JGOFS Planning and Coordination Office, Woods Hole, MA, 1998), pp. 247–253.
17. Our in situ measurements of spectral downwelling irradiance in the APFZ indicated that the 0.1% level of surface irradiance at 490 nm is, on average, 94 m. To a first approximation, we assume the 100 m depth delimits the upper layer where most organic carbon production by photosynthesis occurs. From vertical profiles of POC concentration, we established the relation between the water column POC integrated from the surface to 100 m depth (in milligrams per square meter) and the surface concentration of POC (in milligrams per cubic meter), which is: POC (water column–integrated) = 53.36 (±3.83) · POC (surface concentration) + 2341.41 (±361.56). The standard errors of the regression coefficients are given in parentheses, the squared correlation coefficient is 0.894, and the number of observations is 25.
18. The inherent optical properties (IOPs) are those properties that depend only on the medium, and not on the geometric (directional) structure of the ambient light field within the medium. In the ocean, the IOPs (which include the backscattering coefficient b_b) depend on the concentration and composition of optically significant constituents of seawater. The apparent optical properties (AOPs) are those properties that depend both on the medium (the IOPs) and on the geometric structure of the ambient light field, and that display enough regular features and stability to be useful descriptors of the medium [R. W. Preisendorfer, *Union Geod. Geophys. Inst. Monogr.* **10**, 11 (1961); (*3*)].
19. We thank J. Wieland, B. Bichnevicius, the technical staff of Antarctic Support Associates, and the officers and crew of the R/V *Nathaniel B. Palmer* and R/V *Roger Revelle*. We also thank the SeaWiFS Project and the NASA Distributed Active Archive Center at the Goddard Space Flight Center for the production and distribution of the SeaWiFS data. This research was supported by NASA and NSF as part of the U.S. JGOFS Antarctic Environment Southern Ocean Process Study.

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A Nucleoside Transporter from *Trypanosoma brucei* Involved in Drug Resistance

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Drug resistance of pathogens is an increasing problem whose underlying mechanisms are not fully understood. Cellular uptake of the major drugs against *Trypanosoma brucei* spp., the causative agents of sleeping sickness, is thought to occur through an unusual, so far unidentified adenosine transporter. *Saccharomyces cerevisiae* was used in a functional screen to clone a gene (*TbAT1*) from *Trypanosoma brucei brucei* that encodes a nucleoside transporter. When expressed in yeast, *TbAT1* enabled adenosine uptake and conferred susceptibility to melaminophenyl arsenicals. Drug-resistant trypanosomes harbor a defective *TbAT1* variant. The molecular identification of the entry route of trypanocides opens the way to approaches for diagnosis and treatment of drug-resistant sleeping sickness.

Reduced drug uptake has emerged as a common characteristic of drug-resistant trypanosomes [(*1*); reviewed in (*2*)], rendering the molecular identification of drug transport systems crucial for the understanding of the underlying resistance mechanisms. The main clinical trypanocides are the melaminophenyl arsenical melarsoprol and diamidines. As cellular uptake of these agents has been suggested to occur through a transport system specific for adenosine and adenine [(*3*–*5*); reviewed in (*6*)], we decided to functionally clone the trypanosomal gene or genes encoding an adenosine transporter or transporters. We took advantage of the fact that the yeast *Saccharomyces cerevisiae* do not take up exogenous adenosine and cannot use it as a purine source (Fig. 1, A and B). Yeast cells defective in purine biogenesis (*ade2*) (*7*) were transformed with a *Trypanosoma brucei brucei* bloodstream form cDNA expression library (*8*) and selected for growth in media containing adenosine as sole purine source. Library plasmids conferring the ability to proliferate were isolated (*9*) and found to encode a putative transporter, designated TbAT1. When expressed in yeast, *TbAT1* enabled growth on adenosine as sole purine source (Fig. 1A) and cellular uptake of adenosine (Fig. 1B). Adenosine transport was saturable (Fig. 1C) and conformed to Michaelis-Menten kinetics with an apparent Michaelis constant (K_m) of 2.2 μM (*10*).

Trypanosoma brucei brucei salvage adenosine from their mammalian hosts through two high-affinity transport activities, P1 and P2, that differ in substrate specificity. P1 is specific for adenosine and inosine, whereas P2 transports adenosine, adenine, melaminophenyl arsenicals, and diamidines (*3*, *4*). To determine whether *TbAT1* encodes the P1 or P2 activity, we assessed substrate specificity by the ability of potential substrates to inhibit TbAT1-mediated adenosine transport in yeast (*11*). Adenine caused a strong reduction, whereas inosine, hypoxanthine, guanosine, guanine, uridine, and uracil had no effect (Fig. 2A). Moreover, radioactively labeled inosine was not taken up, and neither inosine nor guanosine could support growth of *ade2* yeast expressing *TbAT1* (*12*). Among the trypanocides tested for inhibition of adenosine transport, the melaminophenyl arsenicals (melarsoprol and melarsen oxide) and isometamidium (a phenanthridine used in veterinary medicine) were most effective (Fig. 2B), suggesting that these drugs are TbAT1 substrates. The experimental compound tubercidin (7-deazaadenosine) produced a smaller but substantial reduction. The diamidines (pentamidine and diminazene aceturate) had no substantial effect. Thus, the apparent substrate specificity of TbAT1 closely matches that of the reported P2 transport activity (*3*–*5*), except for the insensitivity to diamidines. TbAT1 expressed in yeast may lack a trypanosomal cofactor or modification required for diamidine recognition. Future studies of TbAT1 function in genetically engineered trypanosomes will elucidate the role of this transporter in diamidine uptake.

As labeled melaminophenyl arsenicals are unavailable, we could not directly measure transport of these drugs by TbAT1. Instead, we determined whether TbAT1 could mediate uptake of melarsen oxide into cells, measured as susceptibility to the drug (*13*). Expression of *TbAT1* in yeast rendered cell growth sensitive to melarsen oxide (Fig. 2C), suggesting that TbAT1 indeed transports this drug. As expected for competing substrates, the presence of adenosine or adenine in the media abrogated TbAT1-mediated melarsen toxicity (*12*).

Sequencing of the cDNA revealed a protein of 463 amino acids (Fig. 3A) with a predicted structure of 10 transmembrane α-helices, cytosolic NH_3- and CO_2-termini, and a large, negatively charged cytosolic loop between transmembrane domains 6 and 7 (Fig. 3B). Recently, two nucleoside transporter genes (*LdNT1.1* and *LdNT1.2*) have been cloned from the protozoan parasite *Leishmania donovani* (*14*). *LdNT1.1* and *LdNT1.2* are 99.5% identical and tandemly linked (*14*, *15*). In contrast, *TbAT1* appears to be a single-copy gene, as determined by Southern (DNA) blot analysis (*12*). The LdNT1 transporters belong to the ENT (equilibrative nucleoside transporter) family, feature 11 predicted transmembrane domains,

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and accept a broad range of substrates including pyrimidine nucleosides (*16*). LdNT1 and TbAT1 are of similar length and share 30% identical amino acid residues (*15*). Thus, TbAT1 may also be a member of the ENT family, albeit a distant one that shows restricted substrate specificity and, possibly, a distinct membrane topology.

Loss or alteration of the P2-type adenosine transporter has been proposed as a mechanism of resistance to melaminophenyl arsenicals (*3*) and diamidines (*4*, *5*) in trypanosomes. To investigate whether *TbAT1* is involved in drug resistance, we cloned and sequenced the genes from *T. brucei brucei* STIB 777S, a drug-sensitive clone (*17*), and STIB 777R, a melarsenoxide cysteamine–resistant clone derived from STIB 777S by subcurative treatment in mice (*18*). Particular care was taken to avoid the artificial introduction of mutations during cloning of the respective genes (*19*). *TbAT1* from STIB 777S was identical in sequence to the originally cloned gene. Sequencing of the *TbAT1* allele from STIB 777R (*TbAT1^r*) revealed 10 nucleotide differences, six of which manifest at the amino acid level (Fig. 3, A and B), resulting in the changes Leu^{71} → Val (L71V) and Leu^{380} → Pro (L380P) (transmembrane), Ala^{178} → Thr (A178T) and Gly^{181} → Glu (G181E) (extracellular), and Asp^{239} → Gly (D239G) and Asn^{286} → Ser (N286S) (cytosolic). Introduction of *TbAT1^r* into yeast did not enable usage or uptake of exogenous adenosine (Fig. 1, A and B) and did not confer sensitivity to melarsen oxide (Fig. 2C), suggesting that TbAT1^r cannot import adenosine and melaminophenyl arsenicals. In addition, yeast carrying *TbAT1^r* could not grow on other nucleosides as purine source (guanosine, inosine, xanthosine, and purine ribofuranoside) and did not take up radioactively labeled adenine, hypoxanthine, or inosine (*12*), suggesting that TbAT1^r may be a nonfunctional rather than an altered substrate-specificity variant. Not having an antibody against TbAT1, we cannot determine yet whether the amino acid changes in TbAT1^r affect transport activity or proper expression of the protein.

Early diagnosis of drug-resistant strains with a simple, sensitive assay would be of great benefit for successful chemotherapy. *TbAT1* and *TbAT1^r* are distinguishable by digestion with the restriction endonuclease Sfa NI. While the mutation causing A178T abrogates a Sfa NI site, the mutation underlying N286S creates one (Fig. 3C). Amplification of a fragment of the purine transporter gene by polymerase chain reaction (PCR) followed by Sfa NI digestion (*20*) may serve as a convenient means for rapid identification of TbAT1^r-type drug-resistant trypanosomes. A *T. brucei gambiense* isolate from a patient refractory to melarsoprol treatment exhibited the Sfa NI digestion pattern typical of *TbAT1^r* (Fig. 3C), suggesting that *TbAT1^r*-like alleles are present in the field. The presence of Thr^{178} and Ser^{286} was confirmed by direct sequencing of the gene from the *T. brucei gambiense* field isolate. Sequencing also showed that the *T. brucei gambiense* allele differs from both *TbAT1* and *TbAT1^r* by the absence of the trinucleotide repeat encoding Phe^{316}. This sequence difference rules out the possibility of a contamination with DNA from STIB 777R. Larger studies will be necessary to determine the prevalence of specific *TbAT1* alleles and their correlation with melarsoprol treatment failures.

Our findings support the idea that *TbAT1* encodes an adenosine transporter mediating uptake of, and thus susceptibility to, melaminophenyl arsenicals and that defects in TbAT1 contribute to resistance to these agents in *T. brucei* spp. The cloning of *TbAT1* opens prospects for the therapy of sleeping sickness. Current chemotherapy is unsatisfactory because of both the severe side effects of the currently used trypanocides and the considerable fraction of patients that fail to respond to treatment. Functional expression of *TbAT1* in yeast provides a tool for the development of drug derivatives with an increased tropism to the parasite (*21*) and possibly a larger therapeutic window. Iden-

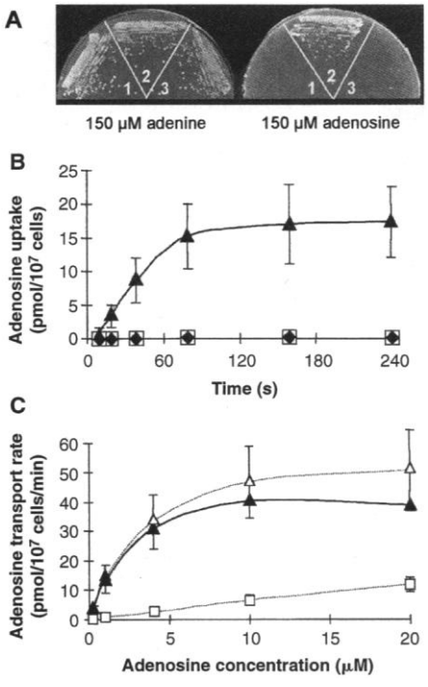

Fig. 1. Adenosine utilization (**A**), uptake (**B**), and transport (**C**) mediated by TbAT1 in *S. cerevisiae*. (A) TbAT1 permits utilization of exogenous adenosine. Yeast carrying a control (1), *TbAT1* (2), or the variant *TbAT1^r* (3) expression vector were grown on synthetic minimal media containing 150 μM adenine (left) or 150 μM adenosine (right). (B) TbAT1 mediates uptake of adenosine. The uptake of 1 μM exogenous [^{3}H]adenosine was measured at the indicated times in yeast carrying a control (□), *TbAT1* (▲), or *TbAT1^r* (◆) expression vector. TbAT1-mediated uptake was linear for 40 s; TbAT1^r showed no activity. (C) Adenosine transport by TbAT1 is saturable. Transport of [^{3}H]adenosine was measured in yeast carrying a control (□) or *TbAT1* (△) expression vector. Specific transport by TbAT1 (transport in TbAT1 expressing cells minus transport in control cells, ▲) conformed to a K_m of 2.2 ± 0.8 μM.

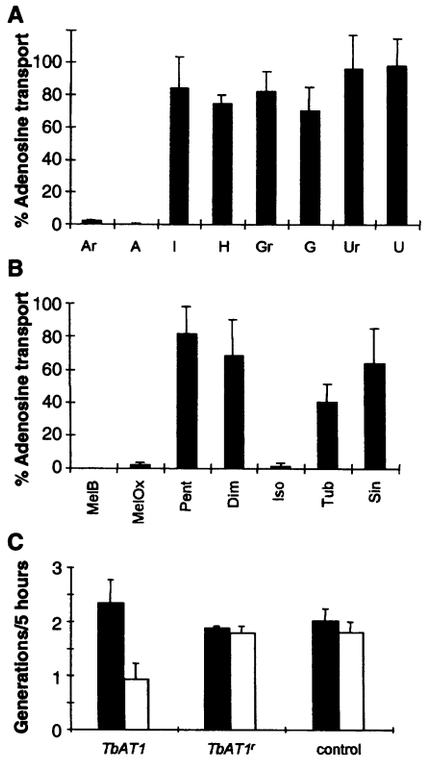

Fig. 2. Specificity of TbAT1-mediated transport in yeast. (**A**) Adenosine and adenine but not other physiological nucleosides and bases compete for adenosine transport. [^{3}H]Adenosine (1 μM) transport was measured in yeast expressing *TbAT1*, in the presence of the indicated compounds (Ar, adenosine; A, adenine; I, inosine; H, hypoxanthine; Gr, guanosine; G, guanine; Ur, uridine; U, uracil; all at 100 μM). [^{3}H]Adenosine transport in the presence of the potential substrates is presented as the percentage of transport in the absence of competitive substrate. (**B**) Melaminophenyl arsenicals and isometamidium inhibit adenosine transport. [^{3}H]Adenosine (1 μM) uptake was measured in yeast expressing *TbAT1*, in the presence of the indicated drugs (MelB, melarsoprol; MelOx, melarsen oxide; Pent, pentamidine; Dim, diminazene aceturate; Iso, isometamidium; Tub, tubercidin; Sin, sinefungin; at 100 μM except for melarsoprol, which was added at 72 μM). (**C**) *TbAT1* expression in yeast renders the cells sensitive to melarsen oxide. Cells carrying *TbAT1*, *TbAT1^r*, or vector only (control) were grown in minimal media containing 1 mM hypoxanthine, either in the presence of 100 μM melarsen oxide (open bars) or without drug (solid bars). Cell growth was quantified as number of generations during 5 hours of incubation time.

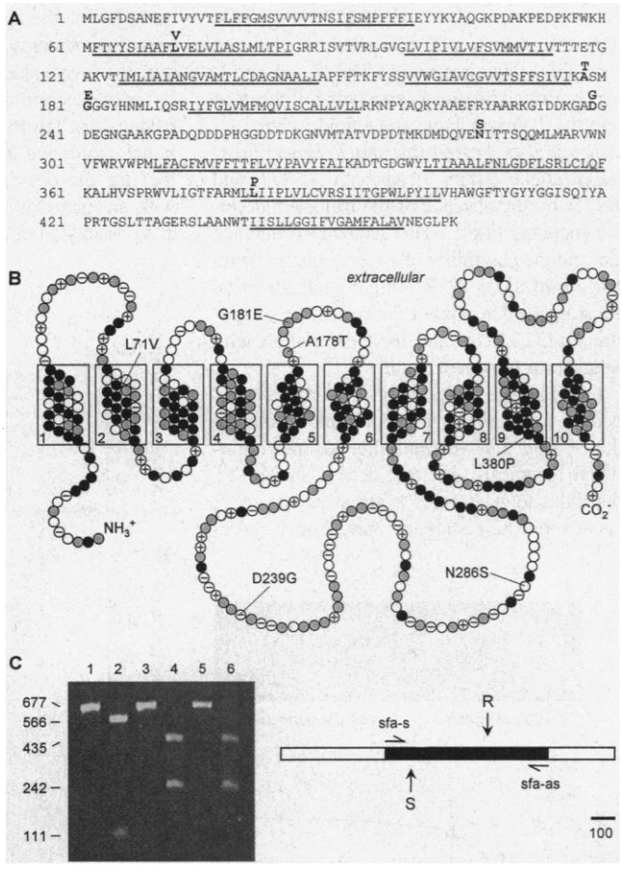

Fig. 3. Sequence and predicted structure of TbAT1 and TbAT1^r. **(A)** Deduced amino acid sequence; predicted transmembrane domains are underlined, and point mutations in TbAT1^r are indicated in bold above the TbAT1 sequence (*22*). **(B)** Predicted TbAT1 topography based on hydropathy and distribution of charges. Potential transmembrane domains are numbered. Hydrophobic residues (Val, Leu, Ile, Phe, and Trp) are represented in black and polar residues (Ser, Thr, Asn, and Gln) in white; positive (Lys, His, and Arg) and negative (Asp and Glu) charges are indicated by symbols. The point mutations in TbAT1^r are indicated. **(C)** Distinction between *TbAT1* alleles by restriction digest. PCR with the primers sfa-s and sfa-as amplifies 677 bp of the purine transporter gene. A diagnostic digest with Sfa NI produces fragment sizes of 566 and 111 bp in the case of *TbAT1* (from STIB 777S; lane 1, undigested; lane 2, digested) and 435 and 242 bp in the case of *TbAT1^r* (from STIB 777R; lane 3, undigested; lane 4; digested). STIB 777S and STIB 777R are homozygous for the respective mutations. The same digestion pattern as STIB 777R was exhibited by a *T. brucei gambiense* isolate from an Angolan patient refractory to melarsoprol treatment (lane 5, undigested; lane 6, digested). S, Sfa NI site present only in *TbAT1* (encoding Ala^{178}); R, Sfa NI site present only in *TbAT1^r* (encoding Ser^{286}).

tification and characterization of the remaining trypanosomal purine transporters (candidate genes homologous to *TbAT1* are present among trypanosome expressed sequence tags) will enable the rational design of drugs and drug combinations that either circumvent drug resistance by using different entry routes or compromise the ability of the parasite to salvage purines.

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9. Out of 10^6 transformants plated on synthetic minimal media lacking uracil, adenine, and methionine but containing 150 μM adenosine, five colonies grew. Library plasmids were isolated from the five isolates and found to harbor identical inserts: *TbAT1* starting at the Eco RI at position +25 from the first ATG (Fig. 3A), a truncated but functional form (*12*). The 5′ part of the *TbAT1* cDNA was obtained by reverse transcriptase PCR with total RNA of *T. brucei brucei* bloodstream forms, a primer complementary to the *TbAT1* coding sequence (GGGCGTAAGGTTCTTCCTTA), and a primer corresponding to the *T. brucei brucei* spliced leader (CGCTATTATTAGAACAGTTTCTGTAC).
10. K_m was determined by nonlinear regression with the Anemona program [A. Hernandez and M. T. Ruiz, *Bioinformatics* **14**, 227 (1998)].
11. Cells were grown to a density of 1.5×10^7 ml^{-1} and washed three times in incubation buffer [6.43 mM KH_2PO_4, 718 μM K_2HPO_4, 1.71 mM NaCl, 7.58 mM $(NH_4)_2SO_4$, 2.46 mM $MgCl_2$, 680 μM $CaCl_2$, and 111 mM glucose]. 10^7 cells in 25 μl of incubation buffer were mixed with an equal volume of [^{3}H]adenosine (final concentration = 1 μM) ± competitive substrate (final concentration = 100 μM), incubated for 20 s (except for the time course, Fig. 1A) at 30°C, and harvested by rapid centrifugation through 300 μl of a mixture of 9 parts dibuthylphtalate to 1 part paraffin in 400-μl microfuge tubes (Semadeni, Ostermundigen, Switzerland). Immediately after centrifugation, the tubes were frozen in liquid nitrogen. Pellets were cut off, equilibrated in 3 ml of Ecosint A (National Diagnostics, Atlanta, GA) for 72 hours, and counted in a liquid scintillation counter (Kontron Instruments AG, Zurich, Switzerland).
12. P. Mäser and R. Kaminsky, unpublished data.
13. Sensitivity to melarsen oxide was assessed at 30°C in liquid minimal media lacking uracil and methionine, with 1 mM hypoxanthine as sole purine source. Hypoxanthine saturated the yeast endogenous purine permease without affecting TbAT1 function. Melarsen oxide (100 μM) or carrier (dimethyl sulfoxide) alone was added to triplicate cultures. The high concentration of melarsen oxide required for the toxic effect is not surprising given the differences in physiology between *S. cerevisiae* and *T. brucei*. The action of melaminophenyl arsenicals is thought to be mediated by conjugation with trypanothione, a biochemical peculiarity of trypanosomatids [A. H. Fairlamb *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2607 (1989)].
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17. *Trypanosoma brucei brucei* STIB 777S was originally referred to as STIB 777-AE and is a cloned derivative of KETRI 1957 [W. C. Gibson and J. K. Gashumba, *Trans. R. Soc. Trop. Med. Hyg.* **77**, 114 (1983)].
18. *Trypanosoma brucei brucei* STIB 777R is a clone from STIB 777-AE-MCR41, a population derived from selection of STIB 777S for resistance to melarsenoxide cysteamine [H. Pospichal *et al.*, *Acta Trop.* **58**, 187 (1994)].
19. *TbAT1* and *TbAT1^r* were amplified by PCR (55°C annealing, 30 cycles) with the primers ant-s (GCCCGGATC CGCTATTATTAGAACAGTTTCTGTAC) and ant-as (GCCCCTCGAGCCGCATGGAGTAAGTCTGA) from genomic DNA of *T. brucei brucei* STIB 777S and STIB 777R, respectively. For each strain, the products of three independent PCR reactions were pooled, and the pooled DNA was sequenced directly, that is, before cloning. This procedure excluded PCR-introduced sequence errors because any such errors would have had to occur independently in more than one of the three PCR reactions and within the first round of DNA amplification to be detectable by direct sequencing. PCR products were cloned into the yeast expression vector p416-MET25 [D. Mumberg, R. Müller, M. Funk, *Nucleic Acids Res.* **22**, 5767 (1994)] by means of the incorporated Bam HI (ant1-s) and Xho I (ant1-as) sites. After transformation and testing of activity, plasmids were recovered from yeast and resequenced to confirm that the observed activity (or lack of activity) was attributed to the expected sequence.
20. A 677–base pair (bp) fragment of the purine transporter gene containing the sites of interest was amplified from genomic DNA by PCR (64°C annealing, 30 cycles) with the primers sfa-s (CGCCGCACTCATCGCCCCGTTT) and sfa-as (CCACCGCGGTGAGACGTGTA). The products of three independent PCR reactions were pooled. Two hundred nanograms of DNA was digested with Sfa NI (New England Biolabs) and analyzed on a 2% agarose gel. The results were reproduced at least three times for each strain, with different DNA preparations and in two different laboratories.
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22. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

23. We thank E. Matovu, M. Fasler, and C. Schmid for technical assistance; F. Dietrich for support with sequencing; H. Riezman for the provision of yeast strains; J. Keiser and C. Burri for provision of a melarsoprol-refractory *T. brucei gambiense* isolate from Angola; V. Eckert and R. Schwarz for the gift of the *T. brucei* cDNA library; and G. Schatz and U. Müller for comments on the manuscript. This work received financial support from the Stanley Thomas Johnson Foundation. P.M. received fellowships from the Emilia Guggenheim-Schnurr and the Roche Research Foundations; A.K. received funding from the Max Cloëtta Stiftung and the Swiss National Science Foundation. DNA and amino acid sequences of TbAT1 and TbAT1^r have been deposited in GenBank under accession numbers AF152369 and AF152370, respectively.

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