24210–24215
24. Plagemann, P. G. W., Wohlhueter, R. M., and Woffendin, C. (1988) *Biochim. Biophys. Acta* 947, 405–443
25. Roos, D. S. (1993) *J. Biol. Chem.* 268, 6369–6380
26. Giachino, M., Beaumont, N., Yao, S. Y. M., Sundaram, M., Boumah, C. E., Davies, A., Kwong, F. Y. P., Coe, I., Cass, C. E., Young, J. D., and Baldwin, S. A. (1997) *Nat. Med.* 3, 89–93
27. Yao, S. Y. M., Ng, A. M. L., Muzyka, W. R., Griffiths, M., Cass, C. E., Baldwin, S. A., and Young, J. D. (1997) *J. Biol. Chem.* 272, 28423–28430
28. Vasudevan, G., Carter, N. S., Drew, M. E., Beverley, S. M., Sanchez, M. A., Seyfang, A., Ullman, B., and Landfear, S. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 7770–7776
29. Westphal, K., Jensen, N., and Munch-Petersen, A. (1987) *Eur. J. Biochem.* 165, 385–391
The Adenosine Transporter of *Toxoplasma gondii*: IDENTIFICATION BY INSERTIONAL MUTAGENESIS, CLONING, AND RECOMBINANT EXPRESSION

Chi-Wu Chiang, Nicola Carter, William J. Sullivan, Jr., Robert G. K. Donald, David S. Roos, Fardos N. M. Naguib, Mahmoud H. el Kouni, Buddy Ullman and Craig M. Wilson

*J. Biol. Chem.* 1999, 274:35255-35261.
doi: 10.1074/jbc.274.49.35255

Access the most updated version of this article at [http://www.jbc.org/content/274/49/35255](http://www.jbc.org/content/274/49/35255)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 21 of which can be accessed free at [http://www.jbc.org/content/274/49/35255.full.html#ref-list-1](http://www.jbc.org/content/274/49/35255.full.html#ref-list-1)