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Molecular and Biochemical Parasitology 104 (1999) 93–105

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

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The *Leishmania donovani* LD1 locus gene *ORFG* encodes a biopterin transporter (BT1)

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Received 9 April 1999; received in revised form 30 June 1999; accepted 30 June 1999

Abstract

We have previously described two genes, *ORFF* and *ORFG*, from the LD1 locus near one telomere of chromosome 35, which are frequently amplified in *Leishmania* isolates. In *Leishmania donovani* LSB-51.1, gene conversion of the rRNA gene locus on chromosome 27 with these two genes resulted in their over-expression, because of their transcription by the RNA polymerase I-mediated rRNA promoter. The predicted ORFG protein has substantial sequence homology to the *ESAG10* gene product from the *Trypanosoma brucei* VSG expression site and both are putative membrane proteins. Using successive rounds of gene replacement of the three *ORFG* genes in *L. donovani* LSB-51.1, *ORFG* null mutants were obtained. These mutant cell lines show a direct relationship between *ORFG* mRNA, protein expression levels and active transport of biopterin into the cells. Transformation of the null mutant with a plasmid containing *ORFG* restores biopterin transport activity. In addition, the null mutants are unable to grow in the absence of supplemental biopterin. Thus, *ORFG* encodes a biopterin transporter and has been renamed *BT1*. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania donovani*; Gene replacement; Pteridines; Biopterin; Transporter

1. Introduction

Leishmania are protozoan parasites that are responsible for substantial public health problems, especially in tropical and subtropical regions. The various species of *Leishmania* cause disease ranging from localized cutaneous lesions to disseminated visceral disease. Worldwide, over

Abbreviations: BT1, biopterin transporter 1; EDTA, ethylenediamine tetraacetic acid; HPLC, high pressure liquid chromatography; ORF, open reading frame; PCR, polymerase chain reaction; PTR1, pteridine reductase.

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PII: S0166-6851(99)00132-2

20 million people are estimated to suffer from leishmaniasis and 400 000 new cases occur each year [1]. Visceral leishmaniasis is currently treated with pentavalent antimonials, pentamidine and amphotericin B, but these drugs are difficult to administer and have considerable toxicity [1]. In addition, resistance to therapy is common. Thus, there is an urgent need for new therapeutic approaches.

The genome of *Leishmania* has substantial plasticity, with different strains containing amplified circular or linear DNAs that vary in size and copy number. Several of these amplified DNAs have been shown to contain genes responsible for resistance to drugs such as methotrexate, tunicamycin, arsenite, DL-α-difluoromethylornithine and mycophenolic acid (for reviews, see Refs. [2–5]). However, amplified DNA elements not obviously associated with drug resistance have also been identified, including circular D DNA in *Leishmania tropica* [6], T DNA in *Leishmania tarentolae* [7], and LD1/CD1 in several species of *Leishmania* [8–10]. The sequences found in circular LD1 also occurred in linear small chromosomes that were amplified in *L. tarentolae* following nutrient stress [11].

The multigenic LD1 locus is located near one telomere on chromosome 35 of *Leishmania* and various portions are amplified as 100–200 copies of 55-kb circular molecules or 20–60 copies of 200–450-kb linear chromosomes in ~15% of *Leishmania* isolates tested [10,12,13]. We cloned and sequenced the circular LD1 molecule from one strain of *Leishmania donovani* (LSB-7.1), and described several open reading frames (ORFs) with potential protein-coding function [14–16]. In another strain of *L. donovani* (LSB-51.1), gene conversion resulted in duplication of two LD1 genes (*ORFF* and *ORFG*) into the *rRNA* gene locus on chromosome 27 and their consequent transcription by RNA polymerase I initiated at the *rRNA* promoter [17]. Steady state mRNA levels of both the *ORFF* and *ORFG* genes are substantially elevated in this strain, and increased ORFF protein levels were found [17].

The predicted ORFG protein has substantial sequence homology to *ESAG10*, which occurs in some *Trypanosoma brucei* VSG expression sites. Both contain 12 putative membrane-spanning domains that are predicted to form amphiphilic α-helix or β-strands typical of type IV integral membrane proteins [16]. Thus, it has been suggested that these proteins are involved in the formation of aqueous channels through the parasite membrane, and are potentially transporters [16]. Transfection with cosmids derived from the *L. donovani* LD1 region, and plasmids expressing only *ORFG*, rescued a methotrexate-resistant folate-transport mutant of *L. donovani* (Moore and Beverley, abstract 107, Woods Hole Molecular Parasitology Meeting, 1996). These transfectant cell lines showed elevated uptake of both biopterin and folate, suggesting that *ORFG* (renamed *BT1*) encodes a biopterin/folate transporter. Subsequent studies expressing *BT1* mRNA in *Xenopus* oocytes confirmed that *BT1* encodes a biopterin transporter (Cunningham et al., abstract 236, Woods Hole Molecular Parasitology Meeting, 1997). In this paper, we report the disruption of the three *BT1* genes in *L. donovani* LSB-51.1 by successive rounds of gene replacement and gene rescue with a plasmid construct containing *BT1*. Using these mutant cell lines, we show a direct relationship between *BT1* mRNA, protein expression levels, and active transport of biopterin into the cells. Null mutants of *BT1* are unable to grow as promastigotes in medium lacking supplemental biopterin, indicating the essential nature of this gene in *Leishmania* under conditions of limited pteridine availability.

2. Materials and methods

2.1. Molecular constructs

Three plasmid constructs (pfdhPacG, pGHygG, pGdhBleGX) were prepared for the targeted replacement of the three *BT1* alleles in *L. donovani* strain LSB-51.1 (MHOM/SD/00/Khartoum) and a fourth (pGGdhNeoG) for introduction of an episomal copy of *BT1*. All constructs were grown in *Escherichia coli* SURE™ cells (Stratagene) and plasmid DNA isolated using Promega Wizard or Qiagen kits.

2.1.1. pFNeoG

pFNeoG, which contains the neomycin phosphotransferase (*NPT)* gene flanked at its 5′ and 3′ ends by the *ORFE-ORFF* and *BT1-ORFH* intergenic regions, respectively, served as the backbone from which the four transformation constructs were derived. DNA from clone pB3FL35 [17] was digested with *Xba*I to release a 2753-bp fragment, and religated to produce plasmid pBX3FL8, which contains the *ORFI-ORFβ* intergenic region in a pBluescriptII SK^- (Stratagene) backbone. The *ORFA-ORFB* intergenic region was PCR amplified from clone pKH6 [14], using primers MR (5′-aacagctatgaccatg) and B-5orfBr (5′-GCTGGaTCcTACAGGAGC), the product digested with *Hin*dIII and *Bam*HI and ligated into *Hin*dIII + *Bam*HI-digested pBX3FL8 DNA to produce p5B3I. The *NPT* gene was amplified from pNEO (Pharmacia) with primers B-5B-5Neo (5′ - taggatccagcaATGATTGAACAAGATGG) and BmN-3Neo (5′-accagcattcgcggcCGCTCAGAAGAACTCG), the product digested with *Bam*HI and *Bsm*I, then ligated into *Bam*HI + *Bsm*I-digested p5B3I DNA to produce clone pBNeoI. The *ORFE-ORFF* intergenic region was amplified from clone pCK1 [15], with primers Hd5EFi (5′-GAGGAGACCACAGaAGCTTCTCTC) and B3EFi (5′-ATGGatcCTGACGAGAAGAATG), the product digested with *Hin*dIII and *Bam*HI, and ligated into *Hin*dIII + *Bam*HI-digested pBNeoI DNA to produce pFNeoI. The *BT1-ORFH* intergenic region was amplified from pK22 [12] with primers N5GHi (5′-AGGTGATGGcGGCCGCACTC) and St3GHi (5′-tcCCGCgGCACCGAAGAGAGC), the product digested with *Not*I and SstII and ligated into *Not*I + *Sst*II-digested pFNeoI DNA, replacing the *ORFI-ORFβ* intergenic region with *BT1-ORFH* intergenic sequence, to generate pFNeoG.

2.1.2. PfdhPacG

PfdhPacG, which contains the puromycin *N*-acetyl transferase (*PAC*) gene flanked by the 5′ and 3′ intergenic regions from the *Leishmania major* dihydrofolate reductase/thymidylate synthetase (*DHFR-TS*) gene, all bounded by the *ORFE-ORFF* and *BT1-ORFH* intergenic regions, was used to replace one allele of both *ORFF* and *BT1* on chr35. pFNeoG DNA was digested with *Not*I and the ends filled with T4 DNA polymerase (Gibco BRL). It was then digested with *Bam*HI to release the *NPT* gene and the ends partially filled with dATP and dGTP using the Klenow fragment of *E. coli* DNA polymerase (Gibco BRL). A DNA fragment containing the *PAC* gene bounded by the *L. major DHFR-TS* flanking regions, was obtained from pX63PAC DNA [18] by digestion with *Bgl*II, followed by complete filling of the digested ends, and *Xho*I digestion, followed by partially filling with dTTP and dCTP. This fragment was ligated with the larger fragment resulting from digestion of pFNeoG DNA with *Not*I, followed by complete filling, and *Bam*HI, followed by partial filling with dGTP and dATP, to obtain pGdhPacG.

2.1.3. pGHygG

pGHygG, which contains the hygromycin B phosphotransferase (*HYG*) gene flanked by the *ORFF-BT1* and *BT1-ORFH* intergenic regions, was used to replace the second allele of *BT1* on chr35. The *ORFF-BT1* intergenic region was amplified from pK27 [12] with primers MF (5′-gtaaaacgacggccagt) and B3FGi (5′-gcggatCCTGGTATGATGGC), and the product digested with *Kpn*I and *Bam*HI. This fragment was then ligated with pFNeoG DNA which was digested with *Kpn*I and *Bam*HI (to remove the *ORFE-ORFF* intergenic region) to produce pGNeoG. The *NPT* gene was removed from pGNeoG by *Not*I digestion and complete filling of the digested ends, followed by *Bam*HI digestion. The larger fragment from this digestion was ligated with a fragment containing the *HYG* gene obtained by *Bam*HI + *Nae*I digestion of pTSA-HYG2 [19] to produce pGHygG.

2.1.4. pGdhBleGX

pGdhBleGX, which contains the bleomycin/phleomycin binding protein (*BLE*) gene bounded on its 5′ side by the *ORFF-BT1* intergenic region and on its 3′ side by the *BT1*-x 3′ flanking sequence [17], was used to replace the *BT1* allele on

chr27. The *NPT* gene with *DHFR-TS* flanking sequences was amplified from pX63Neo [20] with primers 5′dhfrS (5′gcGgAtccCACCCCACCCCTGCATTC) and 3′dhfrAS (5′-AggcGGccGcGCTACGGT) and the product digested with *Bam*HI and *Not*I. This fragment was then ligated with *Bam*HI + *Not*I-digested pGNeoG DNA replacing the existing *NPT* gene (lacking the *DHFR-TS* flanking sequences) to produce pGdhNeoG. The *BT1*-x 3′ flanking sequence in clone pNE78 [17] was amplified using primers N5GHi (5′-AGGTGATGGcGGCCGCACTC) and St3X (5′-atCCGCGgCGCTCCGTG) and the product digested with *Not*I and *Sst*II. The *BT1-ORFH* intergenic region from pGdhNeoG was removed by digestion with *Not*I and *Sst*II and replaced by ligation with the *BT1*-x fragment above to obtain pGdhNeoGX. The *BLE* gene was amplified from pUT333 (Cayla) with primers 5′ble (5′-gacgctcgagaggcctATGGGCGAAATGACCGACCA) and 3′bleEcXb (5′-gcgggcgatatctagACTCATGAGATGCCTGCAAGC) and the product digested with *Xho*I, followed by complete filling of digested ends, then *Eco*RV. pGdhNeoGX DNA was digested with *Spe*I to remove the *NPT* gene, the ends completely filled using Klenow and dephosphorylated using Calf Intestinal Alkaline Phosphatase (Gibco/BRL), and then ligated with the *BLE* fragment above. The resultant clones were screened to obtain one (pGdhbleGX) in which the *BLE* gene was in the appropriate orientation.

2.1.5. pGGdhNeoG

The episomal rescue construct, pGGdhNeoG, contains an intact copy of *BT1* along with its wild-type 5′ and 3′ flanking sequences, upstream of the *NPT* gene bounded by *DHFR-TS* flanking sequences, and second copy of the *BT1-ORFH* intergenic region. A 3250-bp fragment containing *BT1* and its 5′ and 3′ flanking sequences was obtained by digestion of pS85 [12] DNA with *Bgl*II, *Dra*I and *Xho*I. pGdhNeoG DNA by digested with *Bam*HI, the ends completely filled, and further digested with *Bgl*II to remove a 438-bp fragment containing the *ORFF-BT1* intergenic region. The remaining fragment from pGdhNeoG (containing the *NPT* gene and the *BT1-ORFH* intergenic region) was then ligated with the *BT1*-containing fragment from pS85 to obtain pGGdhNeoG.

2.2. Parasite culture and transformation

All parasite cell lines used in this study were derived from LSB-51.1, except for a clonal line of *L. donovani* strain D1700, which was kindly provided by Dr Buddy Ullman (OHSU). Promastigotes stages were grown at 24°C in AM medium as previously described [17], except where otherwise noted. Methods for cell electroporation and cell plating have been described previously [21] and were utilized with only minor changes. Briefly, *Leishmania* promastigotes were grown to late-log phase, washed in phosphate-buffer saline solution (PBS, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 145 mM NaCl), followed by electroporation (EP) buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 6 mM glucose, pH7.4), and suspended in EP buffer at 2×10^8 cells/ml. Purified plasmid DNA from the *BT1* replacement constructs above was linearized (with *Hin*dIII + *Spe*I for pFdhPacG, *Bss*HII for pGHygG, and *Kpn*I + *Sst*II for pGdhBleGX) before addition of 5 μg to 0.4 ml of the cell solution in electroporation cuvettes (BTX). For the episomal *BT1* rescue, cells were incubated on ice for 10 min with 50 μg of undigested (circular) pGGdhNeoG DNA, before electroporation using a BTX Electro Cell Manipulator® 600 with settings of 480 V, 13 Ω, and 500 μF. Cells were cultured for ~18 h in AM medium before spreading on plates containing 0.7% Seaplaque GTG agarose (FMC BioProducts) in AM media with 20–80 μg ml^{-1} neomycin (G418), 25 μg ml^{-1} puromycin, 20 μg ml^{-1} hygromycin, or 12.5 μg ml^{-1} phleomycin to isolate single colonies. For transformations using pGdhBleGX, the medium was supplemented with 7.5 μM each of biopterin, dihydrobiopterin, folate, and dihydrofolate. Biopterin (Sigma) and dihydrobiopterin (Schircks Laboratories, Jona, Switzerland) stock solutions were made in 40 mM Hepes, pH 7.4, 5 mM DTT while folate and dihydrofolate were made in distilled H_2O. After confirmation by Southern blot analysis that recombinant *Leishmania* clones were of the appropriate genotype (see below), they were maintained

in AM media without antibiotic selection, except those containing the episomal construct pGGdh-NeoG which were maintained in 40–88 μg ml^{-1} neomycin. For comparison of growth rates between cell lines, stationary phase promastigotes were inoculated at a density of 2×10^6 cells ml^{-1} into fresh Minimum Essential Medium with α modification (α-MEM) [22], with or without supplementation with biopterin and folate (7.5 μM each of biopterin, dihydrobiopterin, folate and dihydrofolate). Cell densities of duplicate cultures were determined at 24-h intervals and doubling times calculated by regression analysis using an exponential curve fit (SigmaPlot).

2.3. Southern and Northern blot analysis

Chromosome-sized DNA was prepared in agarose blocks, separated by clamped homogenous electric field electrophoresis (CHEF) using a Bio-Rad CHEF Mapper, and transferred to Nylon membranes (GeneScreen, NEN LifeScience) as previously described [17]. Genomic DNA was extracted from *Leishmania* using a method adapted from Bellofatto and Cross [23]. Late log-phase promastigotes were collected by centrifugation, washed in PBSG (PBS with 0.2% glucose), resuspended in EA solution (0.01 M Tris, pH 8.0, 0.25 M NaCl, 0.005% NP-40) and incubated 5 min at room temperature. Lysates were centrifuged and the pellets resuspended in EB solution (0.01 M Tris, pH 8, 0.01 M NaCl, 0.01 M EDTA, 0.5% SDS, 50 μg ml^{-1} proteinase K) and incubated on ice for 5 min. The DNA solution was extracted sequentially with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), then precipitated at −20°C with 0.3 M sodium acetate and two volumes of 95% ethanol. The DNA pellet was collected by centrifugation and resuspended in TE (10 mM Tris, pH 8.0, 1 mM EDTA). DNA was digested with restriction enzymes and separated by conventional agarose gel electrophoresis or field inversion gel electrophoresis (FIGE), stained with ethidium bromide, and transferred to nylon membranes as described previously [17]. Total RNA was extracted from *Leishmania* using an acid guanidinium-phenol-chloroform method, then 10 μg aliquots were separated on MOPS/formaldehyde agarose gels and transferred to nylon filters as described previously [12,17]. Nucleic acids were cross-linked to the filters with a Stratalinker UV-crosslinker (Stratagene) according to the manufacturer's instructions. Filters were hybridized with [α-^{32}P]dATP-labeled probes prepared from gel-isolated DNA fragments using the Random Primers DNA labeling System (Gibco/BRL). The *BT1* probe was a 375-bp fragment from *Bam*HI + *Not*I-digested pK27 [12]. The 577-bp α-tubulin probe was obtained by PCR amplification of genomic DNA using primers 5′Ktubg (5′-atgggtaccGCTATCTGCATCCACATC) and 3′Xtubg (5′-ccgctcgagACAGCACGCAGTTGTAC). Hybridization was carried out for 3 h at 65°C in Rapid Hybe solution (Amersham Life Science). The filters were washed twice in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS for 5 min at room temperature followed by two washes in 0.1 × SSC, 0.1% SDS for 20 min at 65°C.

2.4. Expression of recombinant BT1 and Western blot analysis

The region encoding the C-terminal 63 amino acids of BT1 was amplified from clone pS85 [12] using primers LD1-G31 (5′-CTgaaTTCGACCTCCTCATTTTG) and 3EorfG (5′-cgaattCTAGCTGTCCCGCTTC). The resulting product (488 bp) was digested with *Bcl*I and *Eco*RI, ligated into *Bam*HI + *Eco*RI-digested pRSETC (Invitrogen), and transformed into *E. coli* SURE™ cells (Stratagene) to produce clone pSETCG7B. Plasmid DNA from this clone was transformed into BL21 cells (Novagen) and induced with IPTG (isopropyl-β-D-thiogalactopyranoside) to express recombinant protein. Recombinant pSETCG7b protein (10.5 kDa) was purified using ProBond™ Metal-Binding Resin (Invitrogen) according to the manufacturer's instructions. Protein was eluted from the column in DBB buffer pH 4.0 supplied with the kit, dialyzed in PBS to remove urea, and concentrated with a Centricon 3 concentrator (Micon). Purified protein (∼ 300 μg) was mixed with equal volume of incomplete Freund's adjuvant plus 100 μg mu-

ramyldipeptide (MDP) and injected subcutaneously into a New Zealand White rabbit. The rabbit was boosted with the same amount of recombinant protein/adjuvant mixture six additional times at 2-week intervals after which serum was obtained at sacrifice.

Leishmania promastigotes were grown to late log-phase in M199 medium (Gibco/BRL) supplemented with 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 10% heat inactivated fetal bovine serum (Biological Industries Israel); collected by centrifugation; washed with PBSG; and resuspended in SDS-PAGE buffer (0.15 M Tris, pH 6.8, 4.0% SDS, 20% glycerol, 0.06% bromophenol blue, 1.4 M 2-mercaptoethanol) to a concentration of 5×10^9 cells per ml. Protein samples representing 10^7 cells were boiled and separated on a 10% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane by standard Western blotting methods [24]. Membranes were washed three times for 10 min each in PBS with 0.1% Tween-20 and blocked for 1 h at room temperature in PBS plus 0.1% Tween-20 and 5% low-fat milk. The membranes were washed again, incubated for 1 h at room temperature in 1/200 dilution of rabbit antiserum in PBS with 0.1% Tween-20 before being washed again as described above. The membrane was incubated in 1/8000 dilution of protein A-horseradish peroxidase, washed again, and exposed to ECL System detection solution (Amersham) according to the manufacturer's instruction. Bound antibodies were visualized by autoradiography.

2.5. Biopterin uptake assays

Biopterin uptake studies were carried out essentially as described elsewhere [25,26]. Briefly, promastigotes were grown to mid-log phase ($3–6 \times 10^6$ cells/ml) in M199 medium [21], harvested by centrifugation, washed twice with M199 medium lacking folate and thymidine and containing 0.66% bovine serum albumin (BSA; fdM199, Gibco/BRL), resuspended in fdM199 at 3×10^6 cells/ml and incubated overnight. Cells were then harvested, washed once with transport medium (fdM199 lacking BSA) and resuspended at 5×10^8 cells/ml in transport medium. The cell suspension (100 μl) was mixed with 100 μl HPLC-purified [^{3}H]-biopterin (52 μCi/μmol, batch 127-276-0129, Moravek) [25] on top of a 100 μl dibutyl phthalate cushion in a microfuge tube to a final substrate concentration of 200 μM. Triplicate samples were incubated for 10 min at either 23 or 4°C, and the reaction terminated by spinning the cells through the cushion at $16\,000 \times g$ for 30 s. The aqueous layer was removed and the cushion washed twice with Hank's Buffered Salt Solution. The cell pellet was recovered, lysed with 100 μl 1% Triton X-100 and counted in Scintiverse II scintillation fluid (Fisher). Biopterin uptake due to active transport was determined by subtracting values obtained at 4°C from those obtained at 23°C.

3. Results

3.1. Targeted gene replacement of BT1

L. donovani strain LSB-51.1 (51.1) used in this study contains three copies of *BT1*: one allele in the LD1 locus on each homolog of chr35 and a single copy in the rRNA locus on one chr27 homolog [17]. Thus, the genotype of 51.1 wild type (WT) cells is $BT1/BT1^{chr35}$ $BT1^{chr27}$ and production of *BT1* null mutants requires three successive rounds of targeted gene replacement (Table 1). Electroporation of WT cells with the construct pFdhPacG and selection with puromycin yielded transformants in which one allele of both *ORFF* and *BT1* on chr35 was replaced with the *PAC* gene. Southern blot analysis of genomic DNA from a resultant cloned cell line (SKOΔ35) using probes for *BT1* (Fig. 1, lane 2) and *PAC* (data not shown) confirmed one *BT1* allele on chr35 was replaced with the *PAC* gene. Thus, this cell line retains the other *BT1* allele on chr35 and other copy on chr27 (i.e. the genotype is $\Delta orff\Delta bt1::PAC/BT1^{chr35}$ $BT1^{chr27}$).

The remaining *BT1* allele on chr35 in SKOΔ35 cells was replaced by electroporation with construct pGHygG and isolation of single colonies following hygromycin selection (Table 1). Genomic Southern analyses using probes for *BT1* (Fig. 1, lane 4) and *HYG* (data not shown) confi-

Table 1
Description of recombinant *Leishmania* cell lines

Cell line	WT	SKOΔ35	SKOΔ27	DKOΔ35	TKO	TKO-rescue
Construct[a]						
PfdhPacG		X		X	X	X
PGHygG				X	X	X
PgdhbleGX			X		X	X
PGGdhNeoG						X
BT1 copies[b]						
chr35	2	1	2			
chr27	1	1		1		
Episomal						>1[c]

[a] Cells were electroporated with these constructs, as described in the text.
[b] Number of copies of *BT1* per diploid genome.
[c] Copy number was not determined accurately.

rmed that the second allele of *BT1* on chr35 was replaced with *HYG*. Thus this cell line, termed DKOΔ35, lacked both alleles of *BT1* on chr35, but retained a copy of *BT1* on chr27 (i.e. the genotype is $\Delta orff\Delta bt1::PAC/\Delta bt1::HYG^{chr35}$ $BT1^{chr27}$). *BT1* null mutants were produced by replacing the remaining copy of *BT1* by electroporation of DKOΔ35 with construct pGdhBleGX and selection of single colonies on phleomycin plates (Table 1). Initial attempts to isolate null mutants were unsuccessful until the medium was supplemented with 7.5 μM each of biopterin, dihydrobiopterin, folate, and dihydrofolate. Southern analyses of the resulting cell line, termed TKO, confirmed that it lacked all three copies of *BT1* (Fig. 1, lane 5), having replaced the copy on chr27 with the *BLE* gene (data not shown). Thus, the genotype of the null mutant (TKO) is $\Delta orff\Delta bt1::PAC/\Delta bt1::HYG^{chr35}$ $\Delta bt1::BLE^{chr27}$. As a control, a second single knockout mutant was produced by electroporation of WT cells with construct pGdhBleGX and selection on phleomycin plates. In the resulting cell line, termed SKOΔ27, both copies of *BT1* on chr35 were retained, but the copy of *BT1* on chr27 was replaced by the *BLE* gene (Fig. 1, lane 3 and data not shown). Thus, the genotype of this cell line is $BT1/BT1^{chr35}$ $\Delta bt1::BLE^{chr27}$.

Finally, to assess whether any phenotypic differences observed in the *BT1* null mutants were solely due to the absence of *BT1*, rather than unanticipated genetic changes, TKO cells were electroporated with circular DNA from the con-

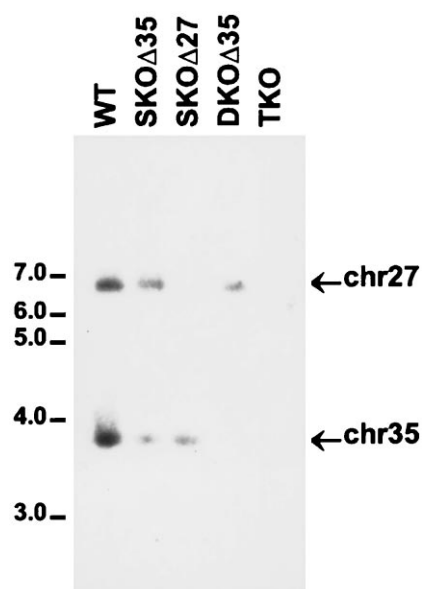

Fig. 1. Sequential gene replacement of *BT1* in *L. donovani* LSB-51.1. Genomic DNA (10 μg) from wild type (WT) and mutant (SKOΔ35, SKOΔ27, DKOΔ35, and TKO) was digested with *Bgl*II and Southern blots probed with an *BT1*-specific probe (derived from pK27). The 3.7-kb band is derived from the copies of *BT1* on chr35, while the 6.8-kb band is derived from the copy on chr27, as indicated to the right of the panel. The molecular size markers (Gibco/BRL) are shown to the left in kb.

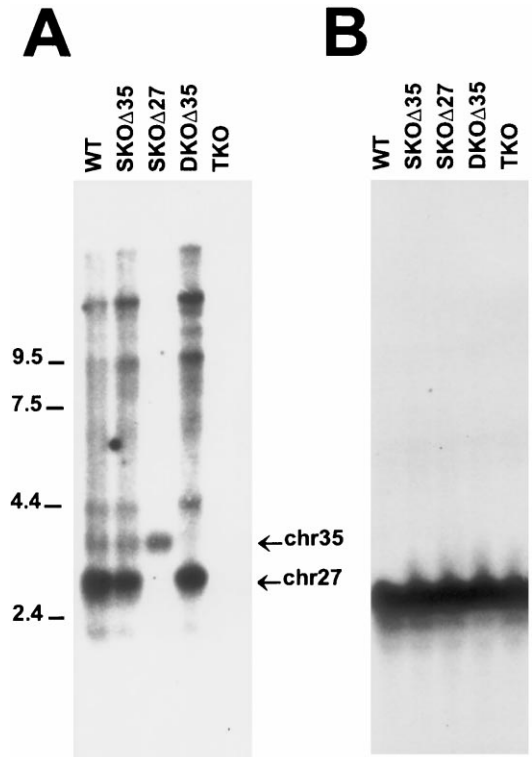

Fig. 2. Northern blot analysis of *BT1* mutants. Total cellular RNA (10 μg) from the cells indicated was separated by agarose gel electrophoresis and probed with the *BT1*-specific probe (panel A). Hybridization of the same blot with a tubulin probe (panel B) confirmed equal loading of RNA in each lane. The chromosomal origin of the 2.8- and 3.5-kb *BT1* transcripts are indicated to the right of panel A. Molecular size markers (Gibco/BRL) are shown to the left in kb.

struct pGGdhNeoG and selected on plates containing neomycin. The resulting cloned cell line, termed TKO-rescue, contain a copy of *BT1* (and *NEO*) on an extrachromosomal element (Table 1). TKO-rescue cells (genotype Δ*orf*Δ*BT1::PAC*/Δ*orfg::HYG*^{chr35} Δ*orfg::BLE*^{chr27} *[pX NEO BT1]*) were grown in media supplemented with neomycin to maintain a high copy number of the plasmid.

3.2. BT1 expression in mutant cell lines

BT1 expression at the RNA level was investigated by Northern blot analysis (Fig. 2). Total cellular RNA was extracted from WT cells and the *BT1* deletion mutants, separated by gel electrophoresis and hybridized with a *BT1*-specific probe (Fig. 2). The pattern of steady state *BT1* mRNA abundance largely reflected the genotype of each cell line. In WT, an abundant ~2.8 kb transcript and a less abundant ~3.5 kb transcript are detected, corresponding to the mature *BT1* transcripts from chr27 and chr35, respectively [17]. This interpretation is confirmed by the results from the other cell lines. The 3.5-kb transcript is absent in DKOΔ35 (which lacks both copies of *BT1* on chr35), indicating that this transcript is derived from chr35. Conversely, the 2.8-kb transcripts and several additional transcripts of ~4, ~9 and greater than 10 kb are no longer present in SKOΔ27, which lacks *BT1* on chr27, indicating their origin from this chromosome in WT. The larger transcripts represent precursors of the *BT1* transcript from the *BT1*/*rRNA* locus on chr27 [17]. Interestingly, the abundance of the 3.5-kb *BT1* transcript is elevated in SKOΔ27, suggesting the existence of regulatory systems controlling mRNA abundance. Nevertheless, total abundance of *BT1* mRNA is substantially decreased in SKOΔ27. As expected, no detectable *BT1* mRNAs are detected in TKO confirming the absence of *BT1* expression in this null mutant.

BT1 is predicted to encode a 627-amino acid protein of 68.9 kDa containing 12 potential membrane-spanning domains [16]. Attempts to express full-length recombinant BT1 fusion proteins in bacterial expression systems were unsuccessful, presumably due to its hydrophobic nature. However, we were able to express the C-terminal 63 amino acids of BT1 fused to an N-terminal His-Tag and raise rabbit polyclonal antisera. Western blot analysis using these antibodies detects a protein with an apparent size of ~45 kDa in all cell lines, except the null mutant TKO (Fig. 3). Similar results were obtained by immunoprecipitation (data not shown). Anomalous migration is not unusual for membrane proteins [27], suggesting that the 45-kDa protein does represent the BT1 protein product.

3.3. BT1 encodes a biopterin transporter

Previously studies using homologous and heterologous expression systems indicated that *ORFG/BT1* encoded a biopterin/folate transporter (Moore and Beverley, abstract 107, Woods Hole Molecular Parasitology Meeting, 1996, Cunningham et al., abstract 236, Woods Hole Molecular Parasitology Meeting, 1997). The inability to obtain *BT1* null mutants in the absence of biopterin and folate supplementation of growth medium (Section 3.1) was consistent with this finding. In order to assess directly the role of *BT1* in pteridine transport, uptake of [^{3}H]-biopterin was determined in wild type 51.1 (WT) and the transgenic *BT1* mutants described above (Fig. 4). WT cells showed the highest biopterin transport levels at 463.0 ± 16.0 pmol min^{-1} $(10^9 cells)^{-1}$, after correction for passive diffusion (i.e. biopterin uptake at 4°C). The levels in the null mutant (TKO) were just above background at 7.9 ± 4.2 pmol min^{-1} $(10^9 cells)^{-1}$. Reintroduction of *BT1* into TKO on an episomal plasmid (TKO-rescue) led to an increase in biopterin uptake to 47.9 ± 0.7 pmol min^{-1} $(10^9 cells)^{-1}$. Thus, *BT1* encodes a biopterin transporter and has been renamed *BT1* (Biopterin Transporter 1). The biopterin uptake level in SKOΔ35 parasites was 452.1 ± 16.7 pmol min^{-1} $(10^9 cells)^{-1}$, indicating that biopterin transport was unaffected by the loss of a single chr35 allele of *BT1*. Loss of the second chr35 *BT1* allele in DKOΔ35 cells caused a modest, but measurable, reduction in biopterin uptake to 383.3 ± 14.4 pmol min^{-1} $(10^9$ cells$)^{-1}$. In contrast, deletion of the single *BT1* gene from chr27 in SKOΔ27 cells led to a dramatic reduction in biopterin uptake to 48.5 ± 3.0 pmol min^{-1} $(10^9 cells)^{-1}$. This level is similar to the value of 53.1 ± 2.8 pmol min^{-1} $(10^9 cells)^{-1}$ obtained with another strain (D1700) of *L. donovani*, which does not contain any amplification of *BT1*.

Comparison of the growth rates of these cells lines in the presence of supplemental biopterin and folate showed doubling times of ~50, 53, 71 and 137 h for WT, SKOΔ35, DKOΔ35, and

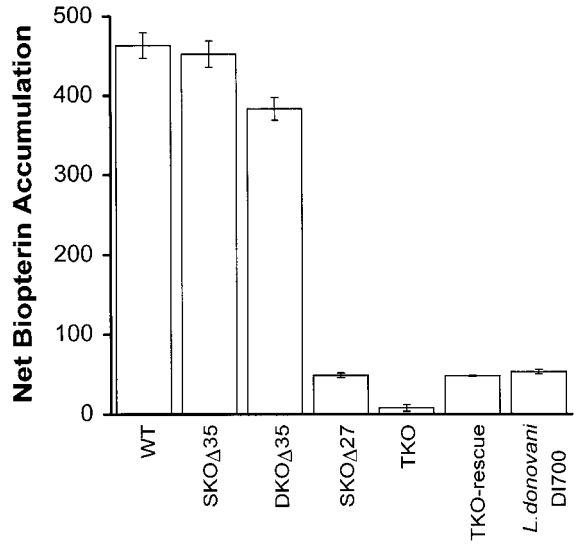

Fig. 4. Biopterin transport in *BT1* mutant cell-lines. Active biopterin transport (in pmol min^{-1} per 10^9 cells) was determined by subtracting the amount accumulated at 4°C from that accumulated at 23°C. All values are the means of triplicate assays and the error bars represent one standard deviation.

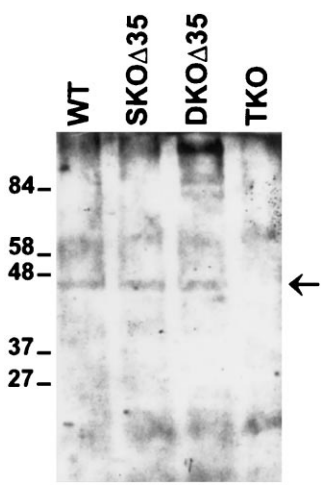

Fig. 3. Western blot analysis of *BT1* mutants. Protein samples representing 10^7 *Leishmania* cells from the cell-lines indicated were separated by SDS-PAGE and probed with rabbit antiserum against recombinant BT1 protein. Bound antibodies were detected using the ECL System (Amersham). The BT1 protein is indicated by the arrow to the right. Molecular weight markers (Pharmacia) are shown to the left in kDa.

TKO, respectively. In the absence of supplemental biopterin and folate, these values increased to ~77, 91, 131 and greater than 186 h, respectively. The TKO cell line died after 1–2 weeks in this medium, and could be maintained only in medium containing supplemental biopterin and folate. Thus, deletion of *BT1* genes has a substantial effect on growth rate, especially in the absence of supplemental biopterin and folate.

4. Discussion

The multigenic LD1 locus that is near one end of chr35 is frequently amplified in several species of *Leishmania* [10,12,13]. While the region that is amplified varies among strains, two genes, *ORFF* and *BT1*, are invariably part of the amplification unit [13], suggesting that one or both have important roles in the parasite. Transfectants containing cosmids or plasmids expressing *BT1* functionally complemented a methotrexate-resistant *L. donovani* mutant deficient in folate transport (Moore and Beverley, abstract 107, Woods Hole Molecular Parasitology Meeting, 1996). These lines showed elevated uptake of both biopterin and folate, suggesting that *BT1* encoded a biopterin/folate transporter. Analogous results were also obtained in *L. tarentolae* [28]. Subsequent studies expressing *L. donovani BT1* mRNA in *Xenopus* oocytes (Cunningham et al., abstract 236, Woods Hole Molecular Parasitology Meeting, 1997) confirmed that *BT1* encodes a biopterin transporter. Here, we use a reverse genetic approach to show that *BT1* encodes the primary transporter of biopterin in *Leishmania*. Analysis of single, double and triple (null) knockout mutants of *BT1* derived from *L. donovani* 51.1 (which contains an allele of *BT1* on each homolog of chr35 and a single copy in one *rRNA* locus on chr27) revealed a direct correlation between expression levels of the *BT1* gene product and active transport of biopterin into the cells. Wild-type 51.1 cells (WT) and mutants with an intact copy of *BT1* on chr27 (SKOΔ35 and DKOΔ35) showed substantially higher (approx. seven to nine-fold) biopterin uptake activity than those with only the chr35 alleles (*L. donovani* D1700, SKOΔ27), confirming that the increased *BT1* expression level due to the transcription of chr27 copy by the RNA polymerase I [17] has phenotypic consequences. TKO-rescue cells, which contain *BT1* only on an episomal plasmid, showed biopterin transport levels similar to cells expressing only the chr35 copies of *BT1*.

The exact role of biopterin in *Leishmania* metabolism remains unresolved [29]. Previous studies have demonstrated its role in hydroxylation of phenylalanine and tyrosine, cleavage of ether-linked lipids, and biosynthesis of nitric oxide in other organisms [30]. However, phenylalanine hydroxylase activity has not been detected in trypanosomatids [31], and ether-linked lipid cleavage in *Leishmania* appears to use NADPH as a co-factor [32]. Biopterin is closely linked to the folate biosynthesis, since biopterin can compensate for folate deficiency in culture medium [33–36]. Furthermore, [^{3}H]-biopterin is converted into tetrahydrofolates in *L. donovani* [35]. However, biopterin is thought to have essential functions independent of the folate pathway [29], since neither folate nor dihydrofolate can rescue null mutants of pteridine reductase (PTR1), the enzyme which catalyzes biopterin reduction [37]. In addition, dihydropteridine reductase (DHPR), which is involved in regeneration of tetrahydrobiopterin from quinonoid tetrahydrobiopterin, is expresed in *Leishmania* [37] and alteration in expression of *PTR1* affects the oxidant sensitivity of *Leishmania* [29]. Thus, biopterin plays a critical, yet undefined, metabolic role in *Leishmania*.

Since *Leishmania* are auxotrophic for pteridines and must acquire them from their host uptake of biopterin would seem critical for the parasites' survival. The role of *BT1* in this uptake is supported by the inability of null mutants (TKO) to survive long-term in culture without biopterin and folate supplementation. In contrast, wild-type parasites are able to grow under these conditions of limited pterin availability. Moreover, we were unable to obtain null mutants by targeted gene replacement in standard growth medium without

added biopterin and folate (Section 3.1). All recombinants from these transformations selected without pteridine supplementation contained amplified copies of the *BT1* gene in addition to integration of the knockout construct at the intended site (data not shown). This is similar to results obtained by others when attempting to create null mutants of essential loci in *Leishmania* [38–40]. Thus, uptake of biopterin by BT1 appears critical for cell survival at physiological concentrations of biopterin. The *BT1* null mutants (TKO), showed significantly slower growth as promastigotes, even in the presence of supplemental biopterin and folate, suggesting that their ability to grow in this medium is due to uptake of biopterin via passive diffusion or a secondary (and lower-affinity) transporter. Conversely, amplification and over-expression of *BT1* confers a significant growth advantage in both naturally-isolated (Dole et al., manuscript in preparation) and recombinant (Moore and Beverley, abstract 107, Woods Hole Molecular Parasitology Meeting, 1996, Cunningham et al., abstract 236, Woods Hole Molecular Parasitology Meeting, 1997) cell lines, suggesting that this may be the selective pressure for the frequent amplification of LD1 seen in $\sim 15\%$ of *Leishmania* isolates [2,5,10].

Targeting the folate pathway with chemotherapeutic agents such as methotrexate has been successful in other organisms but proves ineffective in *Leishmania*. DHFR-TS, the enzyme involved in the reduction of folate to tetrahydrofolate, is inhibited by methotrexate; however, *Leishmania* is also able to carry out these reactions using PTR1, which is resistant to methotrexate inhibition [37]. Thus, PTR1 acts as an alternate pathway for folate reduction; and indeed, methotrexate resistance results from *PTR1* gene amplification [29,37]. Recent attempts to inhibit the folate pathway by targeting PTR1 and DHFR-TS with inhibitors of both enzymes appear promising [41]. Since *BT1* is essential for parasite survival, it provides an attractive candidate for development of novel anti-leishmanial therapeutic agents that inhibit the folate pathway at the site of pteridine entry. This approach may be especially effective if combined with agents targeting the folate transporter and/or other elements in the pathway. Given the ability of the *Leishmania* genome to readily duplicate and amplify genes and to develop drug resistance by compensating with alternative pathways, it is likely that a multi-targeted approach will be needed to create effective anti-leishmanial agents.

Acknowledgements

This work is supported by PHS grant AI 17375 from the National Institutes of Health to K.D. Stuart, NIH R37 AI 21903 to S.M. Beverley, and Indo-US Vaccine Action Programme grant from the Department of Biotechnology, Government of India, New Delhi, India and National Institute of Allergy and Infectious Diseases, USA to R. Madhubala and K.D. Stuart.

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