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Multiple Genetic Mechanisms Lead to Loss of Functional TbAT1 Expression in Drug-Resistant Trypanosomes[▽]

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The P2 aminopurine transporter, encoded by Tb*AT1* in African trypanosomes in the *Trypanosoma brucei* group, carries melaminophenyl arsenical and diamidine drugs into these parasites. Loss of this transporter contributes to drug resistance. We identified the genomic location of Tb*AT1* to be in the subtelomeric region of chromosome 5 and determined the status of the Tb*AT1* gene in two trypanosome lines selected for resistance to the melaminophenyl arsenical, melarsamine hydrochloride (Cymelarsan), and in a *Trypanosoma equiperdum* clone selected for resistance to the diamidine, diminazene aceturate. In the *Trypanosoma brucei gambiense* STIB 386 melarsamine hydrochloride-resistant line, Tb*AT1* is deleted, while in the *Trypanosoma brucei brucei* STIB 247 melarsamine hydrochloride-resistant and *T. equiperdum* diminazene-resistant lines, Tb*AT1* is present, but expression at the RNA level is no longer detectable. Further characterization of Tb*AT1* in *T. equiperdum* revealed that a loss of heterozygosity at the Tb*AT1* locus accompanied loss of expression and that P2-mediated uptake of [^{3}H]diminazene is lost in drug-resistant *T. equiperdum*. Adenine-inhibitable adenosine uptake is still detectable in a ΔTb*at1 T. b. brucei* mutant, although at a greatly reduced capacity compared to that of the wild type, indicating that an additional adenine-inhibitable adenosine permease, distinct from P2, is present in these cells.

Organisms of the genus *Trypanosoma* cause a range of infectious diseases, including human African trypanosomiasis (HAT), nagana in cattle, and dourine in equines (2), and are of major public health and economic importance in sub-Saharan Africa. Chemotherapy is used against many of the human- and animal-infective parasites. However, all drugs currently registered for use carry significant problems related to administration, toxicity, increasing incidence of treatment failure, and in the case of animal trypanosomiasis, drug resistance (1). Previous work has shown that defects in the P2 aminopurine transporter, encoded by the Tb*AT1* gene (the same gene in *Trypanosoma equiperdum* is referred to as Te*AT1* [*Trypanosoma equiperdum AT1*] in this article), are linked to drug resistance in *Trypanosoma brucei brucei* (11, 21), *Trypanosoma brucei gambiense* (22), and *Trypanosoma brucei rhodesiense* (28), as well as in *Trypanosoma evansi* (24, 32) and *T. equiperdum* (3), very close phylogenetic relatives of *T. brucei* (27). The P2 transporter has been shown to be capable of carrying both melaminophenyl arsenical (11) and diamidine (10, 14, 15, 18) classes of drug into African trypanosomes in the *T. brucei* group. Furthermore, a series of drug-resistant parasites from both the laboratory and the field have all been shown to be defective in P2-mediated transport using a novel fluorescence test (28), and a restriction fragment length polymorphism (RFLP)-based approach has been used to infer the presence of drug-resistant alleles in populations of human-infective parasites from Uganda (22). Previous work had indicated that two independent genetic mechanisms, namely, introduction of point mutations (21) and deletion of the Tb*AT1* gene (21, 18), are associated with the acquisition of drug resistance, which implies that several mechanisms can alter P2 transporter activity, causing drug resistance. A more complete understanding of these mechanisms will be essential to underpinning the development of better molecularly based diagnostic tools to monitor drug resistance.

Here we have used three isogenic pairs of resistant lines to investigate alterations within the Tb*AT1* gene that result in a loss of P2 expression and associated drug resistance. STIB 247 (Swiss Tropical Institute Basel 247), a *T. b. brucei* line, and STIB 386, a *T. b. gambiense* line, were selected independently for resistance to the melaminophenyl arsenical drug melarsamine hydrochloride (Cymelarsan). They are resistant to the maximum tolerated dose of melarsamine hydrochloride in animals (25), and the STIB 247 line is cross resistant to diminazene aceturate (26). The third line is a diminazene-resistant *T. equiperdum* line, PBR, derived from the parental sensitive line *T. equiperdum* P (33). A previous biochemical study investigated the role of the P2 transporter in the resistance of *T. equiperdum* PBR to trypanocidal drugs (3), finding that the overall rate of adenosine uptake was reduced. Inhibition studies indicated that the majority of the lost activity was due to a decrease in P2 activity. However, since some adenine-inhibitable adenosine uptake was still present, it was postulated that

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the change in P2 activity was due to a loss in the affinity for the drugs as substrates. This mechanism of drug resistance, where point mutations to a transporter have altered the substrate specificity and conferred drug resistance, has also been reported in *Leishmania donovani* (30).

With the identification of Tb*AT1* encoding P2 activity (21), it was important to determine what changes, if any, occurred to this gene during selection of diminazene resistance in *T. equiperdum* and melarsamine hydrochloride resistance in *T. brucei* and what effect these changes have had on transcript availability and transporter activity. The Tb*AT1* gene does not, however, appear in the published sequence (6) or version 4 of the *T. brucei* genome sequence, suggesting that a region of the sequence is incomplete. As this gene and possibly surrounding regions are important for our understanding of the mechanisms of resistance to the melaminophenyl arsenicals and diamidines, we determined the sequence of a bacterial artificial chromosome (BAC) clone covering the region of the genome surrounding the Tb*AT1* gene.

MATERIALS AND METHODS

Trypanosome strains and cultivation. The *T. b. brucei* STIB 247 cell line was originally isolated from a hartebeest in 1971 in the Serengeti National Park, Tanzania (17). The melarsamine hydrochloride-resistant 247Mr line was derived by serial passage in mice treated with subcurative doses of melarsamine hydrochloride. The resulting clone was found to be 130-fold less sensitive to melarsamine hydrochloride and 16-fold less sensitive to diminazene aceturate *in vivo* (25). The STIB 386 type 2 *T. b. gambiense* line was originally isolated from a man in Daloa in the Ivory Coast. The melarsamine hydrochloride-resistant 386Mr line was derived by serial passage in mice treated with subcurative doses of melarsamine hydrochloride. *T. b. gambiense* strain 386Mr is 20-fold less sensitive to melarsamine hydrochloride *in vivo* (25) and has been shown to have lost P2-mediated pentamidine transport (9). *T. equiperdum* BoTat 1 (Bordeaux trypanozoon antigenic type 1) P originated from a stock held at the Pasteur Institute, Paris. The diminazene aceturate (Berenil)-resistant line *T. equiperdum* PBR was derived from the parental line by serial passage through mice treated with subcurative doses of diminazene aceturate (33). This resistant line is 35.6-fold less sensitive to diminazene aceturate and 4-fold less sensitive to melarsamine hydrochloride *in vitro*. *In vivo*, the resistant line is insensitive to diminazene aceturate up to the maximum tolerated dose in Swiss mice and 4-fold less sensitive to melarsamine hydrochloride. The ΔTb*at1* (P2) null mutant clone was previously described and constructed by sequential replacement of Tb*AT1* with the neomycin and puromycin resistance markers in *T. b. brucei* 427 MiTat 1.2 (BS221). The knockout line was 4-fold less sensitive to melarsoprol and melarsen oxide and 18.6-fold less sensitive to diminazene aceturate (23).

Molecular biology. Bloodstream-form trypanosomes were harvested from culture. RNA was extracted using TRIzol reagent (Life Technologies) or a Qiagen RNeasy kit, according to the manufacturer's protocol. Genomic DNA was extracted from cells using a Qiagen DNeasy kit.

To determine the genomic status of Tb*AT1* in the various lines, the Tb*AT1* open reading frame (ORF) was amplified by PCR using *Taq* polymerase and the primers AT1F (5′ ATG CTC GGG TTT GAC TCA GC 3′) and AT1R (5′ CTA CTT GGG AAG CCC CTC AT 3′). Positive-control primers for amplifying a region of the triose phosphate isomerase (TIM) genes TIM-C and TIM-D were as previously described (9). Thermal cycling was carried out as follows: 1 cycle of 95°C for 2 min and 30 cycles of 95°C for 50 s, 58°C for 50 s, and 65°C for 2 min.

To determine the expression of Tb*AT1* in *T. equiperdum* PBR, Northern blots were prepared by standard methods (20), probed with the full-length Tb*AT1* open reading frame, and radioactively labeled with ^{32}P using the Prime-It II kit (Stratagene). Blots were washed 3× for 30 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65°C before exposure to autoradiography for 1 to 3 days. To detect the presence of transcripts in the 247mr line, first-strand cDNA was synthesized using the Omniscript reverse transcriptase PCR (RT-PCR) kit (Qiagen) with oligo(dT). Total RNA was DNase treated with Turbo DNase (Ambion) prior to cDNA synthesis, and cDNA was amplified by PCR, using *Taq* polymerase and primers AT1F (see above) and AT1seq2 (5′ CAT ACT TGT AGT ACT CGA TG). Primers TIM-E and TIM-F were used as controls (19). Thermal cycling was carried out as follows: 1 cycle of 95°C for 2 min and 35 cycles of 95°C for 50 s, 50°C for 50 s, and 65°C for 1 min.

To sequence the Tb*AT1* ORF from the *T. equiperdum* wild-type and PBR lines, PCR was conducted using the primers P2F (5′ CAT GCG CTT TGG TGG AGG) and P2R (5′ TTG GCG AAT CGG TGT ACG), both of which fall outside the open reading frame (ORF) of Tb*AT1*. All PCRs for sequencing were conducted in the presence of *Pfu* polymerase. PCR products, once purified, were cloned into the pGEM-T Easy vector and sequenced. Both strands were fully sequenced in all cases, using internal primers to give complete coverage of the ORF. For RFLP analysis of Tb*AT1* from the *T. equiperdum* wild-type and PBR lines, PCR was conducted with the primers AT1F and AT1R in the presence of *Taq* polymerase. The resultant PCR products were cleaned using the Qiagen PCR purification kit prior to restriction digestion with the enzymes described in the figure legends and text.

Rapid amplification of cDNA 3′ ends (3′ RACE) was carried out using the 5′/3′ RACE kit from Roche, according to manufacturer's instructions, and a gene-specific primer (5′-CTTCGTTGGCGCCATGTTCGC) for the first amplification reaction. The products of a nested PCR using the anchor primer and a second nested gene-specific primer (5′-GCTGTCAATGAGGGGCTTCCC) were ligated into pGEM-T Easy vector and sequenced with both T7 and SP6 oligos to provide double-stranded reads. The 3′ untranslated regions (3′ UTR) of *T. equiperdum* strains P and PBR were amplified using a primer (5′-GGAC CTTCACACGTTTAAACAAGCG) which anneals beyond the indicated polyadenylation site and a primer (5′-CAAATAGTAACTAGTGGCGAGTAGGC) which anneals at 100 bp into the 3′ UTR. The PCR products were purified, cloned into pGEM-T Easy, and sequenced as previously described.

Identification of the genomic position and surrounding sequence of Tb*AT1*. The genome sequence of the *Trypanosoma brucei* line TREU 927/4 (Trypanosomiasis Research Edinburgh University) (GPAL/KE/70/EATRO 1534) single variant antigen type (VAT) derivative GUTat 10.1 has been previously published (6). To identify a clone containing the Tb*AT1* gene from the *T. brucei* bacterial artificial chromosome (BAC) library RPCI93 for sequencing, the Tb*AT1* gene was amplified from a sheared genomic DNA clone (47M12.TF; EMBL accession number AQ947989). The PCR product was used as a probe to screen a high-density filter containing the gridded BAC library. The fingerprinting pattern of several positive BAC clones confirmed that these BACs are anchored in the same genomic region as BACs 25N21 and 29K2, which overlap in this region of sequence release 4 of the chromosome 5 BAC tile path. The presence of the Tb*AT1* gene was confirmed by PCR, and BAC 26D11 was chosen for sequencing by random sequencing of small insert libraries using the BigDye Terminator cycle sequencing kit from Applied Biosystems. Sequence readings were assembled using Phrap (P. Green, unpublished data; www.phrap.org). Manual base calling and finishing were carried out using Gap4 software (8). Gaps and low-quality regions of the sequence were resolved by primer walking and targeted PCRs. Additional PCRs (primers used: r1, 5′-GATTCGGTGGGAGGACTG, paired with r2, 5′-CTGGTAAGGGAAGCAATAAG; r3, 5′-GAAGGGGAAG CAGCTATG, paired with r4, 5′-CAAAGGCGTGTAAAAACTTC; r5, 5′-CGT CTGTCGTTGTCATTTC, paired with r6, 5′-CCAACTCTACGAGGAACG; r7, 5′-GGTTTTCTTCACCACATTTC, paired with r8, 5′-CAGGTCAACGGA GAAACAC; r9, 5′-CATGACGGTGACATACAATG, paired with r10, 5′-GCT GTTGTCGTTGTTGTGC) were designed to confirm consistency between the assembled BAC sequence and the genomic DNA.

The assembled contiguous BAC sequence was annotated using Artemis software (7). Protein-coding sequences were predicted, and putative functions were assigned as previously described (6). Pseudogenes were annotated using a Blastx search against the UniProtKB database (29). This identified sequences with translations of high sequence similarity to known proteins but which are interrupted by stop codons and/or frameshifts. The full annotation of BAC 26D11 can be viewed and searched via GeneDB (http://www.genedb.org/). Sequence comparisons (tBlastx) between version 4 of *T. brucei* chromosome 5 and the 26D11 BAC sequence were visualized using the Artemis Comparison Tool (12).

Transport assays. Parasites were separated from blood using a DEAE cellulose column. Uptake assays were performed using the centrifugation through oil procedure essentially as previously described (10, 14, 15, 18).

The uptake of 20 μM diminazene and 10 μM adenosine was measured over 10 min at 25°C. [^{3}H]diminazene and [^{3}H]adenosine were used as described in reference 15. Concentrations (1 mM) of adenine, inosine, and hypoxanthine, either singly or together, were combined in 100 μl of CBSS (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.55 mM CaCl, 0.4 mM $MgSO_4$, 5.6 mM Na_2HPO_4, 11.1 mM D-glucose; pH 7.4) containing radiolabeled substrate. A total of 100 μl of trypanosomes at 2×10^8 cells/ml was added to the tubes, which were then incubated for 30, 60, 120, 300, or 600 s at 25°C. The cells were then spun through the oil layer to form a pellet separated from the excess radioactivity in the

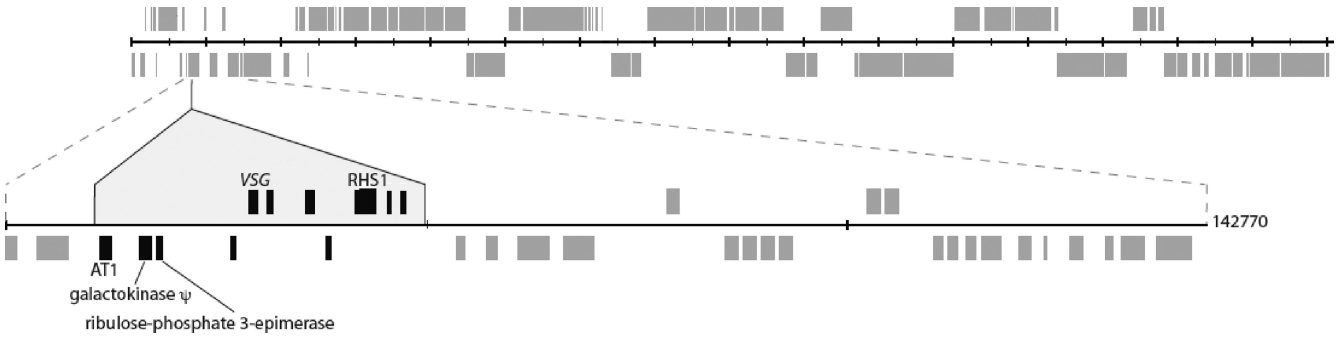

FIG. 1. Genomic location of Tb*AT1*. Alignment of BAC 26D11 against *T. brucei* chromosome 5. Diagrammatic representation of chromosome 5, with gray boxes indicating directional gene clusters. The region of BAC 26D11 homologous to chromosome 5 is shown schematically below. The region of sequence unique to 26D11 is shaded, genes shared by both the BAC and chromosome sequences are indicated in gray, while the novel genes, including Tb*AT1*, are colored in black. VSG, variant surface glycoprotein; RHS1, retrotransposon hot spot 1.

aqueous phase. The entire reaction tube was flash frozen in liquid nitrogen, the pellet was cut off into a scintillation vial, and the radioactivity was measured in a scintillation counter. Each experiment was conducted in triplicate.

Nucleotide sequence accession number. The sequence data from this study have been submitted to EMBL under accession number FM160648.

RESULTS

Genomic location of TbAT1. Sequence release 4 of the *Trypanosoma brucei* 927 genome lacks the Tb*AT1* gene, and so, clones from a genomic DNA BAC library were screened with a Tb*AT1*-specific probe. One of the hybridizing clones, clone 26D11, was chosen for sequencing. The assembled sequence totals 142,770 nucleotides and encompasses 34 genes, the majority of which are syntenic with the left end of the published chromosome 5 sequence between nucleotide positions 71500 and 161900 (Fig. 1). However, in addition to the common regions, the BAC carries a further 43,453 nucleotides of novel sequence (indicated by the shaded areas in Fig. 1). In addition to the Tb*AT1* protein, this segment encodes a number of other proteins, including six proteins of unknown function, and one of which (Tb927.5.292b) has a predicted signal sequence, suggestive of surface targeting. No functional clues were discerned from sequence similarity or protein domain search results for the other coding sequences. The remaining genes include a galactokinase pseudogene, a variant surface glycoprotein-related gene, and genes encoding a retrotransposon hot spot protein family member, as well as a putative ribulose-phosphate 3-epimerase. These data now provide a corrected version of the chromosome 5 sequence and confirm the location of the Tb*AT1* gene on this chromosome.

Three drug-resistant trypanosome lines are defective in the P2 transporter. *T. b. brucei* 247Mr and *T. b. gambiense* 386Mr, both selected for resistance to melarsamine hydrochloride, and *T. equiperdum* PBR, selected for resistance to diminazene, have previously been shown by a fluorescence-based assay to lack a functional P2/TbAT1 transporter (28). This lack has also been shown for *T. equiperdum* PBR (3) and *T. b. gambiense* 386Mr (9) through studies of P2-mediated transport. As it has been shown that the 427 arsenical drug-resistant line has deleted the Tb*AT1* gene, we investigated the status of the Tb*AT1* gene in these lines, along with their drug-sensitive parental cell lines, by PCR analysis of genomic DNA. Results are shown in Fig. 2a. An amplicon of the predicted size using DNA from all three of the drug-sensitive parental lines and in the *T. b. brucei* 247Mr and the *T. equiperdum* PBR resistant lines is observed, showing that the gene is present and that deletion is not the mechanism of resistance in these cases. In contrast, the 386Mr

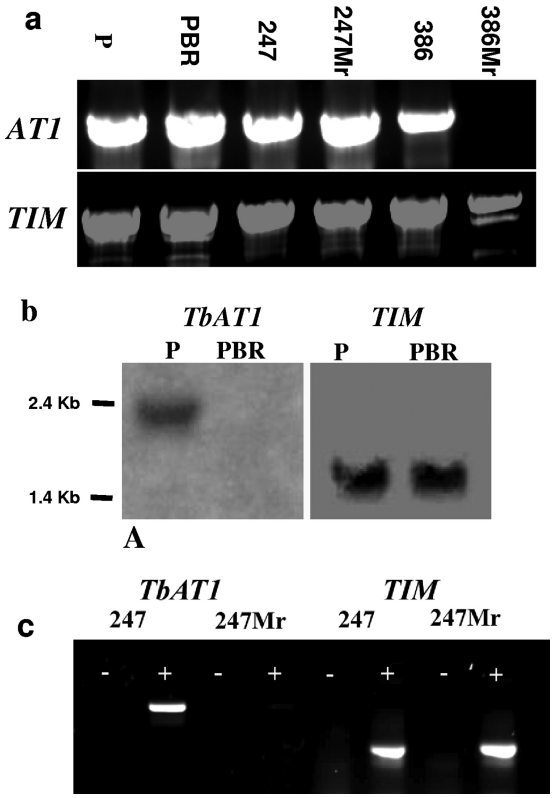

FIG. 2. Tb*AT1* gene and its expression in wild-type and drug-resistant trypanosome lines. (a) Tb*AT1* was amplified from genomic DNA prepared from *T. equiperdum* P, *T. equiperdum* PBR, STIB 247 (247), 247Mr, STIB 386 (386), and 386Mr. (b) Northern blot assay of *T. equiperdum* strains P and PBR, probed with the Tb*AT1* ORF and subsequently with a control probe for triosephosphate isomerase (TIM) as a loading control. (c) Reverse transcriptase PCR with cDNA from STIB 247 and 247Mr, confirming the lack of product in the drug-resistant derived STIB 247 line. A minus sign indicates a no-reverse-transcriptase control. TIM primers were used as positive controls.

TABLE 1. Point mutations in the Tb*AT1* nucleotide sequence and changes to the amino acid sequence in the *T. equiperdum* P and PBR cell lines compared to the *T. brucei* 927 sequence[a]

Type of sequence	Position	Point mutation in indicated cell line			Related mutation (position of mutation)
		927	P	PBR	
Nucleotide	21	C	T	T	
	151	A	C/A	A	K/Q (51)
	542	G	A	A	G/E (181)
	625	T	C	C	
	627	A	C/A	A	
	699	T	C	C	
	716	A	G	G	D/G (239)
	1122	A	G	G	
Amino acid	51	K	Q/K	K	C/A (151)
	181	G	E	E	G/A (542)
	239	D	G	G	A/G (716)

[a] Six of nine *T. equiperdum* P clones contained the additional point mutations at positions 151 and 627. SNPs that code for nonsynonymous changes are as follows: position 151, A = K and C = Q; position 542, G = G and A = E; and position 716, A = D and G = G.

line shows no amplification (Fig. 2a), although the control gene is amplified, thus showing that the Tb*AT1* gene has been deleted.

As both the 247Mr and *T. equiperdum* PBR lines had previously been shown to lack P2 transporter activity, we tested whether these lines had lost expression of the Tb*AT1* gene. Northern blot analysis of RNA from the drug-sensitive and -resistant lines of *T. equiperdum* showed that Tb*AT1* RNA was no longer stably expressed in the PBR line (Fig. 2b). RT-PCR analysis of RNA from the 247Mr line also showed the loss of stable expression of the transporter gene (Fig. 2c). In both sets of analyses, the parental sensitive lines were shown to express TbAT1, and transcripts of the positive-control (TIM) gene were detected in the preparations from the resistant lines. Thus, it can be concluded that selection for resistance results in a loss of stable RNA expression of the Tb*AT1* gene. This demonstrates a novel mechanism of drug resistance associated with the P2 transporter.

Te*AT1* ORF and 3′ UTR of *T. equiperdum* P/PBR. To investigate the possible basis of the loss of transcript in the PBR resistant lines, the Te*AT1* ORF was sequenced from both the sensitive and resistant lines. The entire Te*AT1* ORF (1,389 bp) and short flanking regions (PCR product size, ~1,500 bp) were amplified from both *T. equiperdum* lines P and PBR. Multiple independent PCR products were cloned and then sequenced from each line in order to identify the presence of any mutations or deletions in the gene. All 10 *T. equiperdum* PBR Te*AT1* clones had identical sequences, and three *T. equiperdum* P clones also had sequences identical to those in the resistant line. However, six other clones from the sensitive line had identical sequences, except for two nucleotide changes, compared to those of the other clones from this line. Comparison of these sequences with the 927 reference sequence identified eight single nucleotide polymorphisms (SNPs), three of which lead to nonsynonymous substitutions (Table 1). The sequence data indicate that the wild-type *T. equiperdum* P line is heterozygous for the Te*AT1* locus, whereas *T. equiperdum* PBR has lost heterozygosity at this locus and become homozygous for one of the alleles (Table 1). To confirm this finding, the A/C SNP present at position 627 was investigated by PCR-RFLP using the diagnostic enzymes BfaI (cutting the "A" allele) and BssSI (cutting the "C" allele). Using genomic DNA, Te*AT1* was PCR amplified from the wild-type line, and the amplicon was digested with BfaI and BssSI to generate three fragments, with sizes of 1,392 bp (uncut allele), 626 bp, and 766 bp (Fig. 3), indicating the presence of two alleles. Restriction digestion with BfaI from the amplicon derived from the PBR line, however, lacked the 1,392-bp band, whereas the two lower bands were present, indicating the presence of only the A allele. Digestion with BssSI yielded the converse result, where only the uncut 1,392-bp band was present in the resistant line (Fig. 3). This indicated that BssSI did not cut PBR Te*AT1*, and therefore, the C allele is absent. Thus, loss of heterozygosity at the Te*AT1* locus has accompanied selection of resistance in *T. equiperdum* and may be associated with a loss of expression of this gene.

3′ RACE was performed on *T. equiperdum* P (wild-type) cDNA. Of 8 clones from the nested PCR product, 5 indicated that polyadenylation springs from an A residue at 719 bp from the termination codon. Two further clones point to polyadenylation sites within 30 bp downstream of this site, and a final clone appears to indicate that polyadenylation may occur at 640 bp from the termination codon. This last site coincides with a poly(A) stretch in the 3′ UTR and could be an artifact due to mispriming of the oligo(dT) anchor primer on this poly(A) stretch. The double-stranded sequencing also indicated a heterozygosity in the 3′ UTR of *T. equiperdum* P, which has been lost in the PBR line. To investigate this further, we amplified and sequenced a 750-bp fragment of the 3′ UTR from both *T. equiperdum* lines P and PBR covering this area of heterozygosity. Of 9 *T. equiperdum* P clones, 6 carried an A residue at a site that is 514 bp into the 3′ UTR, and the remaining 3 carried a G residue. Sequencing of the *T. equiperdum* PBR 3′ UTR once again indicates that a loss of heterozygosity has accompanied loss of expression, as all 10 clones carried the A residue at this site.

The coding sequences of the Tb*AT1* genes from *T. brucei* 247 and 247Mr were amplified by PCR, and the amplicons

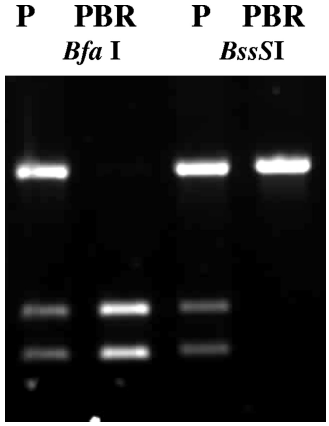

FIG. 3. Loss of heterozygosity in drug-resistant *T. equiperdum*. PCR-amplified products of Tb*AT1* ORF from *T. equiperdum* strains P and PBR digested with BfaI and BssSI.

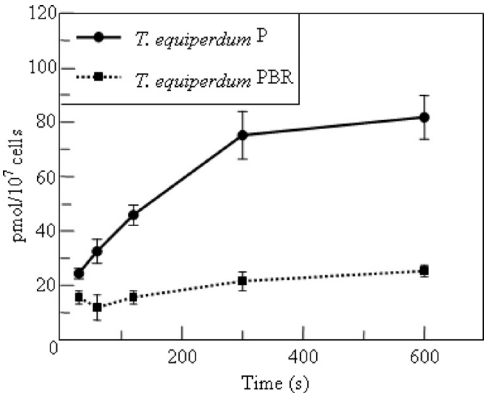

FIG. 4. Uptake of diminazene in *T. equiperdum* strains P and PBR. Accumulation of 20 μM diminazene was measured over time in wild-type *T. equiperdum* and its diminazene-resistant derivative, PBR.

were cloned and sequenced from both lines (data not shown). Comparison of the sequences (two clones from each line) showed that they were identical, which indicates that the genes in both lines are homozygous and identical.

Diminazene uptake is lost in *T. equiperdum*-selected PBR. Previous studies have shown the presence of a P2-like adenine-inhibitable adenosine transport function in the *T. equiperdum* PBR cell line (3). However, by fluorescence assay, P2 transport function itself has been shown to be absent (28). Furthermore, there is no detectable transcript for Tb*AT1* in this cell line (Fig. 2b). As diminazene has been shown to be accumulated in *T. brucei* predominantly via the P2 transporter (15), uptake of 20 μM diminazene was measured in both wild-type and diminazene-resistant *T. equiperdum* to resolve this apparent contradiction. While diminazene uptake in wild-type *T. equiperdum* is robust, uptake in *T. equiperdum* PBR is detectable only at low levels over 10 min, indicating that P2 transport is indeed lost (Fig. 4). The low level of uptake indicates the presence of another low-capacity diminazene transporter. Indeed, while the *T. equiperdum* PBR line is considered diminazene resistant, it is still sensitive to low micromolar concentrations of the drug, as is the ΔTb*at1* line, indicating that a secondary route of uptake is probably important for diminazene, which is analogous to the situation reported for furamidine (18).

A residual adenine-inhibitable adenosine uptake component has been previously identified in *T. equiperdum* PBR, yet all other data indicate a functional loss of Te*AT1*/P2 transport. Therefore, the question remains as to the identity of this residual adenine-sensitive adenosine uptake component. To investigate this, we compared the uptake of adenosine in both wild-type and diminazene-resistant *T. equiperdum* lines and also in the *T. b. brucei* ΔTb*at1* line, along with the wild-type parental line from which it was derived (23). The uptake of 10 μM [^{3}H]adenosine in the presence of various inhibitors was followed. A total of 1 mM inosine was used to block the P1 transporter, which takes up adenosine with a high affinity. A total of 1 mM adenine was used to block any P2 or P2-like adenosine transport, and 1 mM hypoxanthine was used to block any transport of adenosine mediated by the H2 nucleobase transporter (31). The results of these uptake assays are shown in Fig. 5. The uptake of adenosine is clearly reduced in both the PBR line (Fig. 5a and b) and the ΔTb*at1* line (Fig. 5c and d) compared to that in their respective wild-type parental lines. Blockage of adenosine uptake with either 1 mM inosine or inosine and hypoxanthine leads to a similar reduction in adenosine uptake in both the wild-type and mutant lines but did not abolish uptake completely, thus showing that additional routes of uptake were present. Addition of adenine (in the presence of inosine and hypoxanthine) completely inhibited adenosine uptake in all four lines, indicating that both the PBR and ΔTb*at1* lines retain a P2-like uptake activity that is not encoded by the Tb*AT1* gene.

DISCUSSION

In this study, we have characterized various genetic changes associated with the Tb*AT1* gene in trypanosomes selected for resistance to the diamidine drug diminazene and the melaminophenyl arsenical melarsamine hydrochloride, as well as correction of the genome sequence of chromosome 5.

The original *T. brucei* genome assembly did not contain Tb*AT1*. We therefore identified a BAC (26D11) containing the gene in order to identify its genomic localization. The assembly strategy for chromosome 5 was based on the mapping of a seed BAC from the RPCI93 library, followed by identification of overlapping and outwardly extending BACs through sequence similarity searches against a database of BAC end sequences. A number of candidate BACs were subsequently chosen for fingerprinting and selected for sequencing only if the fingerprint data were consistent and shared by others in the set. The region of chromosome 5 to which BAC 26D11 is homologous is located toward the extreme 5′ end of the assembly, where the consensus was generated based on two BACs (25N21 and 29K2) that overlap in this region. The sequence unique to BAC 26D11 is flanked by ~7.5 kb of duplicated sequence, encompassing an adenylyl cyclase gene. Given that the genetic data showed this locus to be homozygous and diploid, it is possible that BACs 25N21 and 29K2 each contain a deletion around the Tb*AT1* locus. An alternative explanation is that BACs 25N21 and 29K2 encoded the 5′ and 3′ ends of the duplicon, respectively, and hence, neither of these BACs carries the sequence unique to 26D11 including the Tb*AT1* gene. Whether the location of Tb*AT1* in the subtelomeric region of chromosome 5 in fact contributes to a more rapid generation of resistant cells under subcurative drug pressure due to the greater rates of recombination in these areas (4) is a matter for future investigation.

Deletion of the Tb*AT1* gene is associated with the selection of resistance to melarsamine hydrochloride in *T. b. gambiense* strain 386 (25), as identified by PCR analysis. Gene deletion was previously reported in a field isolate of arsenical-resistant *T. b. rhodesiense* (22) and also in a *T. b. brucei* line selected for resistance to the diamidine furamidine (18). In the case of *T. b. brucei* 247, a similar melarsamine hydrochloride-driven selection procedure (25) led to derivation of a line in which the gene is still identified by PCR, but in which no transcript was detected by RT-PCR and from which P2 transport activity is lacking. A further strain, *T. equiperdum* PBR selected for resistance to diminazene, has also retained the Te*AT1* gene, as detected by PCR, but in this line as well, no transcript was identified in Northern hybridization experiments. We went on

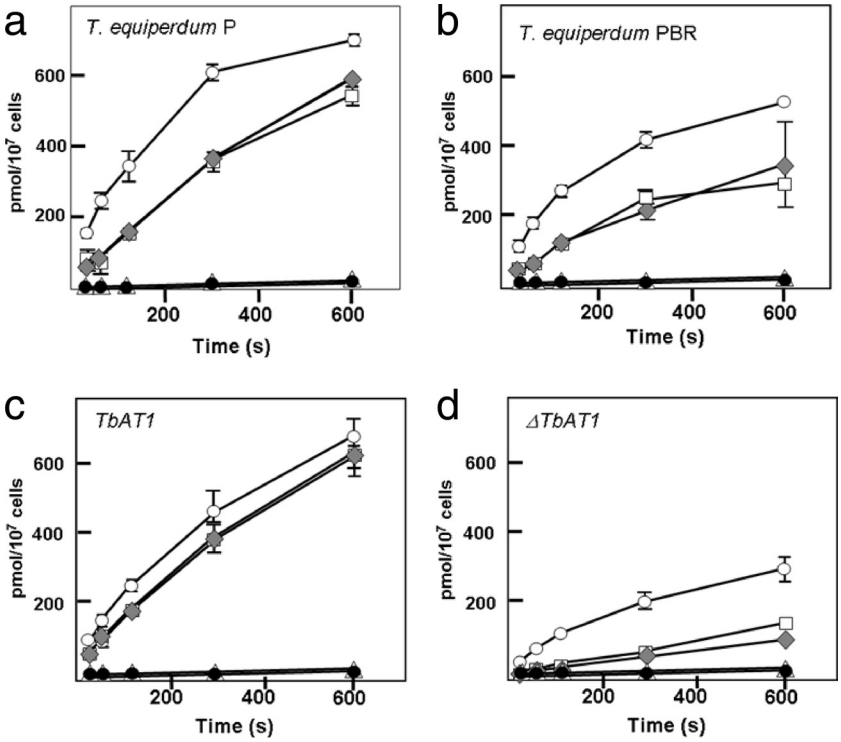

FIG. 5. Adenosine uptake via a P2-independent adenine-inhibited carrier. Inhibition of 10 μM adenosine uptake in *T. equiperdum* P (a), *T. equiperdum* PBR (b), Tb*AT1* (wild type) (c), and ΔTb*AT1* (d). Bars indicate standard deviations ($n = 3$). Open circles show uptake in the absence of inhibitor, open squares show uptake in the presence of 1 mM inosine (which blocks the P1 transporter), gray diamonds represent uptake in 1 mM inosine and 1 mM hypoxanthine (blocking the P1 adenosine transporter and the H2 hypoxanthine carrier), filled circles show 1 mM inosine and adenine, which inhibits both the P1 and P2 transporters, and open triangles represent uptake with all three of the aforementioned inhibitors.

to investigate the status of Te*AT1* in the paired *T. equiperdum* lines.

Of the six nucleotide differences identified here between the 927 sequence and the PBR line, three of these mutations have also been identified in STIB 777R, a laboratory-derived, melarsen oxide-resistant cell line (21). Two of these mutations code for changes in the amino acid sequence of TbAT1, and one is a silent mutation. These same three mutations also occur, among others, in the Tb*AT1* gene in *T. b. gambiense* cerebrospinal fluid isolates from patients in Uganda, in a *T. b. rhodesiense* isolate (STIB 871) that shows some resistance to diminazene aceturate and melarsoprol, and in a *T. b. gambiense* stock (K 003) from Angola (22). Further investigation showed that Te*AT1* in the *T. equiperdum* wild-type cell line is heterozygous, but the *T. equiperdum* PBR line contains only one of these alleles, implying that the loss of P2 transport may be linked to a loss of heterozygosity at the Te*AT1* locus. This finding was also upheld in the 3′ UTR, where heterozygosity is apparent in the *T. equiperdum* P line and is lost in the *T. equiperdum* PBR line.

The loss of heterozygosity associated with the Te*AT1* locus in PBR may account for the lack of P2 transcript and hence the loss of P2 function in this resistant line. If the A allele in the heterozygous P cell line were to be transcriptionally silent or to produce a nonfunctional transporter, drug resistance could have been acquired solely by a loss of heterozygosity in the PBR line, which carries only the A allele. However, the sequence of the A allele at positions 151 and 627 of the ORF is identical to that of Tb*AT1* from both the *T. brucei* 927 (Table 1) and 427 wild-type strains, which have fully functional P2 transport systems and a typical sensitivity to drugs. This would suggest that these mutations within the coding sequence of the gene, followed by loss of heterozygosity alone, are unlikely to bring about drug resistance.

Another possibility for the loss of drug sensitivity is that a silent copy of Tb*AT1* has been generated by changes up- or downstream of the coding sequence, which regulate the expression of the A allele. In this model, a mutation occurring in one allele (the A allele) might silence that gene, allowing the loss of heterozygosity to lead to silencing of the entire Tb*AT1* locus. In the PBR cell line, it would appear that the active C allele has been selected against, and a subsequent loss of heterozygosity has resulted in a loss of expression of the Te*AT1* gene. Gene expression in trypanosomes is frequently associated with regulatory elements present within the 3′ UTR of genes, including AU-rich elements (AREs) (5, 13, 16). However, the 3′ UTR of *T. equiperdum* has been established in this study, and the closest canonical AREs in the 3′ sequence occur at a position that is 1,283 bp from the termination codon, well beyond the polyadenylation site of this sequence and, as such, should not be influencing expression in any conventional way. The influence of any other UTRs remains to be elucidated, including the influence of the SNP at position 514 of the UTR. The finding of loss of expression of Tb*AT1* at the mRNA level is also seen for the *T. brucei* 247 line selected for melarsamine hydrochloride resistance. This represents a novel genetic

mechanism for drug resistance in *T. brucei* group trypanosomes.

Loss of expression of Te*AT1* would be expected to effectively eliminate all P2 transport function, defined as high-affinity adenine-inhibitable adenosine transport (11), in a trypanosome line. However, adenine-sensitive adenosine transport was still present in the drug-resistant *T. equiperdum* line (3), and this had led to the original hypothesis that drug resistance had arisen from a change in affinity of the P2 transporter for the substrate. The absence of expression of Te*AT1* in this line clearly eliminates this hypothesis. Blockage of the uptake of adenosine with inosine and subsequently with adenine shows that there is still an adenine-sensitive adenosine transport component in the *T. equiperdum* PBR cell line. This activity is also apparent in the ΔTb*at1* cell line, which has been confirmed to have no P2 transport activity (23). Thus, our data support the occurrence of a further permease, capable of adenine and adenosine uptake, for which a physiological role is not yet known.

The accumulation of point mutations, gene deletion, loss of heterozygosity, and loss of a stable transcript of the Tb*AT1* gene are now all shown to be genetic mechanisms leading to net loss of activity of the P2 aminopurine/drug transporter. It remains to be shown whether all these alterations are in play in generating resistance in a field setting, but any test for drug resistance based on genetic profiles will need to incorporate multiple alterations if they are to be of use in identifying drug resistance in the field. We have recently developed a fluorescence-based test that reports P2 transporter activity (28), independent of the genetic basis of its loss. This test appears to hold great promise as a diagnostic tool for the detection of drug resistance in patients treated for human African trypanosomiasis.

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